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# МЕДИЦИНСКАЯ ИММУНОЛОГИЯ

*Данный выпуск реализован при финансовой поддержке Правительства Санкт-Петербурга*

**ИЛЛЮСТРАЦИИ К СТАТЬЕ «ОСОБЕННОСТИ ИММУНОЛОГИЧЕСКИ АКТИВНОГО И «ХОЛОДНОГО» ФЕНОТИПОВ ИНВАЗИВНОЙ КАРЦИНОМЫ ШЕЙКИ МАТКИ РАННИХ СТАДИЙ ПО ДАННЫМ СЕКВЕНИРОВАНИЯ ТРАНСКРИПТОМА» (АВТОРЫ: КУРМЫШКИНА О.В., КОВЧУР П.И., ВОЛКОВА Т.О. [с. 1141-1150])**

ILLUSTRATIONS FOR THE ARTICLE "CHARACTERISTICS OF IMMUNE-ACTIVE AND IMMUNESILENT PHENOTYPES OF EARLY-STAGE CERVICAL CARCINOMA AS REVEALED BY TRANSCRIPTOME SEQUENCING" (AUTHORS: KURMYSHKINA O.V., KOVCHUR P.I., VOLKOVA T.O. [pp. 1141-1150])

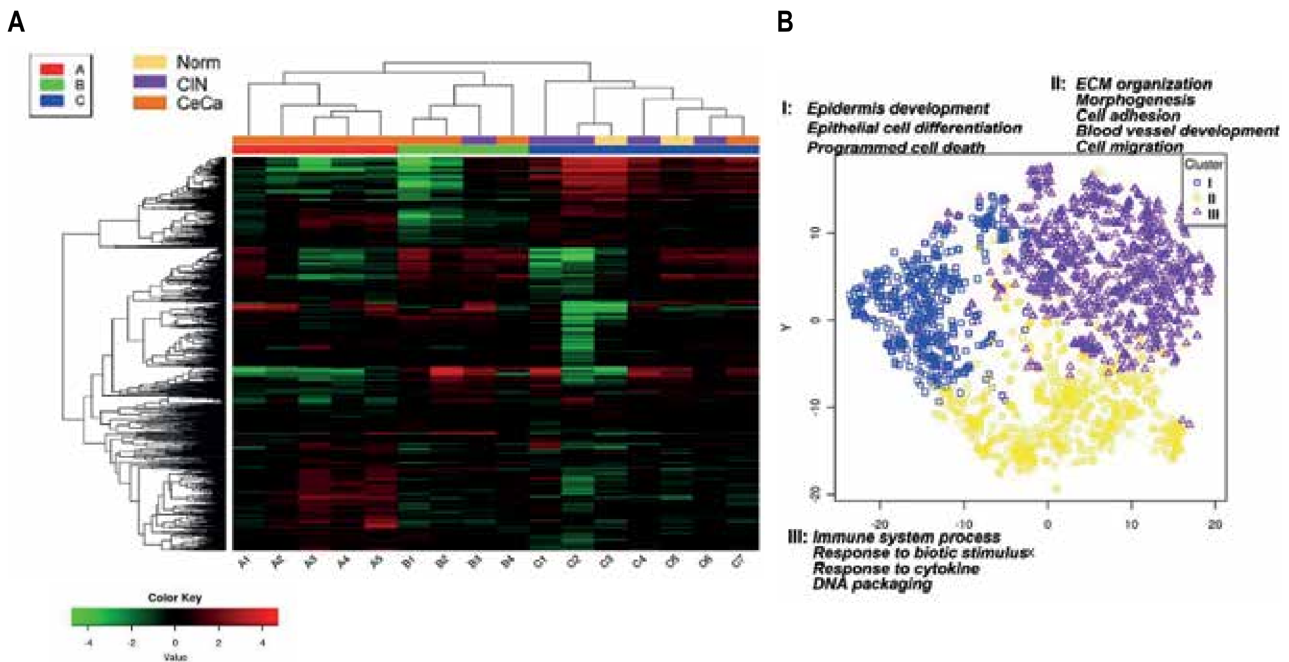


**Figure 1. Cluster analysis and Gene Ontology enrichment performed on gene expression profiles of cervical lesions**

Note. (A) Hierarchical clustering identified 3 clusters (‘A’, ‘B’, and ‘C’) comprising invasive CeCa, precursor CINs, and normal epitheli. (B) t-SNE plot visualizing distribution of groups ‘A’, ‘B’, and ‘C’ between the three patterns of coordinately expressed genes.

**ИЛЛЮСТРАЦИИ К СТАТЬЕ «ЭКСПРЕССИЯ МОЛЕКУЛ CD56 И TIM-3 НА РАЗНЫХ СУБПОПУЛЯЦИЯХ МОНОЦИТОВ ПЕРИФЕРИЧЕСКОЙ КРОВИ ПРИ БЕРЕМЕННОСТИ» (АВТОРЫ: ОРЛОВА Е.Г., ЛОГИНОВА О.А. [с. 1177-1182])**

ILLUSTRATIONS FOR THE ARTICLE "CD56 AND TIM-3 MOLECULE EXPRESSION IN DIFFERENT MONOCYTE SUBSETS IN PHYSIOLOGICAL PREGNANCY" (AUTHORS: ORLOVA E.G., LOGINOVA O.A. [pp. 1177-1182])

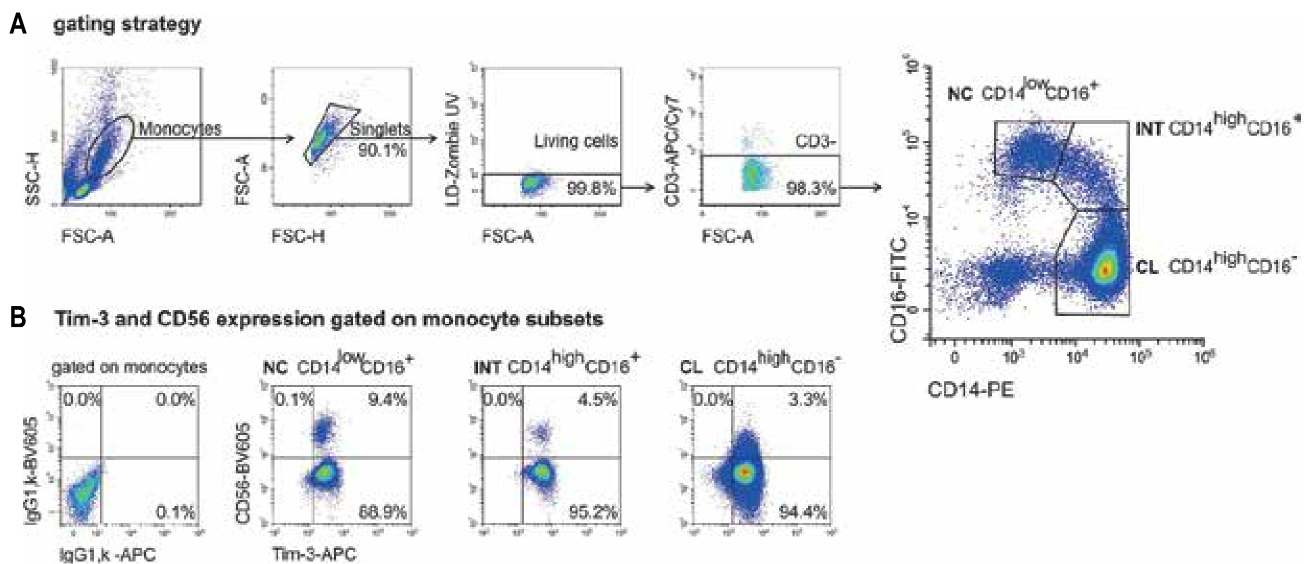


**Figure 1. Gating strategy for monocytes subsets and assessment of Tim-3 and CD56 expression**

Note. (A) Monocytes gate selection according to the forward (FSC-A) and side (SSC-H) scattering parameters; discrimination of doublets according to the FSC-A/FSC-H parameters; discrimination between dead and live cells by LIVE/DEAD-ZOMBIE UV stained; selection of CD3- cells in the peripheral blood PBMC living cell gate; the number of the monocytes subsets was determined as a percentage of $CD14^{low}CD16^{+}$ (non-classical, NC), $CD14^{high}CD16^{+}$ (intermediate, INT) and $CD14^{high}CD16^{-}$ (classical, CL) in the gate of CD3-negative monocytes. (B) Expression of Tim-3 and CD56 in monocytes subsets.

