PERFIL DA CITOCINA (TNF α , IL1 β , IL6, IL10) E SUA ASSOCIAÇÃO COM CARACTERÍSTICAS CLÍNICAS NA ARTRITE IDIOPÁTICA JUVENIL**CYTOKINE PROFILE (TNF α , IL1 β , IL6, IL10) AND ITS ASSOCIATION WITH CLINICAL FEATURES IN JUVENILE IDIOPATHIC ARTHRITIS****ВЗАИМОСВЯЗЬ ЦИТОКИНОВОГО ПРОФИЛЯ (TNF α , IL1 β , IL6, IL10) С КЛИНИЧЕСКИМИ ОСОБЕННОСТЯМИ ЮВЕНИЛЬНОГО ИДИОПАТИЧЕСКОГО АРТРИТА**

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RESUMO

Acredita-se que diferentes citocinas desempenham um papel importante na patogênese da artrite idiopática juvenil (AIJ). Alguns deles podem servir como marcadores prognósticos para a gravidade da doença, a lesão ocular e a eficácia da terapia em pacientes com AIJ. Avaliar a concentração sérica de TNF α , IL1 β , IL6 e IL10, seus níveis de produção espontânea e induzida por mitógenos na cultura de células sanguíneas totais de pacientes com AIJ e correlacionar esses indicadores com as características da doença e com a eficácia da terapia com metotrexato. Vinte e oito pacientes russos com AIJ, sem exposição prévia para drogas antirreumáticas modificadoras da doença, foram incluídos no estudo. Os níveis séricos de citocinas (TNF α , IL1 β , IL6 e IL10); os níveis de sua produção espontânea e induzida por mitógeno na cultura de células sanguíneas completas foram estudados por imunoensaio enzimático em fase sólida. Os níveis séricos e os níveis de produção espontânea de TNF α , IL1 β , IL10 foram baixos em pacientes com AIJ. Os níveis de TNF α , IL1 β , IL6, IL10 na produção induzida por mitógeno foram significativamente maiores do que aqueles na produção espontânea (pcor < 0,05). A associação de algumas características clínicas (duração da doença, uveíte, VHS elevada, hiperimmunoglobulinemia M com os níveis de citocinas (TNF α , IL6 e IL10) foi estabelecida na AIJ. O mais promissor é a associação entre a uveíte em pacientes com AIJ e um aumento na produção espontânea de IL6 (pcor = 0,004), bem como uma diminuição na produção de TNF α induzida por mitógeno (pcor = 0,029) na cultura de células sanguíneas completas. Associações estatisticamente significativas com uma resposta à terapia com metotrexato e gravidade da doença em pacientes com AIJ não foram observadas. Como resultado do estudo dos níveis de citocinas (TNF α , IL1 β , IL6 e IL10) em pacientes com AIJ, a relação de TNF α , IL6 e IL10 foi estabelecida com o número de manifestações clínicas da doença. Se confirmado em amostras maiores, o estudo da produção espontânea de IL6 na cultura de células sanguíneas completas pode ser usado para o diagnóstico precoce da uveíte em pacientes com AIJ..

Palavras-chave: *artrite idiopática juvenil, uveíte, citocinas, soro sanguíneo, cultura celular, produção espontânea, prognóstico.*

ABSTRACT

Different cytokines are believed to play an important role in the pathogenesis of juvenile idiopathic arthritis (JIA). Some of them may serve as the prognostic markers for the disease severity, the eye lesion, and

the effectiveness of therapy in JIA patients. To evaluate the serum concentration of TNF α , IL1 β , IL6 and IL10, their spontaneous and mitogen-induced production levels in the whole blood cell culture of JIA patients and to correlate these indicators with the disease features and with the effectiveness of methotrexate therapy. Twenty-eight Russian patients with JIA, naive for disease-modifying antirheumatic drugs, were enrolled in the study. The serum levels of cytokines (TNF α , IL1 β , IL6, and IL10); the levels of their spontaneous and mitogen-induced production in the whole blood cell culture were studied by solid-phase enzyme immunoassay. The serum levels and spontaneous production levels of TNF α , IL1 β , IL10 were low in JIA patients. The levels of TNF α , IL1 β , IL6, IL10 in mitogen-induced production were significantly higher than those in spontaneous production ($p_{\text{cor}} < 0,05$). The association of some clinical features (disease duration, uveitis, elevated ESR, hyperimmunoglobulinemia M with the cytokines (TNF α , IL6, and IL10) levels was established in JIA. The most promising is the association between the uveitis in JIA patients and an increase of spontaneous IL6 production ($p_{\text{cor}} = 0,004$), as well as a decrease of mitogen-induced TNF α production ($p_{\text{cor}} = 0,029$) in the whole blood cell culture. Statistically significant associations with a response to methotrexate therapy and disease severity in JIA patients were not observed. As a result of studying levels of cytokines (TNF α , IL1 β , IL6, and IL10) in JIA patients, the relationship of TNF α , IL6 and IL10 was established with the number of clinical manifestations of the disease. If confirmed in larger samples, the study of the spontaneous IL6 production in the whole blood cell culture might be used for the early diagnosis of the uveitis in JIA patients.

Keywords: *juvenile idiopathic arthritis, uveitis, cytokines, blood serum, cell culture, spontaneous production, prognosis.*

АННОТАЦИЯ

Предполагается важная роль цитокинов, в том числе TNF α , IL1 β , IL6 и IL10, в патогенезе ювенильного идиопатического артрита (ЮИА), однако сведения об уровне их продукции при данном заболевании противоречивы. Большое значение в предотвращении осложнений ЮИА и инвалидизации пациентов имеет поиск маркеров прогноза тяжести течения, формирования увеита, а также эффективности терапии. Целью данного исследования стало изучение уровня цитокинов TNF α , IL1 β , IL6, IL10 в сыворотке крови, их спонтанной и митоген-индуцированной продукции в культуре клеток цельной крови пациентов с ЮИА, а также поиск ассоциаций данных показателей с особенностями течения заболевания и эффективностью терапии метотрексатом. Методом твердофазного иммуноферментного анализа изучено содержание TNF α , IL1 β , IL6, IL10 в сыворотке крови и уровень спонтанной и митоген-индуцированной продукции данных цитокинов в культуре клеток цельной крови 28 пациентов с ЮИА из России, наивных по базисной противоревматической терапии. Установлена слабая обратная взаимосвязь между длительностью заболевания и уровнем митоген-индуцированной продукции TNF α ($R = -0,47$, $p_{\text{cor}} = 0,012$). Показано наличие ассоциаций между формированием увеита и повышением спонтанной продукции IL6 ($p_{\text{cor}} = 0,004$), а также снижением митоген-индуцированной продукции TNF α ($p_{\text{cor}} = 0,029$); между повышением СОЭ и снижением уровня IL10 в сыворотке крови ($p_{\text{cor}} = 0,024$), а также снижением спонтанной продукции IL10 ($p_{\text{cor}} = 0,024$); между гипериммуноглобулинемией класса М и повышением спонтанной продукции IL6 ($p_{\text{cor}} = 0,013$). Статистически значимых ассоциаций с ответом на терапию метотрексатом и тяжестью течения заболевания у пациентов с ЮИА не наблюдалось ($p_{\text{cor}} > 0,05$). Таким образом, в результате проведенного исследования установлено наличие взаимосвязи TNF α , IL6 и IL10 с формированием ряда клинических проявлений ЮИА. Впервые, по нашим сведениям, показана ассоциация между формированием увеита у пациентов с ЮИА и повышением спонтанной продукции IL6, а также снижением митоген-индуцированной продукции TNF α в культуре клеток цельной крови, что может быть использовано для ранней диагностики соответствующей патологии. Наиболее информативным методом является исследование показателей спонтанной продукции цитокинов в культуре клеток цельной крови.

Ключевые слова: *ювенильный идиопатический артрит, увеит, цитокины, сыворотка крови, культура клеток цельной крови, спонтанная продукция, ранняя диагностика.*

1. INTRODUCTION

Juvenile idiopathic arthritis (JIA) is one of the most frequent and most disabling rheumatological diseases in children. The development of JIA is based on an autoimmune process, accompanied by a change in the secretion of pro- and anti-inflammatory mediators (Petty *et al.*, 2015; Alekseeva and Litvitsky, 2007; Hahn and Kim, 2010; Woo, 2002).

One of the main pro-inflammatory cytokines is the tumor necrosis factor alpha (TNF α). This mediator stimulates activating transcription factors and their signaling pathways, which leads to increased production of arachidonic acid, leukotrienes, prostaglandins, and pro-inflammatory cytokines (Sedger and McDermott, 2014). TNF α has a pleiotropic effect on cells of virtually all physiological systems, including immune cells; in particular, it promotes the differentiation of monocytes/macrophages and is able to enhance the proliferation of activated B cells and fibroblasts (Petty *et al.*, 2015).

Interleukin 1 beta (IL1 β) is also a powerful pro-inflammatory cytokine, the local effect of which is manifested by vasodilation, the recruitment of granulocytes in the inflammatory focus and induction of prostaglandin expression. This cytokine also affects T cells, presumably acting as a link between the systems of innate and adaptive immunity. In addition to affecting immune cells, IL1 β enhances the expression of matrix enzymes involved in the destruction of articular cartilage, promotes osteoclast differentiation and stimulates bone resorption. At the systemic level, IL1 β acts on the hypothalamus and induces a fever response and hyperalgesia (Schett *et al.*, 2016). It has been shown that stimulation of monocytes, fibroblasts, and endothelial cells with IL1 β causes an increase in the level of IL6 (Tosato and Jones, 1990).

Interleukin 6 (IL6) is a pleiotropic mediator that influences the functioning of the immune system and homeostatic processes, often demonstrating hormone-like action (Hunter and Jones, 2015). Under various conditions, this cytokine exhibits both pro- and anti-inflammatory properties. IL6 has a clear pro-inflammatory effect in the course of an acute innate immune response. However, it also coordinates the anti-inflammatory processes necessary to resolve inflammation and prevent excess tissue damage. IL6 plays an important role in the mechanisms of adaptive immunity; in particular, it induces

maturation of B-lymphocytes into antibody-producing cells and promotes the survival and maintenance of a pool of long-lived plasma cells. At the same time, IL6, in combination with IL1 β , promotes the formation of regulatory B-lymphocytes secreting IL10. In addition, IL6 controls the proliferation, survival, and differentiation of T lymphocytes and modulates the production of cytokines. Thus, IL6 plays an important role in the generation of type 17 T helper cells, and, at the same time, enhances the secretion of IL10 by certain subtypes of T lymphocytes (Hunter and Jones, 2015).