САНКТ-ПЕТЕРБУРГСКОЕ РЕГИОНАЛЬНОЕ ОТДЕЛЕНИЕ
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(СПб РО РААКИ)

# МЕДИЦИНСКАЯ ИММУНОЛОГИЯ

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# СОДЕРЖАНИЕ МЕДИАТОРОВ ВРОЖДЕННОГО ИММУНИТЕТА В СЛЕЗНОЙ ЖИДКОСТИ ПАЦИЕНТОВ С СОСУДИСТЫМИ И НЕЙРОДЕГЕНЕРАТИВНЫМИ ПРОЯВЛЕНИЯМИ ДИАБЕТИЧЕСКОЙ РЕТИНОПАТИИ

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**Резюме.** По результатам последних исследований диабетическую ретинопатию можно рассматривать не только как сосудистое заболевание, но и как нейродегенеративный процесс. Изучение состава слезной жидкости используется для оценки состояния локального иммунитета при развитии глазных заболеваний. Однако исследования, изучающие влияние состава слезы при диабетической ретинопатии немногочисленны. Цель исследования – определить уровни IL-1β, IL-10, TGF-β3, MMP-7, TIMP-2, белка S100b, BDNF и NGF в слезной жидкости пациентов сосудистыми и нейродегенеративными проявлениями диабетической ретинопатии. В исследование были включены 80 пациентов с диагнозом сахарный диабет 2 типа, которые были разделены на 2 группы: группа 1 – 40 пациентов, у которых не было клинических признаков диабетической ретинопатии на глазном дне, группа 2 – 40 пациентов с начальными признаками непролиферативной диабетической ретинопатии. Всем включенным в исследование проводилось обследование на оптическом когерентном томографе RTVue-100 (США), определяли объем фокальных потерь ганглиозных клеток сетчатки (FLV). Увеличение FLV выше показателей нормативной базы прибора расценивали как ОКТ-признак нейродегенерации сетчатки. По результатам ОКТ участников первой и второй группы дополнительно разделили на 4 подгруппы: 1А – пациенты без сосудистых изменений на глазном дне и без ОКТ-признаков нейродегенерации сетчатки (n = 12), 1Б – пациенты без сосудистых изменений на глазном дне и с наличием ОКТ-признаков нейродегенерации сетчатки (n = 28), 2А – пациенты с начальной непролиферативной ДР и без ОКТ-признаков нейродегенерации сетчатки (n = 10), 2Б – пациенты с начальной непролиферативной ДР и с ОКТ-признаками нейродегенерации сетчатки (n = 30). Уровень IL-1β, IL-10, TGF-β3, MMP-7, TIMP-2, белка S100b, BDNF и NGF в слезной жидкости определяли с помощью иммуноферментного анализа. Уровни IL-1β и IL-10 в слезной жидкости во всех подгруппах были сопоставимы с контролем на протяжении всего исследования. Содержание TGF-β3 в слезной

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жидкости пациентов группы с начальными признаками непролиферативной ДР (группа 2) было достоверно (p = 0,001) ниже в сравнении с контролем и группой 2. Однако отсутствовала достоверная разница (p > 0,05) между подгруппами А и Б внутри групп. Концентрация MMP-7 в слезной жидкости во всех подгруппах была достоверно ниже чем в контроле (p < 0,05), однако, в подгруппах с ОКТ-признаками нейродегенерации сетчатки (1Б и 2Б) дефицит данной металлопротеиназы был более выражен (p = 0,0001). Уровни исследуемых нейропептидов NGF, BDNF и S100B в слезной жидкости не отличались от контроля во всех подгруппах.

*Ключевые слова: нейродегенерация, цитокины, нейропептиды, матриксные металлопротеиназы, диабетическая ретинопатия, слеза*

# CONTENT OF MEDIATORS OF INNATE IMMUNITY IN THE TEARS OF PATIENTS WITH VASCULAR AND NEURODEGENERATIVE MANIFESTATIONS OF DIABETIC RETINOPATHY

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**Abstract.** According to the results of recent studies, diabetic retinopathy can be considered not only as a vascular disease, but also as a neurodegenerative process. Study of the composition of the tear fluid is used to assess the state of local immunity in the development of eye diseases. However, studies examining the effect of tear composition in diabetic retinopathy are few. The aim of the study is to determine the levels of IL-1β, IL-10, TGF-β3, MMP-7, TIMP-2, protein S100b, BDNF and NGF in the tear fluid of patients with vascular and neurodegenerative manifestations of diabetic retinopathy. The study included 80 patients diagnosed with type 2 diabetes which were divided into 2 groups: the 1st group included 40 patients who had no clinical signs of diabetic retinopathy on the fundus; the 2nd group included 40 patients with initial signs of non-proliferative diabetic retinopathy. All those included in the study were examined on an optical coherent tomograph RTVue-100 (USA); the volume of focal losses of retinal ganglion cells (FLV) was determined. An increase in FLV above the normative base of the device was regarded as an OCT-sign of retinal neurodegeneration. According to the results of OCT, the participants of the first and second groups were additionally divided into 4 subgroups: 1A – patients without vascular changes in the fundus and without OCT signs of retinal neurodegeneration (n = 12); 1B – patients without vascular changes in the fundus and with the presence of OCT signs of retinal neurodegeneration (n = 28); 2A – patients with initial non-proliferative DR and without OCT signs of retinal neurodegeneration (n = 10); and 2B – patients with initial non-proliferative DR and with OCT signs of retinal neurodegeneration (n = 30). The levels of IL-1β, IL-10, TGF-β3, MMP-7, TIMP-2, protein S100 b, BDNF, and NGF in tear fluid were determined by enzyme-linked immunosorbent assay. Levels of IL-1β and IL-10 in tear fluid in all subgroups were comparable to controls throughout the study. TGF-β3 content in the tear fluid of patients in the group with initial signs of non-proliferative DR (group 2) was significantly (p = 0.001) lower compared with control and group 2. However, there was no significant difference (p > 0.05) between subgroups A and B within groups. The concentration of MMP-7 in the tear fluid in all subgroups was significantly lower than in the control (p < 0.05). However, in the subgroups with OCT signs of retinal neurodegeneration (1B and 2B), the deficiency of this metalloproteinase was more pronounced (p = 0.0001). The levels of the neuropeptides under study NGF, BDNF and S100 B in tear fluid did not differ from controls in all subgroups.

*Keywords: neurodegeneration, cytokines, neuropeptides, matrix metalloproteinases, diabetic retinopathy, tear*

## Introduction

Currently, the influence of metabolic disorders that occur in diabetes mellitus, on the neurosensory apparatus of the retina, is being actively discussed. According to the results of both our research and a number of studies by other authors, diabetic retinopathy (DR) can be considered not only as a vascular disease, but also as a neurodegenerative process [1, 2]. At the same time, damage to retinal neurons can lead to visible vascular changes and reduced visual function.