Interleukin 10 (IL10) is a key anti-inflammatory cytokine that plays an important role in the inflammation resolution phase (Ouyang *et al.*, 2011; Ip *et al.*, 2017). It suppresses the pro-inflammatory response and is involved in the autoregulation of T helpers type 1 and activated macrophages, preventing excessive inflammation and potential tissue destruction (Ouyang *et al.*, 2011). A recent study by Ip *et al.* (2017) showed that the anti-inflammatory effect of IL10 is partially mediated by its effect on metabolic processes in activated macrophages (Ip *et al.*, 2017). At the same time, IL10 stimulates the growth and differentiation of B-lymphocytes and also affects the production of different classes of immunoglobulins (St Clair, 1999).

Thus, considering the important role of the cytokines TNF α , IL1 β , IL6 and IL10 in the regulation of the immune response and in the pathogenesis of JIA, it is relevant to study their possible effects on the clinical features of the disease and the effectiveness of therapy, which can be used to develop prognosis markers in the implementation of the approaches of a personified medicine (Pralhad *et al.*, 2008; Duurland and Wedderburn, 2014).

The aim of this research was to study the level of cytokines TNF α , IL1 β , IL6 and IL10 in blood serum and their spontaneous and mitogen-induced production in the whole blood cell culture of JIA patients; associations of these indicators with the disease features and with the effectiveness of methotrexate therapy were also assessed.

2. MATERIALS AND METHODS

2.1. Patients

The objects of the study were 28 children aged 1,5 to 16 years, residents of the Republic of Bashkortostan, Russia, with the established diagnosis of JIA, who were to initiate the

methotrexate therapy. According to the ILAR criteria, the following JIA subtypes were presented: rheumatoid factor positive polyarthritis (n=1), rheumatoid factor negative polyarthritis (n=11), persistent oligoarthritis (n=13), extended oligoarthritis (n=1), enthesitis-related arthritis (n=1), undifferentiated arthritis (n=1). Patients previously did not receive any nonbiologic and biologic disease-modifying antirheumatic drugs (DMARDs) and no systemic glucocorticosteroids during the previous 6 months. All the patients underwent examination and treatment in the cardiorheumatology department of the Republican Children's Clinical Hospital (RCCH) (Ufa) in 2015-2017. The study was approved by the expert council on biomedical ethics of Bashkir State Medical University. The parents of all the patients signed the voluntary informed consent. When considering the features of the JIA course, patients' sex, the debut age, the duration and severity of the disease, the presence of uveitis and the changes in laboratory parameters were taken into account. The severity division was based on the classification of treatment groups defined in the recommendations of the American College of Rheumatology (ACR, 2011) for the treatment of JIA (Table 1) (Beukelman *et al.*, 2011). The relationship with the cytokine levels was evaluated only for the extreme variants of the trait – for severe and mild JIA. The effect of methotrexate therapy was assessed according to the ACR criteria for pediatric patients (ACR Pedi) (Giannini *et al.*, 1997). Achieving the ACR Pedi 30 improvement was considered as a presence of the response to the therapy, otherwise – as the absence. At the same time, the response was regarded as sufficient only in the case the patient achieved a clinical remission on medication (Wallace *et al.*, 2011), otherwise – as insufficient (Wallace *et al.*, 2004, 2011). The JIA patients clinical characteristics studied for the relationship with the cytokine levels are presented in Table 2.

2.2. Laboratory methods of research

All the parameters were evaluated in the clinical laboratory of the RCCH during the period of the active articular syndrome, prior to the initiating of methotrexate therapy. The erythrocyte sedimentation rate (ESR) was measured by Panchenkov method and was considered elevated if its value exceeded the upper limit of the normal range two or more times (Panchenkov, 1924). Serum concentration of immunoglobulins classes A, M, G (IgA, IgM, IgG) was estimated by radial immunodiffusion in agar

gel by Mancini ('Microgen', Russia); of C-reactive protein (CRP) by latex immunoturbidimetric method ('Vector-Best', Russia, biochemical analyzer 'Random Access A-25', 'BioSystems S.A.', Spain) (Mancini *et al.*, 1965; Imelbaeva *et al.*, 2004).