In the pathogenesis of the development of retinal neurodegeneration in diabetic retinopathy, one of the basic roles is played by a violation of the balance between damaging and neuroprotective factors [3]. We identified systemic disorders in the levels of cytokines, matrix metalloproteinases and their inhibitors, and neuropeptides in patients with retinal neurodegeneration against the background of type 2 diabetes mellitus, and their role as predictors of the development of this process was also determined.

Study of the composition of the tear fluid is used to determine the effect of local immunity on the development of eye diseases [4]. So the change in the content of various biologically active substances in the tear were found in patients with diabetic retinopathy had a correlation with its severity [5]. However, complex studies studying the composition of tears in diabetic retinopathy are few.

**The aim of the study** is to determine the levels of IL-1β, IL-10, TGF-β3, MMP-7, TIMP-2, protein S100b, BDNF and NGF in the tear fluid of patients with vascular and neurodegenerative manifestations of diabetic retinopathy.

## Materials and methods

The study included 80 patients with an endocrinologist-verified diagnosis of type 2 diabetes mellitus, who, after an ophthalmological examination, were divided into 2 groups: group 1 – 40 patients who did not have clinical signs of diabetic retinopathy on the fundus; and group 2 – 40 patients with initial signs of non-proliferative diabetic retinopathy (the presence of single microhemorrhagia and microaneurysms on the fundus). All participants in the main group took oral hypoglycemic drugs. The average experience of diabetes was 7.5 years. The level of glycated hemoglobin averaged 7.7%. The control group consisted of 30 practically healthy volunteers comparable in sex and age with the main group. Sex distribution in the main group: men 42.5% (n = 34), women 57.5% (n = 46), average age 60.8±6 years. All persons participating in the study provided informed consent. The study was approved by the ethics committee of the Pacific State Medical University (of 16.12.2019 protocol No. 4).

All those included in the study were examined on an optical coherent tomograph RTVue-100 (USA); the volume of focal losses of retinal ganglion cells (FLV) was determined. An increase in FLV above the normative base of the device was regarded as an OCT-sign of retinal neurodegeneration. According to the results of OCT, the participants in the first and second groups were additionally divided into 4 subgroups: 1A – patients without vascular changes in the fundus and without OCT signs of retinal neurodegeneration (n = 12); 1B – patients without vascular changes in the fundus and with the presence of OCT signs of retinal neurodegeneration (n = 28); 2A – patients with initial non-proliferative DR and without OCT signs of retinal neurodegeneration (n = 10); and 2B – patients with initial non-proliferative DR and with OCT signs of retinal neurodegeneration (n = 30).

The levels of IL-1β, IL-10, TGF-β3, MMP-7, TIMP-2, protein S100b, BDNF and NGF in the tear fluid were determined using specific reagents from R&D Diagnostics Inc. (USA) by the sandwich version of the solid-phase enzyme-linked immunosorbent assay, according to the attached instructions. Recording of results was performed using the enzyme-linked immunosorbent assay "Multiscan" (Finland). Quantification of the measured parameters was expressed in pg/mL or ng/mL.

Clinical-instrumental and laboratory examination of patients of the main group was carried out at the initial treatment, as well as after 6 months.

Statistical processing of the results obtained was carried out using the SPSS Statistics 23 program (IBM, USA). Indicators are presented in the form of medians (Me), as well as lower and upper quartiles ($Q_{0.25}$-$Q_{0.75}$). Comparison of quantitative values in unrelated samples was carried out using the Mann–Whitney U test. The Wilcoxon T-test was used in the bound samples. The Spearman rank coefficient was used for correlation analysis. The differences were considered significant at $p \leq 0.05$. The sensitivity and specificity of changes in the studied indicators were assessed by linear regression with the construction of ROC curves.

## Results and discussion

The results of a laboratory study of the tear fluid of the study participants are presented in Table 1.

Levels of IL-1β and IL-10 in tear fluid in all subgroups were comparable to controls throughout the study. Given the fact that in other studies serum levels of these cytokines were altered in patients with retinal neurodegeneration in diabetic retinopathy, it is possible that IL-1β and IL-10 play a role. In these

**TABLE 1. LEVELS OF THE STUDIED INDICATORS IN THE TEAR FLUID OF THE EXAMINED CONTINGENT, Me ($Q_{0.25}$-$Q_{0.75}$)**

| Index | Subgroup 1A (n = 12) | Subgroup 1B (n = 28) | Subgroup 2A (n = 10) | Subgroup 2B (n = 30) | Control (n = 30) |
|---|---|---|---|---|---|
| **IL-1β pg/mL primary** | 1.62 (1.23-1.95) | 1.79 (1.49-2.09) | 1.67 (1.39-1.76) | 1.86 (1.39-1.98) | 1.78 (1.57-2.09) |
| **IL-1β pg/mL after 6 months** | 1.66 (1.21-1.88) | 1.84 (1.49-2.07) | 1.79 (1.33-1.72) | 1.81 (1.27-2.08) | |
| **IL-10 pg/mL primary** | 15.21 (10.33-17.85) | 15.19 (10.02-18.95) | 16.01 (10.32-18.25) | 15.68 (10.12-17.71) | 16.33 (10.04-18.87) |
| **IL-10 pg/mL after 6 months** | 16.12 (10.45-18.13) | 15.91 (10.65-17.78) | 15.89 (10.43-18.93) | 16.11 (10.15-18.19) | |
| **TGF-β3 pg/mL primary** | 102.24 (79.14-131.00) | 99.7 (81.38-115.99) | 70.82 (37.54-101.50)* | 74.8 (35.68-91.87)* | 98.49 (84.19-112.35) |
| **TGF-β3 pg/mL after 6 months** | 103.33 (73.23-119.86) | 100.52 (77.14-117.39) | 71.89 (38.23-92.6)* | 67.33 (34.13-96.34)* | |
| **MMP-7 ng/mL primary** | 1.88 (1.71-2.66)* | 1.34 (1.11-2.16)* # | 1.95 (1.82-2.62)* | 1.29 (1.15-2.11)* # | 2.74 (2.56-2.95) |
| **MMP-7 ng/mL after 6 months** | 1.86 (1.77-2.65)* | 1.38 (1.09-2.11)* # | 1.96 (1.77-2.75)* | 1.32 (1.13-2.14)* # | |
| **TIMP-2 pg/mL primary** | 0.15 (0.09-0.23) | 0.16 (0.12-0.26) | 0.13 (0.07-0.21) | 0.17 (0.12-0.24) | 0.12 (0.07-0.23) |
| **TIMP-2 pg/mL after 6 months** | 0.18 (0.12-0.26) | 0.13 (0.09-0.23) | 0.16 (0.11-0.27) | 0.15 (0.09-0.21) | |
| **S100B pg/mL primary** | 12.55 (3.29-17.56) | 11.61 (4.29-16.16) | 13.11 (4.33-18.41) | 11.65 (5.09-17.33) | 13.19 (3.14-17.15) |
| **S100B pg/mL after 6 months** | 11.53 (3.11-18.42) | 11.23 (5.01-16.12) | 13.77 (4.11-19.21) | 11.37 (5.51-17.94) | |
| **NGF pg/mL primary** | 8.54 (6.23-11.79) | 9.02 (6.23-11.96) | 9.13 (6.78-12.03) | 9.01 (6.14-11.98) | 8.14 (5.99-12.02) |
| **NGF pg/mL after 6 months** | 8.56 (6.28-12.05) | 9.05 (6.25-11.85) | 9.16 (6.66-12.05) | 9.01 (6.18-12.01) | |
| **BDNF pg/mL primary** | 10.14 (6.61-15.32) | 10.25 (6.98-14.93) | 10.64 (7.01-15.33) | 10.05 (6.98-15.17) | 10.31 (6.91-14.79) |

Note. *, significant difference with control group ($p < 0.05$); #, reliable difference between groups A and B within groups ($p < 0.05$).

patients, it would be more indicative in the study of intraocular fluids.