2.3. Determination of cytokine levels

The study of the levels of TNF α , IL1 β , IL6, IL10 in blood serum, as well as levels of their spontaneous and mitogen-induced production in the whole blood cell culture was performed by solid-phase enzyme immunoassay ('Cytokine', Russia, analyzer 'Stat Fax 2100' by Awareness Technology, Inc.). To investigate spontaneous and mitogen-induced cytokine production, the 'CYTOKINE-STIMUL-BEST' kits ('Vector-Best', Russia) were used. The kits include two types of vials: No. 1 – containing 4 ml of sterile DMEM medium with heparin (2.5 U/ml), gentamycin (100 μ g/ml) and intended for the evaluation of spontaneous cytokine production; No. 2 – containing sterile lyophilized complex mitogen (phytohaemagglutinin – 4 μ g, concanavalin A – 4 μ g, lipopolysaccharide – 2 μ g) and intended for evaluation of mitogen-induced cytokine production. Immediately after the intake, 1 ml of peripheral venous blood was introduced into vial No. 1 (preheated to 37°C) with a syringe. After careful mixing, 1 ml of diluted blood from vial No. 1 was transferred to vial No. 2 (preheated to 37°C) and again mixed carefully. The vials were incubated at 37°C for 24 hours, after that the blood preparations were transferred to microtubes. After centrifugation at 3000 G for 10 min, the supernatant was aliquoted and stored at -80°C.

2.4. Statistical data analysis

Mathematical processing of the results was carried out using Microsoft Excel, R v.3.4.0, STATISTICA v.10 (StatSoft, Inc). Due to the fact that the distribution of all quantitative characteristics was different from normal, the median (Me) and interquartile range (25th percentile (Q1); 75th percentile (Q3)) were indicated in their description, and the differences in the samples were determined using the Mann-Whitney U test (for 2 independent samples) or Wilcoxon test (for 2 dependent samples). Considering that the levels of spontaneous production of TNF α , IL1 β , and IL10 in the whole blood cell culture, as well as the serum IL1 β and IL10 levels in most cases, had the same zero values, and the total sample quantity was more than 20 people, the U statistic was adjusted for

ties and the respective significance level was evaluated. Simultaneously, when comparing the indices of mitogen-induced production of TNF α , IL1 β , and IL10, as well as of all IL6 indicators, the U statistic was not adjusted for ties, and a significance level was calculated in an exact way. To assess the relationship between the two quantitative characteristics, a Spearman Rank Order correlation analysis was carried out: the relationship was considered to direct at $R > 0$, inverse at $R < 0$, strong at $|R| \geq 0.7$, moderate at $0.5 \leq |R| < 0.7$ and weak at $0.3 \leq |R| < 0.5$. In all the cases, differences were considered statistically significant at $p < 0.05$. Taking into account the multiple comparisons, the values of p were corrected by applying Monte Carlo (MC) 'resampling' method with the number of iterations being 10^7 (p_{cor}) (Hothorn *et al.*, 2008).

3. RESULTS AND DISCUSSION

3.1. Cytokine levels in JIA patients

Levels of cytokines TNF α , IL1 β , IL10 in blood serum and in spontaneous production in JIA patients were low, with 50% to 100% of the values being below the detection threshold of the technique (Table 3). At the same time, the levels of TNF α , IL1 β , IL6, IL10 in mitogen-induced production were significantly higher than those in spontaneous production ($p_{\text{cor}} < 0.05$). Due to the large number of values that exceeded the maximum threshold recorded by the analyzer, and to the limited volume of the primary material for study, the level of mitogen-induced IL6 production was not included in the subsequent analysis. All the serum TNF α values were below the detection threshold of the technique, so this indicator was not analyzed further either.

3.2. Interrelation between cytokine levels

Correlation analysis was used to study the interrelation of cytokine levels. The presence of a statistically significant weak direct relationship between IL6 and IL10 levels in blood serum ($R = 0.497$, $t = 2.86$, $p = 0.008$, $p_{\text{cor}} = 0.007$), as well as between levels of IL6 in blood serum and in spontaneous production ($R = 0.485$, $t = 2.77$, $p = 0.010$, $p_{\text{cor}} = 0.011$) was detected.

A statistically significant moderate direct relationship was found between the indices of spontaneous production of TNF α , IL1 β and IL6 ($R = 0.54$, $t = 3.29$, $p = 0.003$, $p_{\text{cor}} = 0.002$ for TNF α &IL1 β ; $R = 0.54$, $t = 3.26$, $p = 0.003$, $p_{\text{cor}} = 0.002$ for TNF α &IL6; $R = 0.57$, $t = 3.551$, $p = 0.001$, $p_{\text{cor}} = 0.002$ for IL1 β &IL6). The level of

spontaneous production of IL10 significantly correlated only with the same index for TNF α , the relationship turning out to be direct, yet weak ($R = 0.41$, $t = 2.30$, $p = 0.030$, $p_{\text{cor}} = 0.048$). A tendency towards a direct relationship between the values of spontaneous production of IL10 and IL6 ($R = 0.36$, $t = 1.97$, $p = 0.060$, $p_{\text{cor}} = 0.059$) was observed. When analyzing the indices of mitogen-induced production of cytokines, no correlations were established. There was only a tendency towards a direct relationship between IL1 β levels in spontaneous and mitogen-induced production ($R = 0.36$, $t = 1.95$, $p = 0.063$, $p_{\text{cor}} = 0.063$).

3.3. Associations of the cytokine profile indicators with clinical characteristics of JIA

There were no significant correlations between the age of patients and the studied parameters of cytokines ($p > 0.1$). At the same time, a statistically significant weak inverse relationship was established between the duration of the disease and the level of mitogen-induced TNF α production ($R = -0.47$, $t = -2.72$, $p = 0.011$, $p_{\text{cor}} = 0.012$) (Table 4).