The content of TGF-β3 in the tear fluid of patients in the group with initial signs of non-proliferative DR (group 2) was significantly lower ($p = 0.001$) compared with the control and group 2. However, the presence of OCT signs of retinal neurodegeneration did not affect this indicator, which shows the absence of a significant difference ($p > 0.05$) between subgroups A and B within the groups. TGF-3 β under physiological conditions increases the survival of endothelial cells and pericytes in the vessels of the retina [6]. It is possible that a local decrease in TGF-β3 potentiates the damaging effect of chronic hyperglycemia on retinal vessel cells.

The concentration of MMP-7 in the tear fluid in all subgroups was significantly lower than in the control ($p < 0.05$). However, in the subgroups with OCT signs of retinal neurodegeneration (1B and 2B), the deficiency of this metalloproteinase was more pronounced ($p = 0.0001$). A decrease in the activity of MMP-7 at the systemic and local level in patients with diabetes mellitus was found in many studies [7]. According to some reports, MMP-7 is involved in the activation of some neuroproteins, and a decrease in its activity can lead to a decrease in the neurotrophic support of retinal neurons and potentiates their apoptosis [8, 9].

The content of TIMP-2 in the tear fluid in all subgroups was comparable to control both during the initial examination and after 6 months.

In contrast to the expected results, the levels of the neuropeptides NGF, BDNF and S100B in the tear fluid did not differ from the control in all subgroups. Since these proteins are actively involved in maintaining the vital activity of retinal neurons, it may be more indicative to study their level in intraocular fluids [8].

Correlation analysis showed a significant negative relationship between FLV and the concentration of MMP-7 in the tear fluid ($r = -0.3\ 38$, $p = 0.02$), the level of which was reduced in subgroups with retinal neurodegeneration. However, ROC analysis showed unsatisfactory model quality (AUC = 0, 47) for this indicator as a prognostic marker for the development of retinal neurodegeneration. There was a positive correlation between the levels of MMP-7 and TGF-β3 in the tear fluid ($r = 0.311$, $p = 0.03$).

## Conclusion

This study showed a decrease in local levels of MMP-7 and TGF-β3 in the tears of patients with diabetic retinopathy. The presence of vascular symptoms had an effect on the level of TGF-β3. In turn, patients with OCT signs of retinal neurodegeneration showed greater deficiency of MMP-7. Despite the existence of an association between FLV and MMP-7 levels. in the tear, this laboratory indicator for ROC analysis does not have sufficient conjugation with retinal neurodegeneration and cannot be used as a marker of this process.

Thus, further study of the state of the local level of various factors of innate immunity in patients with diabetic retinopathy is required to search for predictors of the development of vascular and neuronal retinal lesions, which will provide a basis for personalized management and treatment of these patients.

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# ДИНАМИКА ПОКАЗАТЕЛЕЙ КЛЕТОЧНОГО ИММУНИТЕТА ПРИ КОМПЛЕКСНОМ ЛЕЧЕНИИ ОСТРОГО ОПТИЧЕСКОГО НЕВРИТА

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**Резюме.** Увеличение частоты развития оптического неврита среди населения трудоспособного возраста, а также неутешительный прогноз для зрения ввиду развития атрофии зрительного нерва определяет высокую социальную значимость данной проблемы. Целью работы является анализ влияния «Имунофана» на показатели клеточного иммунитета и клинические симптомы болезни в комплексном лечении оптического неврита, ассоциированного с герпесвирусной инфекцией. В исследовании приняли участие 37 человек (37 глаз) с острым оптическим невритом, ассоциированным с герпесвирусной инфекцией. Схема лечения включала назначение раствора дексаметазона по убывающей схеме, 1% раствора препарата «Эмоксипин» 0,5 мл и 12,5% раствора препарата «Дицинон» 0,5 мл через ирригационную систему, имплантированную в ретробульбарное пространство, в комбинации с назначением лекарственных средств нейропротекции («Пикамилон» и «Семакс») в течение 10 дней. Все пациенты были разделены на 2 группы. Основная группа – 20 пациентов, которым в схему лечения был добавлен препарат «Имунофан». Группа сравнения – 17 пациентов, лечение которых проводили только по вышеописанной методике. Курс лечения составил 10 дней. Анализ данных продемонстрировал более значимую положительную динамику показателей клеточного иммунитета, получавших иммунотерапию. Наши исследования показали эффективность данного препарата в комплексном лечении оптического неврита, ассоциированного с герпесвирусной инфекцией, что подтверждено ускорением купирования воспаления, более значимым повышением зрительных функций у пациентов, получавших «Имунофан», и меньшим процентом развития атрофии зрительного нерва. В этой группе пациентов в более ранние сроки произошли и оставались стабильными на протяжении всего срока наблюдения изменения показателей клеточного звена иммунитета. По нашим данным, меж-

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групповая оценка иммунорегуляторного индекса показала его более быстрое нарастание у пациентов группы сравнения, получавших препарат «Имунофан», и достигала нормальных значений уже спустя 6 месяцев после лечения. Клиническая эффективность препарата «Имунофан» в комплексной терапии оптического неврита, ассоциированного с герпесвирусной инфекцией, характеризовалась сокращением сроков купирования признаков воспаления в зрительном нерве в 2 раза и более, увеличением максимально корригированной остроты зрения 4,5 раза, снижением частоты возникновения рецидивов оптического неврита в 2 раза при сроках наблюдения 12 месяцев.

*Ключевые слова: оптический неврит, герпесвирусная инфекция, кортикостероидная терапия, иммуномодулирующая терапия, показатели клеточного иммунитета, имунофан*

# DYNAMICS OF CELLULAR IMMUNITY INDICATORS IN THE COMPLEX TREATMENT OF ACUTE OPTIC NEURITIS

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**Abstract.** An increase in the incidence of optic neuritis among the working-age population, as well as an unpromising prognosis for vision due to the development of optic nerve atrophy, determines the high social significance of this problem. The aim of the work is to analyze the effect of Imunofan at the parameters of cellular immunity and clinical symptoms of the disease in the complex treatment of optic neuritis associated with herpes virus infection. The study involved 37 people (37 eyes) with acute optic neuritis associated with herpes infection. The treatment regimen included the appointment of a dexamethasone solution according to a decreasing scheme, a 1% solution of the drug Emoxipin 0.5 mL and a 12.5% solution of the drug Dicynone 0.5 mL through an irrigation system implanted in the retrobulbar space, in combination with the neuroprotection drugs (Pikamilon and Semax) for 10 days. All patients were divided into 2 groups. The main group consisted of 20 patients who received Imunofan to the treatment regimen in addition. The comparison group included 17 patients who were treated only according to the method described above. The course of treatment lasted 10 days. The analysis of the data showed a more significant positive dynamics of cellular immunity parameters in those who received immunotherapy. Our studies showed the effectiveness of this drug in the complex treatment of optic neuritis associated with herpes infection, what is confirmed by the acceleration of inflammation relief, a more significant increase in visual functions of patients treated with Imunofan, and a lower percentage of optic nerve atrophy. In this group of patients, changes in the parameters of the cellular link of immunity occurred earlier and remained stable throughout the entire period of observation. According to our data, an intergroup assessment of the immunoregulatory index showed its faster increase in patients of the comparison group who received Imunofan, and reached normal values already 6 months after treatment. The clinical effectiveness of Imunofan in the complex therapy of optic neuritis associated with herpes infection was characterized by a reduction in the period of relief of signs of inflammation in the optic nerve by 2 times or more, by an increase in the maximum corrected visual acuity by 4.5 times, and by a decrease in the incidence of recurrence of optic neuritis by 2 times over a 12 months observation period.

*Keywords: optic neuritis, herpes virus infection, corticosteroid therapy, immunomodulatory therapy, indicators of cellular immunity, imunofan*

## Introduction

According to the literature, optic neuritis (ON) reaches 30-40% in the structure of inflammatory diseases of the visual pathway [1]. An increase in the incidence of ON among the working-age population and a disappointing prognosis for vision due to the development of optic nerve atrophy (ONA) determines the high social significance of this problem [1].

Along with CNS diseases, including autoimmune damage to the optic nerve, acute and chronic bacterial and viral infections, including herpesvirus infection (HVI) [5, 6, 10, 11], are of great importance in the etiology of ON.

HVI causes changes in the systemic and local immune status, which is determined by the characteristics of the antigenic structure of the pathogen, the level of antigenic load, the production of antibodies, and the rate of elimination of the antigen by the immune system.

Glucocorticoid therapy is the main and rapid way to block immune-mediated inflammation mechanisms in the optic nerve, but at the same time it can lead to a weakening of the immune defense and increased replication of the herpes simplex virus [3, 4, 13].

In connection with the immune imbalance, the possible formation of resistance to antiviral chemotherapy, most authors adhere to the principles of complex treatment for ophthalmic herpes and the appointment of etiotropic chemotherapy and immunocorrective therapy [4, 8, 9].

**The aim of the work** is to analyze the effect of Imunofan in the complex treatment of ON associated with HVI at the parameters of cellular immunity and clinical symptoms of the disease.

## Materials and methods

We examined 37 people (37 eyes) with acute unilateral ON associated with HVI, aged 17 to 36 (average 26.5) years. Inflammation of the optic nerve proceeded in the form of intraocular neuritis.

The clinical study did not include patients with ON within the background of multiple sclerosis and other neurodegenerative diseases of the CNS, Devic's disease and severe concomitant somatic diseases.

According to the results of laboratory serological studies of blood serum by ELISA method in the process of clarifying the etiological diagnosis of ON IgM antibodies to herpes simplex virus were identified in 23% of cases, in all 37 patients HVI was confirmed with an increase in the titer of IgG antibodies to herpes simplex virus by 4-5 times.

The avidity index of IgG antibodies at the time of admission to the hospital in 23 (61.5%) patients with ON was 31-49%, which indicated that they had a late stage of primary infection; in the remaining 14 patients (38.5%) the avidity index IgG antibodies ranged from 56 to 72% and indicated a chronic persistent HVI.

The standard treatment regimen included the introduction for 10 days through an irrigation system implanted in the retrobulbar space of Dexamethasone solutions in a decreasing pattern (course dose of 60 mg), 1% solution of Emoxipin 0.5 mL and 12.5% Dicynone 0.5 mL [13].

Antiviral chemotherapy with Acyclovir (orally 0.4 mg 5 times a day) was prescribed 2-3 days after receiving positive laboratory results of the study for the presence of HVI. In 7 patients with concomitant chronic inflammatory pathology of the upper respiratory tract, the treatment was supplemented with intravenous injections of the antibiotic Ciprofloxacin 100 mg daily for 7 days.

The patients were divided into 2 observation groups: the 1st main group (n = 20), which standard treatment was supplemented by intramuscular injections of Imunofan 50 mcg daily for 10 days. 2nd comparison group (n = 17), which patients received standard treatment without Imunofan. The formed groups were comparable in terms of sex, age, severity of the inflammatory process in the optic nerve ($p > 0.05$).

Ophthalmological examination methods included: visometry (sign projector Carl Zeiss Jena, Germany); ophthalmoscopy (indirect non-contact lens with a lens of 90 diopters) with the calculation according to the severity of the clinical signs of the inflammatory process in the optic nerve in scores of the total clinical inflammation index (TCII), which is characterized by a score of the degree of severity of the ophthalmoscopy picture (0 – no symptom; 1 – mild; 2 – moderately expressed; 3 – sharply expressed); static computer perimetry (Humphrey, Germany). The morphological status of the optic disc (OD) was studied by optical coherence tomography (OCT) (Cirrus HD-OKT 4000, Carl Zeiss Meditec AG, Germany), the thickness of the peripapillary retinal nerve fiber layer (RNFL) was measured.

Immunophenotyping of lymphocytes was performed with an assessment of $CD3^+$, $CD4^+$, $CD8^+$, $CD22^+$. The studies were carried out on the basis of the Research Institute for the Protection of Motherhood and Childhood in Khabarovsk. The results of an immunological blood test, obtained at the regional blood transfusion station in 20 healthy people who donated blood for the first time, were taken as normal indicators.

Cellular immunity testing was performed before the start of treatment, 1.5 months after treatment, then after 6 and 12 months of observation.

Statistical analysis of the obtained results was carried out using the computer program Microsoft Excel with the identification of the significance of the difference according to the Student's criterion.

## Results and discussion

When evaluating the results of immunoenzymatic typing of lymphocytes in patients of both groups, a deficiency in the absolute and relative content of $CD3^+$ lymphocytes and $CD4^+$ lymphocytes was initially determined. In turn, most of the examined patients (75%) had an increase in the absolute and relative content of $CD8^+$ and $CD22^+$ lymphocytes.

Comparative dynamics of clinical-functional and morphometric parameters in patients with ON with various treatment methods is shown in Table 1.

Analysis of the data presented in Table 1 showed that after 10 days of inpatient treatment, more sig-

nificant positive dynamics was noted in the clinical course of ON in patients of the main group. Thus, the best corrected visual acuity (BCVA) by this observation period increased by 4.5 times against the initial one, while in patients of the comparison group it increased only by 3.25 times ($p < 0.05$).

In the comparison group, 10 days after treatment, the thickness of RNFL was 116.32±4.51 μm, which is statistically significantly higher than that of the main group – 96.5±3.4 μm ($p < 0.05$).

One month after treatment, in patients of the main group, the RNFL thickness indicator reached values that was not significantly different from that of the intact eye ($p > 0.05$), while in the comparison group, only by the 3rd month of observation. At the same time, it should be noted that in 4 patients of the comparison group, by the 3rd month of observation, the thickness of the RNFL tended to decrease by 10-15 μm compared with the intact eye, which indicated the development of partial optic nerve atrophy (ONA).

A more rapid completion of the inflammatory process in the optic nerve in patients of the main group compared to the comparison group was also evidenced by the dynamics of the decrease in TCII. So, after 1 month of observation, TCII in patients who did not receive immunotherapy still remained on average level of 4.5±0.1 points, in patients of the main group, TCII was significantly lower (1.9±0.1 points), while after 2 months after treatment, clinical signs of inflammation of the OD were already completely absent in patients of both observation groups.

At the end of 12 months after treatment, the majority of patients (85%) of the main group had stable results. Only 1 patient in this group showed a trend towards a decrease in BCVA by 0.1-0.2 of the previously achieved level. Ophthalmoscopically, these patients were diagnosed with decoloration of the temporal half of the OD, according to OCT, a decrease in the thickness of RNFL by 8-10 μm, which was regarded by us as evidence of the development of postneuritic partial ONA, which was later confirmed by the results of electrophysiological studies.