Statistically significant differences in the studied indicators of cytokine profile depending on the presence of such JIA peculiarities as female sex, the severe disease course, the mild disease course, the absent response to methotrexate, the insufficient response to methotrexate, were not observed ($p > 0.05$). There was a tendency towards a decrease in the level of spontaneous production of IL6 in mild JIA course patients in comparison with the other patients (2,8 (1,5;4,1) vs 17,7 (6,5;50,4) pg/ml respectively, $p = 0.055$, $p_{\text{cor}} = 0.058$) (Table 5).

At the time of the examination, uveitis was detected in only one patient, and after 2 months, the eye lesion was also detected in a second patient. When analyzing the cytokine levels, it was found that the level of spontaneous production of IL6 was increased in both patients with uveitis more than 100 times in comparison with the rest of patients ($p = 0.007$, $p_{\text{cor}} = 0.004$) (Table 4). At the same time, the level of mitogen-induced TNF α production in the presence of eye lesion was significantly lower than in its absence ($p = 0.029$, $p_{\text{cor}} = 0.029$). The duration of the disease in both groups did not differ significantly ($p = 0.133$).

The ESR elevation in JIA patients was associated with a statistically significant decrease in the serum IL10 level and in the IL10 spontaneous production level ($p = 0.040$ ($p_{\text{cor}} = 0.024$) and $p = 0.010$ ($p_{\text{cor}} = 0.024$) respectively) (Table 4). At the same time, the

relationship of cytokine indicators with the CRP elevation was not established ($p > 0,05$).

Hyperimmunoglobulinemia M in JIA patients was associated with a significant increase in the IL6 spontaneous production level ($p = 0,013$, $p_{\text{cor}} = 0,013$) (Table 4). It should be noted that in case of hypoimmunoglobulinemia A, an increase in levels of spontaneous production of TNF α and IL10 was observed, and in case of hyperimmunoglobulinemia A there was an increase in the serum level of IL1 β , but after correction the differences proved to be insignificant ($p = 0,035$ ($p_{\text{cor}} = 0,059$), $p = 0,046$ ($p_{\text{cor}} = 0,072$), $p = 0,042$ ($p_{\text{cor}} = 0,053$) respectively). Moreover, in JIA patients with hyperimmunoglobulinemia G, there was a tendency to decrease the IL10 spontaneous production level ($p = 0,052$, $p_{\text{cor}} = 0,045$).

3.4. Discussion

JIA is one of the most common rheumatological diseases in pediatric practice. Pro- and anti-inflammatory cytokines, including TNF α , IL1 β , IL6, and IL10, are believed to play an important role in the disease pathogenesis. The level of cytokines TNF α , IL1 β , IL6, IL10 in the serum, their spontaneous and mitogen-induced production levels in the whole blood cell culture of JIA patients were studied, and the associations of these indicators with the JIA peculiarities and with the effectiveness of methotrexate therapy were analyzed.

The study revealed that the levels of TNF α , IL1 β , and IL10 in the blood serum and in spontaneous production in JIA patients before the initiating of DMARDs were low. This fact probably indicates no or weak activation of immune cells at the time of the examination. Simultaneously, a significant increase in the levels of all the examined cytokines in mitogen stimulation was noted, which indicates that the immune cells have a high potential for secreting the corresponding products.

The study of cytokine levels in JIA patients was conducted in a number of works, yet the data are contradictory. According to Muller *et al.* (1998), levels of IL1 β , IL6, and TNF α in spontaneous production in the whole blood cell culture of patients with systemic juvenile chronic arthritis were found to be lower than the threshold, however, as in the present study, there was an increase in the corresponding parameters after stimulation with lipopolysaccharide (Muller *et al.*, 1998; Fedorov *et al.*, 2009). At the same time, the authors did not reveal statistically significant correlations between the levels of spontaneous or

induced production of TNF α , IL1 β , IL6, and IL10 with indices of disease activity. It should be noted that some patients in this sample received immunosuppressive therapy and glucocorticosteroids. In the work by Pashnina *et al.* (2014) medians of serum levels of IL1 β and TNF α in JIA patients were equal to zero, while the study of cytokines in supernatants of peripheral blood cell cultures, as in the present work, was more informative (medians of the corresponding parameters after cultivation: 8,0 pg/ml (0,2; 22,0) and 3,0 pg/ml (1,0; 5,0)) (Pashnina *et al.*, 2014). According to Zakirov *et al.* (2016), the medians of levels of spontaneous production of IL6, IL10, and TNF α in the whole blood cell culture of JIA patients who received biologic DMARDs were 356,4 pg/ml (34,1; 303,7), 1,5 pg/ml (1,8; 7,9) and 33,0 pg/ml (6,8; 67,9) respectively (measurements by flow fluorescence). In this case, stimulation with phytohemagglutinin was accompanied by an increase in cytokine levels (Zakirov *et al.*, 2016), which agrees with the results of the paper present.