In the comparison group, 12 months after treatment, partial ONA occurred in 4 cases (23.5%), which was supported by the data of ophthalmoscopy, OCT and electrophysiological studies. This condition was accompanied by a decrease in BCVA by 0.2-0.4 from the previously achieved level. Only one patient in the comparison group had a relapse of ON and one case of acute ON in the fellow eye in the absence of such cases in the main group.

Table 2 shows the indicators of cellular immunity of patients in groups.

Analyzing the data in Table 2, it can be seen that in patients with OH who received Imunofan as part of complex treatment, the indicators of cellular immunity normalized already 6 months after treatment and remained stable throughout the entire observation period, while in patients who did not receive immunotherapy, data results were achieved only 12 months after treatment. Intergroup assessment of the immunoregulatory index showed its faster increase in patients of the main group.

An analysis of the literature data points to the huge role of the herpesvirus family in the development of human infections common in nature [2, 4, 9, 10, 12].

The etiological role of HVI in the occurrence of ON is insufficiently presented in the ophthalmological literature, which significantly limits the possibilities of effective therapy. There is an opinion that in patients with infectious and inflammatory diseases of herpetic etiology, due to concomitant secondary immunodeficiency, antiviral chemotherapy without restoring the adaptive-compensatory capabilities of the immune system is ineffective. In such clinical situations, according to researchers, a positive result

**TABLE 1. DYNAMICS OF FUNCTIONAL AND MORPHOMETRIC INDEXES IN DIFFERENT METHODS OF IMMUNOTHERAPY, M±m**

| Index | Before treatment | After treatment | | | | Intact eye control |
|---|---|---|---|---|---|---|
| | | 10 days | 1 month | 3 months | 12 months | |
| **Main group, n = 20 people** | | | | | | |
| **BCVA** | 0.16±0.02 | 0.63±0.02 | 0.72±0.03 | 0.75±0.02 | 0.77±0.03 | 1.00±0.05 |
| **Thickness of PRNFL, μm** | 129.5±5.5 | 96.5±3.4* | 92.3±2.1* | 88.5±5.2* | 87.2±5.1 | 87.5±5.6 |
| **TCII, points** | 13.1±0.3 | 3.7±0.2* | 1.9±0.1* | – | – | |
| **Comparison group, n = 17 people** | | | | | | |
| **BCVA** | 0.14±0.02 | 0.52±0.05* | 0.65±0.01* | 0.72±0.05 | 0.71±0.03 | 1.00±0.05 |
| **Thickness of PRNFL, μm** | 130.7±3.3 | 116.32±45.00 | 110.0±3.2 | 90.1±4.9 | 87.3±3.2 | 87.5±5.6 |
| **TCII, points** | 13.2±1.4 | 6.5±0.1 | 4.5±0.1 | – | – | |

Note. *, reliability of intergroup differences $p < 0.05$; BCVA, best corrected visual acuity; PRNFL, peripapillary retinal nerve fiber layer; TCII, total clinical inflammation index.

**TABLE 2. INDEXES OF CELLULAR IMMUNITY OF PATIENTS DEPENDING ON THE THERAPY**

| Index | Before Treatment | After treatment | | | Control group |
|---|---|---|---|---|---|
| | | 1.5 months | 6 months | 12 months | |
| **Main group, n = 20 people** | | | | | |
| T lymphocytes (CD3), % | 61.95±0.08# | 68.72±0.71* | 68.98±0.34* | 68.88±0.11* | 70.02±1.41 |
| T helpers (CD4), % | 30.25±0.16# | 33.01±0.14# | 38.01±0.32* | 37.82±0.10* | 38.41±1.22 |
| T cytotoxic (CD8), % | 27.91±0.02# | 25.99±0.05* | 24.97±0.02# | 25.72±0.07 | 25.94±0.05 |
| IRI | 1.05±0.01# | 1.38±0.02# | 1.45±0.01* | 1.44±0.02 | 1.46±0.05 |
| **Comparison group, n = 17 people** | | | | | |
| T lymphocytes (CD3), % | 61.28±0.04# | 64.21±0.29* # | 65.04±0.09* # | 65.91±0.04* # | 70.02±1.41 |
| T helpers (CD4), % | 31.06±0.23# | 33.16±0.32# | 34.23±0.19* # | 33.70±0.06* # | 38.41±1.22 |
| T cytotoxic (CD8), % | 28.00±0.08# | 26.40±0.02* | 24.21±0.03* # | 25.28±0.03 | 25.94±0.05 |
| IRI | 1.19±0.21# | 1.22±0.01* # | 1.37±0.01* # | 1.35±0.02 | 1.46±0.05 |

Note. *, statistically significant differences between the compared groups; #, statistically significant differences from the control group, $p < 0.05$; IRI, immunoregulatory index.

of antiviral etiotropic therapy can only be guaranteed by immunomodulatory therapy [4, 8, 10, 11].

There are a few works in the literature concerning the use of Imunofan in ophthalmology in the treatment of endogenous uveitis in adults [6] and children. However, we have not found any works on the use of Imunofan in the treatment of patients with optic neuritis of HVI etiology in the available literature.

Our studies have shown the effectiveness of this drug in the complex treatment of ON associated with HVI, which was confirmed by the acceleration of inflammation relief, a more significant increase in visual functions in patients treated with Imunofan, and a lower percentage of ONA. In this group of patients, changes in the parameters of the cellular link of immunity occurred earlier and remained stable throughout the entire period of observation. According to our data, an intergroup assessment of the immunoregulatory index showed its faster increase in patients of the comparison group who received Imunofan, and reached normal values already 6 months after treatment.

## Conclusion

The clinical effectiveness of Imunofan in the complex therapy of ON associated with HVI was characterized by a reduction in the period of relief of inflammation signs in the optic nerve by 2 times or more, by an increase in the BCVA by 4.5 times, and by a decrease in the incidence of recurrence of ON by 2 times over a 12 months observation period.

Restoration of cellular immunity indicators was detected already by the 3rd month of observation.

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# ОСОБЕННОСТИ ПОКАЗАТЕЛЕЙ КЛЕТОЧНОГО ИММУНИТЕТА В ЗАВИСИМОСТИ ОТ АКТИВНОСТИ ОЧАГОВ ДЕМИЕЛИНИЗАЦИИ У ДЕТЕЙ С РАССЕЯННЫМ СКЛЕРОЗОМ

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**Резюме.** РС является распространенным заболеванием центральной нервной системы, которое приводит к инвалидизации и снижению качества жизни. Дебют заболевания у 3-5% пациентов приходится на детский возраст и имеет менее благоприятное течение, по сравнению со взрослыми. РС вызывается активацией аутореактивных эффекторных Т-клеток при срыве периферической толерантности, которая в норме контролируется Т-регуляторными клетками (Treg). Перспективным является исследование экспрессии эктонуклеотидаз CD39 и CD73 в популяциях Treg и Th17 для оценки их супрессивной активности. Цель – оценить содержание основных и малых популяций лимфоцитов и экспрессию эктонуклеотидаз CD39 и CD73 в популяции $CD4^+$ лимфоцитов у детей с РС. Обследовано 111 детей с РС, 66 детей – с контраст негативными очагами на МРТ (1-я группа), 45 детей – с контраст позитивными очагами (2-я группа). Группу сравнения составили 46 условно здоровых детей, сопоставимых по возрасту (3-я группа). Содержание Т-лимфоцитов, В-лимфоцитов, NK-лимфоцитов, Treg ($CD4^+CD25^{high}CD127^{low}$), Thact ($CD4^+CD25^{high}CD127^{high}$), Th17 клеток ($CD3^+CD4^+CD161^+$); экспрессию CD39 и CD73 в Treg, Th17 и Thact проводили методом проточной цитофлуориметрии. Выявлено увеличение содержания Т-хелперов, снижение NK-клеток у пациентов во 2-й группе. Получено увеличение количества Thact и Th17 лимфоцитов у пациентов обеих групп с РС. Количество Treg в 1-й группе было достоверно выше, чем в 3-й группе. Соотношение клеток с экспрессией CD39 и CD73 у пациентов с РС зависело от популяции лимфоцитов также, как и в 3-й группе. Наибольшее содержание $CD39^+$ клеток отмечалось в популяции Treg, а наименьшее в популяции Thact. Для экспрессии CD73 наоборот, наибольшая экспрессия CD73 наблюдалась в Thact клетках, наименьшая в Treg. При