A number of studies have reported a high or, at least, detectable levels of TNF α , IL1 β , and IL6 in the blood serum/plasma of patients with juvenile arthritis (Pralhad *et al.*, 2008; Kutukculer *et al.*, 1998; Yilmaz *et al.*, 2001; Mangge *et al.*, 1995; De Jager *et al.*, 2007; Keltsev *et al.*, 2015). According to Ou *et al.* (2002) and Walters *et al.* (2016), in the study of levels of IL1 β , TNF α , and IL6 in the blood serum of JIA patients, only the concentration of IL1 β was below the detectable level (Ou *et al.*, 2002; Walters *et al.*, 2016). The level of IL10 in the plasma/blood serum of JIA patients was positive in the works of de Jager *et al.* (2007), Prahalad *et al.* (2008), Keltsev *et al.* (2015), Walters *et al.* (2016). The observed inconsistency of the results is probably due to the peculiarities of the experiment design (including the method of measuring the indicators, the differences in patient samples, the dose, and type of the mitogen, the type of cultured cells) and the presentation form of the results (mean / median).

In this study, a statistically significant direct relationship was found between the following parameters of the cytokine profile: serum levels of IL6 and IL10; levels of IL6 in blood serum and in spontaneous production; indices of spontaneous production of TNF α , IL1 β , and IL6; levels of spontaneous production of IL10 and TNF α . The presence of a direct relationship between the levels of spontaneous production of TNF α , IL1 β , IL6, and IL10 is consistent with the information available to date on their function and

effects on each other. TNF α is an auto- and paracrine inducer of IL1 and IL6 (Choy and Panayi, 2001). In turn, IL1 β contributes to an increase in the level of IL6, and IL6 is able to enhance IL10 production (Hunter and Jones, 2015). In addition, the correlation of pro-inflammatory cytokines with an anti-inflammatory mediator (IL10) is probably due to the compensatory response of the immune system (St Clair, 1999; Asadullah *et al.*, 2003). Prahalad *et al.* (2008) also showed a direct relationship between levels of IL1 β , IL6, IL10, and TNF α in the serum of JIA patients (Pralhad *et al.*, 2008).

The relationship of certain clinical parameters with the characteristics of the cytokine profile in JIA patients was also established in this study. An inverse relationship between the level of mitogen-induced TNF α production in the whole blood cell culture and the duration of the disease was shown; this fact may indicate a gradual depletion of the secretory potential of cells during a prolonged inflammatory process in the absence of DMARDs. In the work of Goryunov *et al.* (2012), the level of stimulated (and spontaneous) production of TNF α in the culture of lymphocytes and monocytes of JIA patients was also decreased with an increase in the duration of the disease (more than a year) (Goryunov *et al.*, 2012).

In addition, this study showed a sharp (more than a hundredfold) increase in the level of spontaneous IL6 production and a slight decrease in the level of mitogen-induced TNF α production in the early stages of uveitis formation in JIA patients. In a number of studies, an increase in the IL6 level in the aqueous humor was observed in experimental uveitis, as well as in patients with uveitis of various origins, including those with JIA (Lin, 2015; Sijssens *et al.*, 2007; Ohta *et al.*, 2000). A marked increase in the IL6 level in the aqueous humor of mice was noted in the early stages of experimental uveitis, with the values of this cytokine reaching several nanograms, while the levels of TNF α and IL1 β did not exceed 100 pg (Ohta *et al.*, 2000)³⁹, which is consistent with the results of this study. A significant increase of the IL6 level in blood serum was detected in patients with active uveitis of heterogeneous etiology and in spondyloarthritis patients with active anterior uveitis (Kramer *et al.*, 2007; Przepiera-Będzak *et al.*, 2016). According to a number of authors, the level of TNF α was elevated in the aqueous humor both in experimental uveitis (with the level of IL6 declined) and in JIA patients with uveitis (Ohta *et al.*, 2000; Sijssens *et al.*, 2007). Concerning the serum level of TNF α in patients

with uveitis of heterogeneous etiology, there is conflicting information: one study showed a significant increase in the level of this cytokine (Santos Lacomba *et al.*, 2001), while another study found no associations (Kramer *et al.*, 2007). It is assumed, however, that IL6 can suppress the production of TNF α and IL1 β (Ohta *et al.*, 2000; Kramer *et al.*, 2007), and this is consistent with the results of the present study. These data indicate the important role of IL6 in the formation of uveitis in JIA. If confirmed in larger samples, the study of the IL6 spontaneous production in the whole blood cell culture might be used for the early diagnosis of eye lesion in JIA patients.

In addition, the relationship between the decrease in the levels of IL10 in the serum and in spontaneous production with the ESR elevation was established in this study, as well as the relationship between the increase in the IL6 spontaneous production level and hyperimmunoglobulinemia M. These associations are consistent with the known properties of IL10 as an anti-inflammatory cytokine (Ouyang *et al.*, 2011; Ip *et al.*, 2017), and of IL6 as a B-lymphocytes differentiation factor, capable of enhancing the production of immunoglobulins (Faris *et al.*, 1997; Mihara *et al.*, 2012); this confirms the assumption of the important role of these mediators in the pathogenesis of JIA. Several studies previously showed the correlation of the level of ESR and / or CRP with IL6 indices in plasma/serum of patients with juvenile arthritis (Pralhad *et al.*, 2008; Kutukculer *et al.*, 1998; Mangge *et al.*, 1995; Ozen *et al.*, 1997).