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сравнении групп пациентов получено, что у пациентов 1-й группы было достоверно увеличено количество клеток, экспрессирующих эктонуклеотидазу CD39, а количество supTh17 было сравнимо с 3-й группой. В обеих группах пациентов с РС наблюдалось увеличение количества CD73 в Treg, Thact и Th17. Таким образом, выявлены информативные популяции лимфоцитов ($CD4^+$ клетки, Treg, $CD39^+$Treg, supTh17), которые могут быть использованы для мониторинга состояния детей с рассеянным склерозом.

*Ключевые слова: популяции лимфоцитов, $CD4^+$ лимфоциты, Treg, Th17, Thact, CD39, CD73, рассеянный склероз, дети*

# FEATURES OF PARAMETERS OF CELLULAR IMMUNE DEPENDING ON THE ACTIVITY OF FOCI OF DEMYELINATION IN CHILDREN WITH MULTIPLE SCLEROSIS

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**Abstract.** MS is a common disease of the central nervous system that leads to disability and reduced quality of life. The debut of disease in 3-5% of patients occurs in childhood and has a less favorable course compared to adults. MS is caused by the activation of autoreactive T cells in the breakdown of peripheral tolerance, which is normally controlled by regulatory T cells (Tregs). It is promising to study expression of CD39 and CD73 in Treg and Th17 populations to assess their suppressive activity. Aim is to evaluate content of major and minor lymphocyte populations and expression of CD39 and CD73 in $CD4^+$ lymphocyte population in children with MS. 111 children with MS were examined, 66 with contrast-negative lesions on MRI (Group 1), 45 with contrast-positive lesions (Group 2). The comparison group consisted of 46 healthy children (Group 3). Content of T, B, NK lymphocytes, Treg ($CD4^+CD25^{high}CD127^{low}$), Thact ($CD4^+CD25^{high}CD127^{high}$), Th17 cells ($CD3^+CD4^+CD161^+$); expression of CD39 and CD73 in Treg, Th17 and Thact was performed by flow cytometry. An increase in content of T helpers, a decrease in NK cells in patients in group 2 was revealed. An increase in number of Thact and Th17 lymphocytes was obtained in patients of both groups with MS. Number of Tregs in group 1 was significantly higher than in group 3. Ratio of cells expressing CD39 and CD73 in MS patients depended on lymphocyte population as well as in the group 3. The highest content of $CD39^+$ cells was observed in Treg population, and the lowest in Thact population. For CD73 expression, on the contrary, the highest expression of CD73 was observed in Thact cells, the lowest in Treg. When comparing groups of patients, it was found that in patients of group 1, number of cells expressing CD39 ectonucleotidase was significantly increased, and number of supTh17 was comparable with group 3. In both groups of MS patients, an increase in CD73 counts in Treg, Thact and Th17 was observed. Thus, informative populations of lymphocytes ($CD4^+$ cells, Treg, $CD39^+$Treg, supTh17) have been identified, which can be used to monitor condition of children with multiple sclerosis.

*Keywords: lymphocytes subsets, $CD4^+$ subsets, Treg, Th17, Thact, CD39, CD73, multiple sclerosis, children*

## Introduction

Multiple sclerosis (MS) is a chronic, demyelinating disease, which is based on a complex of autoinflammatory and neurodegenerative processes leading to multiple focal and diffuse lesions of the central nervous system (CNS), resulting in disability of patients. There are about 2.8 million people with MS in the world. Since 2013, the prevalence of MS has increased in all regions of the world, in Russia it ranges from 51 to 100 cases per 100,000 population [15]. There are 2 main forms of MS: relapsing-remitting (RR) and primary progressive. The onset of MS disease in 3-5% of patients begins before the age of 16 [8]. MS in children is different from the disease in adults and has its own characteristics. Children are less likely to develop primary or secondary progressive multiple sclerosis; 98% of children with MS have a RR form of the course. Children have more frequent relapses and disability occurs 10 years earlier than adults [1, 8].

The etiopathogenesis of MS is not fully understood. It is believed that the disease occurs in a genetically determined individual under the influence of adverse

environmental factors [5]. There are over 200 genes associated with MS. Understanding of the process and the significance of certain genes is proven only for a few associated loci, such as variants of HLA-A*02:01 and HLA-DRB1*15:01, as well as genes encoding the α-chains of IL-2 and IL-7 receptors [11]. MS is caused by the activation of peripheral autoreactive effector T cells (Teff), which migrate to the CNS and initiate the pathological process [5]. Most Teff is discarded in the thymus (central tolerance), a small amount of Teff is released to the periphery. Normally, regulatory T cells (Treg) control peripheral tolerance mechanisms by downregulating the activity of various cell types, including Teff, cytotoxic $CD8^+$T cells, and antigen presenting cells (APCs), through intercellular contact and the secretion of suppressor cytokines [10].

When peripheral tolerance is disrupted, which involves genetic factors, environmental factors, including infectious agents (Epstein-Barr virus) and microbiota, cell clones are formed that, due to molecular mimicry, are capable of causing damage in the CNS [12, 14]. After entering the CNS, Teff ($CD8^+$T cells, Th1 and Th17 cells) and B lymphocytes activate cellular and humoral immunity reactions, secreting cytokines that cause the activation of CNS resident immune cells (microglia, astrocytes, macrophages), which also produce cytokines, amplifying functions of APC [8].

It is known that Teff in the CNS, in addition to Tregs, can be regulated by other regulatory cells: type 1 T regulatory cells (Tr1), $CD8^+$Tregs, NK cells, and B regulatory cells. Thus, an increase in the number of NK cells is associated with remission with effective therapy with daclizumab and IFNβ [3]. In patients with MS, it has been shown that Treg, as well as Tr1, have a reduced suppressor activity in relation to the inhibition of Teff proliferation [3]. The suppressor activity of Tregs can be assessed by the amount of CD39 and CD73 ectonucleotidase expressed on their surface.

CD39 and CD73 are enzymes expressed on the surface of immune cells that sequentially dephosphorylate pro-inflammatory ATP to adenosine, which has anti-inflammatory properties. To date, there are conflicting data regarding the expression of CD39 in Treg in patients with MS. Some studies have shown that the frequency of $CD39^+$ cells in the Treg population is reduced [4], in others, on the contrary, both their content and ATPase activity are increased, regardless of immunomodulatory treatment [2, 7]. In this regard, **the aim of this study** is to evaluate the content of major and minor lymphocyte populations, as well as the expression of CD39 and CD73 in the $CD4^+$ lymphocyte population in children with MS.