4. CONCLUSIONS

Thus, as a result of studying the levels of cytokines (TNF α , IL1 β , IL6, and IL10) in JIA patients, the relationship of TNF α , IL6, and IL10 was established with the number of clinical manifestations of the disease. Several research groups studied the cytokine profile in JIA before. Nevertheless, to our knowledge, this is the first report for the associations between some cytokines characteristics (in particular in spontaneous production in the whole blood culture) and the disease features (uveitis, elevated ESR, hyperimmunoglobulinemia M), which is important for understanding the JIA pathogenesis. On the other hand, the sample size is relatively small, particularly for some clinical features. If confirmed in larger samples, the study of the spontaneous IL6 production in the whole blood cell culture might be used for the early diagnosis of the uveitis in JIA patients. Statistically significant associations with the

response to methotrexate therapy and the severity of the course of the disease in JIA patients were not observed. However it may be related to the small sample size and warrants further study.

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6. STATEMENTS

6.1. Disclosure of potential conflicts of interest:

None declared.

6.2. Ethical approval (Research involving Human Participants and/or Animals)

The study was approved by the expert council on biomedical ethics of Bashkir State Medical University.

6.3. Informed consent

The parents of all the patients signed voluntary informed consent.

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Table 1. The JIA severity subdivision

Severity of the disease	Categories defined in '2011 American College of Rheumatology Recommendations for the Treatment of Juvenile Idiopathic Arthritis'		
	Disease activity	Treatment groups ^a	Features of poor prognosis
Severe	High	History of arthritis of 5 or more joints Active sacroiliac arthritis	irrespective
Moderate	High	History of arthritis of 4 or fewer joints	irrespective
		History of arthritis of 5 or more joints Active sacroiliac arthritis	irrespective
	Low	History of arthritis of 4 or fewer joints	present
		History of arthritis of 4 or fewer joints ^b	absent
		History of arthritis of 5 or more joints Active sacroiliac arthritis	present
Mild	Moderate	History of arthritis of 4 or fewer joints	
	Low	History of arthritis of 4 or fewer joints ^b	absent
		History of arthritis of 5 or more joints Active sacroiliac arthritis History of arthritis of 4 or fewer joints	absent

^a Treatment groups 'Systemic arthritis with active arthritis (and without active systemic features)' and 'Systemic arthritis with active systemic features (and without active arthritis)' were not presented in the studied sample.

^b If ESR or CRP level was elevated in patients with moderate activity from the group 'History of arthritis of 4 or fewer joints' in the absence of 'features of poor prognosis', the disease course was also considered moderate, otherwise – mild.

Table 2. Clinical characteristics of JIA patients

Symptom	Variants/gradations	Value
Age, Me (Q1;Q3), yrs	Debut age	3,84 (2,69;7,49)
	Age of inclusion in the study	4,42 (3,43;8,66)
Sex, n (%)	Female	19 (67,86)
	Male	9 (32,14)
Uveitis, n (%)	Present	2 (7,14)
	Absent	22 (78,57)
	NA	4 (14,29)
	Severe	6 (21,43)
Severity of the disease, n (%)	Moderate	18 (64,29)
	Mild	2 (7,14)
	NA	2 (7,14)
	Present	17 (60,71)
Elevated ESR, n (%)	Absent	8 (28,57)
	NA	3 (10,71)
Elevated CRP, n (%)	Present	12 (42,86)
	Absent	14 (50,00)
	NA	2 (7,14)
IgA deficiency, n (%)	Present	4 (14,29)
	Absent	24 (85,71)
Elevated IgA, n (%)	Present	9 (32,14)
	Absent	19 (67,86)
Elevated IgM, n (%)	Present	8 (28,57)
	Absent	19 (67,86)
	NA	1 (3,57)
Elevated IgG, n (%)	Present	19 (67,86)
	Absent	9 (32,14)
Response to methotrexate, n (%)	Present	21 (75,00)
	Absent	4 (14,29)
	NA	3 (10,71)
Sufficient response to methotrexate, n (%)	Present	5 (17,86)
	Absent	11 (39,29)
	NA	12 (42,86)

Note: Me (Q1;Q3) – median with interquartile range (25th percentile (Q1); 75th percentile (Q3)); n – the number of patients; NA – the symptom is not differentiated (the anamnesis data are inconsistent or insufficient for the analysis); ESR – erythrocyte sedimentation rate, CRP – C-reactive protein; IgA, IgM, IgG – immunoglobulins classes A, M, G.

Table 3. *TNF α , IL1 β , IL6, IL10 serum levels, their spontaneous and mitogen-induced production levels in the whole blood cell culture of JIA patients*

Cytokine	Serum level	Spontaneous production	Mitogen-induced production	Comparison of spontaneous and mitogen-induced production (Wilcoxon test)		
	Me (Q1;Q3) min-max, pg/ml n	Me (Q1;Q3) min-max, pg/ml n	Me (Q1;Q3) min-max, pg/ml n	Z	p	p _{cor}
TNF α	0,0	0,0	80,0	4,577	0,000005	<0,000001
	(0,0;0,0)	(0,0;0,0)	(40,0;112,0)			
	0,0-0,0	0,0-46,1	15,2-200,0			
IL1 β	28	28	28	4,517	0,000006	<0,000001
	0,0	0,35	750,0			
	(0,0;1,15)	(0,0;6,85)	(510,8;1073,6)			
IL6	0,0-154,0	0,0-522,4	240,4-1264,0	2,366	0,018	0,017
	28	28	27			
	4,2	17,7	4536,0			
IL10	(1,5;11,4)	(5,8;71,1)	(4360,0;7412,0)	4,457	0,000008	<0,000001
	0,0-467,6	1,4-4496,0	4114,0-7606,0			
	27	28	7			
IL10	0,0	0,0	109,3	4,457	0,000008	<0,000001
	(0,0;0,0)	(0,0;0,0)	(47,2;149,6)			
	0,0-81,8	0,0-21,5	0,0-334,8			
	28	28	28			

Table 4. Analysis of the relationship between the cytokine profile *indicators* and clinical characteristics of JIA

Clinical characteristics (specific features) of JIA	Cytokine profile indicators	Change in the cytokine profile indicators in the presence of clinical feature in comparison with its absence (vs)		p p _{cor}
		qualitatively	quantitatively, Me(Q1;Q3), pg/ml	
Disease duration	mitogen-induced production of TNF α	NA (decrease)	NA (R=-0,47)	0,011 0,012
Uveitis	spontaneous production of IL6	increase	2827,5 (1159,0;4496,0) vs 17,7 (4,2;61,6)	0,007 0,004
	mitogen-induced production of TNF α	decrease	32,2 (27,6;36,8) vs 85,6 (52,0;112,0)	0,029 0,029
	serum level of IL10	decrease	0,0 (0,0;0,0) vs 0,0 (0,0;39,8)	0,040 0,024
Elevated ESR	spontaneous production of IL10	decrease	0,0 (0,0;0,0) vs 0,0 (0,0;10,45)	0,010 0,024
Elevated IgM	spontaneous production of IL6	increase	92,7 (16,5;634,35) vs 7,0 (4,1;24,1)	0,013 0,013

Table 5. Supplement table. Comparative analysis of cytokine profile indicators in relation to the sex of JIA patients and the disease severity

Indicators	Sex		Severe JIA				Mild JIA			
	Female Me (Q1;Q3), pg/ml	Male Me (Q1;Q3), pg/ml	U n ² /n	p-value (adj)/ p exact	Present Me (Q1;Q3), pg/ml	Absent Me (Q1;Q3), pg/ml	U n ² /n	p-value (adj)/ p exact	Present Me (Q1;Q3), pg/ml	Absent Me (Q1;Q3), pg/ml
TNF α	spontaneous production	0.0 (0.0;0.0)	67.5 19/9	0.157 0.383	0.0 (0.0;0.0)	0.0 (0.0;0.0)	59.5 6/20	1.00 0.976	0.0 (0.0;0.0)	0.0 (0.0;0.0)
	mitogen-induced production	52.4 (36.8;102.0)	51.5 19/9	0.099 0.095	83.6 (36;100.4)	70.6 (47;112)	55 6/20	0.784 0.790	105 (42.8;167.2)	80 (44.2;108.6)
	serum level	0.0 (0.0;1.9)	78.5 19/9	0.688 0.735	0.0 (0;12.3)	0.0 (0;0.55)	50 6/20	0.459 0.573	0.0 (0.0;0.0)	0.0 (0;1.15)
	spontaneous production	0.7 (0.0;7.8)	84.5 19/9	0.979 0.962	6.55 (0.7;12.1)	0.0 (0.0;5.3)	35.5 6/20	0.112 0.139	0.0 (0.0;0.0)	0.35 (0.0;9.95)
IL1 β	mitogen-induced production	727.6 (510.8;1092)	73 19/8	0.894 0.897	912.6 (580.8;1092)	727.6 (478.4;1073.6)	48 6/19	0.589 0.598	675.4 (600.8;750)	894.0 (478.4;1092)
	serum level	4.3 (0.9;11.4)	71.5 18/9	0.643 0.631	1.55 (0.9;5.4)	6.45 (2.3;11.75)	31.5 6/20	0.088 0.083	1.85 (1.5;2.2)	4.9 (1.7;11.5)
	spontaneous production	17.5 (6;61.6)	85 19/9	1.00 1.00	15.3 (5.6;19.7)	17.7 (5.1;50.4)	55.5 6/20	0.808 0.79	2.8 (1.5;4.1)	17.7 (6.5;50.4)
	serum level	0.0 (0.0;0.0)	82 19/9	0.808 0.885	0.0 (0.0;0.0)	0.0 (0.0;0.0)	59.5 6/20	1.00 0.976	0.0 (0.0;0.0)	0.0 (0.0;0.0)
IL10	spontaneous production	0.0 (0.0;0.0)	83 19/9	0.872 0.923	0.0 (0.0;0.0)	0.0 (0.0;0.0)	51 6/20	0.351 0.614	0.0 (0.0;0.0)	0.0 (0.0;0.0)
	mitogen-induced production	109 (26.6;159)	79.5 19/9	0.787 0.772	142.6 (78.8;187.6)	99.5 (30.0;136.2)	44.5 6/20	0.361 0.355	67.5 (0.0;135)	106.4 (47.2;149.6)

Note: n²/n^b – the number of observations corresponding to the columns No. a and No. b.