## Materials and methods

We examined 111 children with MS at the age of 16 (14.2-17.5) years. MS patients were divided into groups based on clinical and anamnestic data and magnetic resonance imaging (MRI) results: group 1 – patients with contrast-negative demyelination foci (without active foci), n = 66; Group 2 – with contrast-positive foci of demyelination (with active foci), n = 45. We also examined 46 conditionally healthy children, comparable in age and with no deviations in the results of standard clinical and biochemical blood tests. The examination of all groups of children was carried out in accordance with the ethical and regulatory documents of the Russian Federation. The study was approved by the local ethical committee. Before the study, informed consent was obtained from the parents in accordance with the Declaration of Helsinki. Venous blood samples for the study were obtained by taking from the cubital vein on an empty stomach in BDVacutainer® tubes with $K_2EDTA$ anticoagulant.

The content of the main and small populations of lymphocytes, as well as the study of the number of lymphocytes with the expression of CD39 and CD73 ectonucleotidase, was carried out by laser flow cytometry (Novocyte, ACEA Biosciences, USA). A panel of monoclonal antibodies conjugated to various fluorochromes was used: CD4-FITC (cat. A07750, Beckman Coulter, USA), CD127-PE (cat. IM 10980U, Beckman Coulter, USA), CD25-PC7 (cat. A52882, Beckman Coulter, USA), CD161-PE (cat. IM 3450, Beckman Coulter, USA), CD3-PC5 (cat. A07749, Beckman Coulter, USA), CD39-APC-Cy7 (Clone A1, cat. RT2241130 Sony Biotechnology, USA), CD73-APC-Cy7 (Clone AD2, cat. RT2320110, Sony Biotechnology, USA). The number of cells with $CD39^+$ and $CD73^+$ in Treg ($CD4^+CD25^{high}CD127^{low}$), activated T helpers (Thact – $CD4^+CD25^+CD127^{high}$), Th17 lymphocytes ($CD4^+CD161^+CD3^+$) was assessed using stepwise gating. To isolate Tregs and Thacts carrying CD39 and CD73, first, the “lymphoid” region was isolated according to the parameters of direct (FSC) and side (SSC) light scattering, $CD4^+$ positive lymphocytes were isolated, among $CD4^+$ cells, Tregs were isolated by markers ($CD4^+CD25^{high}CD127^{low}$) and Thact ($CD4^+CD25^{high}CD127^{high}$). Within the selected Treg and Thact regions, the percentage of cells carrying CD39 and CD73 was evaluated. Within the isolated region of Th17 cells ($CD3^+CD4^+CD161^+$), the number of cells expressing CD39 and CD73 was also evaluated.

The obtained data were statistically processed using the Statistica 10.0 program (StatSoft; USA). Descriptive statistics of quantitative traits are presented in the format: median (lower and upper quartiles) – Me ($Q_{0.25}$-$Q_{0.75}$). The significance of differences between groups was assessed using the nonparametric Mann–Whitney U test. Differences were considered statistically significant at $p < 0.05$.

## Results and discussion

The content of lymphocyte populations in patients with MS in groups without active foci and with active foci was assessed in comparison with apparently healthy children (Table 1). A significant increase in the relative content of T lymphocytes due to the population of T helpers was found in patients with active foci compared with healthy children. The content of NK cells was significantly reduced in patients with active lesions compared with apparently healthy children and compared with patients without active lesions.

In patients with MS in both groups, the content of small helper populations was also significantly increased. An increase in the population of Thact lymphocytes and an increase in the absolute number of Th17 lymphocytes were obtained. It is interesting to note that the number of Tregs in patients without active lesions was significantly higher than in the group of apparently healthy children. While in patients with active foci, the number of Treg was significantly lower than the comparison group.

As mentioned above, both Treg lymphocytes and NK cells are involved in the regulation of autoreactive lymphocyte clones in MS patients. In our study, in patients without active lesions, the number of Treg significantly increased and the number of NK lymphocytes was increased relative to patients with active lesions, which is a good prognostic sign for patients with MS [3].

**TABLE 1. RELATIVE AND ABSOLUTE CONTENT OF MAJOR AND MINOR POPULATIONS OF LYMPHOCYTES IN CHILDREN WITH MS COMPARED WITH HEALTHY CHILDREN, Me ($Q_{0.25}$-$Q_{0.75}$)**

| Parameter | Patients without active foci n = 66 | Patients with active foci n = 45 | Comparison group n = 46 |
|---|---|---|---|
| CD3, % | 74.6 (69.4-78.8) p = 0.14 | 76.9 (72.4-80.3) p = 0.003 | 72.4 (67.9-75.3) |
| CD3, cells/μL | 1302 (986-1744) p = 0.014 | 1344 (1134-1794) p = 0.089 | 1617 (1237-1850) |
| CD4, % | 39.8 (37.0-48.6) p = 0.103 | 43.3 (39.3-48.9) p = 0.004 | 38.4 (35.2-44.6) |
| CD4, cells/μL | 730 (535-945) p = 0.036 | 804 (616-1094) p = 0.52 | 857 (717-1016) |
| CD8, % | 26.4 (22.2-31.0) p = 0.42 | 25.9 (23.3-30.3) p = 0.45 | 26.3 (24.1-31.9) |
| CD8, cells/μL | 500 (337-633) p = 0.01 | 473 (383-630) p = 0.013 | 618 (415-804) |
| B lymphocytes, % | 13.2 (10.7-17.5) p = 0.44 | 12.2 (9.2-17.4) p = 0.17 | 13.5 (12.1-17.5) |
| B lymphocytes, cells/μL | 239 (189-367) p = 0.012 | 215 (137-319) p = 0.003 | 304 (252-370) |
| NK, % | 11.9 (8.9-16.7) p = 0.62 | 9.4 (6.5-12.0)* p = 0.012 | 14.2 (8.3-17.2) |
| NK, cells/μL | 212 (133-316) p = 0.046 | 180 (102-270)* p = 0.000 | 263.9 (177-402) |
| Treg, % from CD4 | 9.3 (7.5-10.9) p = 0.024 | 3.7 (3.1-4.6) p = 0.004 | 7.7 (7.2-9.4) |
| Treg, cells/μL from CD4 | 63 (48-86) p = 0.46 | 76 (53-95) p = 0.09 | 67.3 (54-89) |
| Thact, % from CD4 | 21.7 (15.5-27.3) p = 0.000 | 18.9 (12.9-24.9) p = 0.000 | 14.1 (9.9-16.5) |
| Thact, cells/μL from CD4 | 149 (104-196) p = 0.016 | 153 (92-207) p = 0.014 | 117.1 (83-139) |
| Th17, % from CD4 | 21.2 (16.9-25.7) p = 0.000 | 20 (15.8-25.6) p = 0.004 | 17.1 (14.0-19.4) |
| Th17, cells/μL from CD4 | 151 (109-188) p = 0.81 | 167 (131-208) p = 0.21 | 150.2 (118-172) |

Note. *, p < 0.05 between groups of patients with MS.

In children with MS, an analysis of the number of cells expressing CD39 and CD73 ectonucleotidase showed that the content of populations with the highest and lowest number of $CD39^+$ and $CD73^+$ cells in lymphocyte populations is comparable to that in conditionally healthy children [13]. Thus, the highest content of $CD39^+$ cells was noted in the Treg population and amounted to Me-34.5 (29.5-44.6) %, and the smallest number in the Thact population was Me-3.5 (1.8-5.7) %. The reverse situation was observed for CD73 expression. The highest expression of CD73 was in Thact cells -Me-21.9 (16.4-28.9) %, and the lowest in Treg -Me-13.2 (9.6-20.9) %.

An analysis of the expression of ectonucleotidase in the populations of Th17, Thact and Treg lymphocytes in patients without active foci (n = 42), with active foci (n = 26) and in the group of apparently healthy children (n = 34) is shown in Figure 1.

When comparing the expression of ectonucleotidase in groups of patients with MS with a group of apparently healthy children, a significant increase in cells expressing CD39 in Treg in patients without

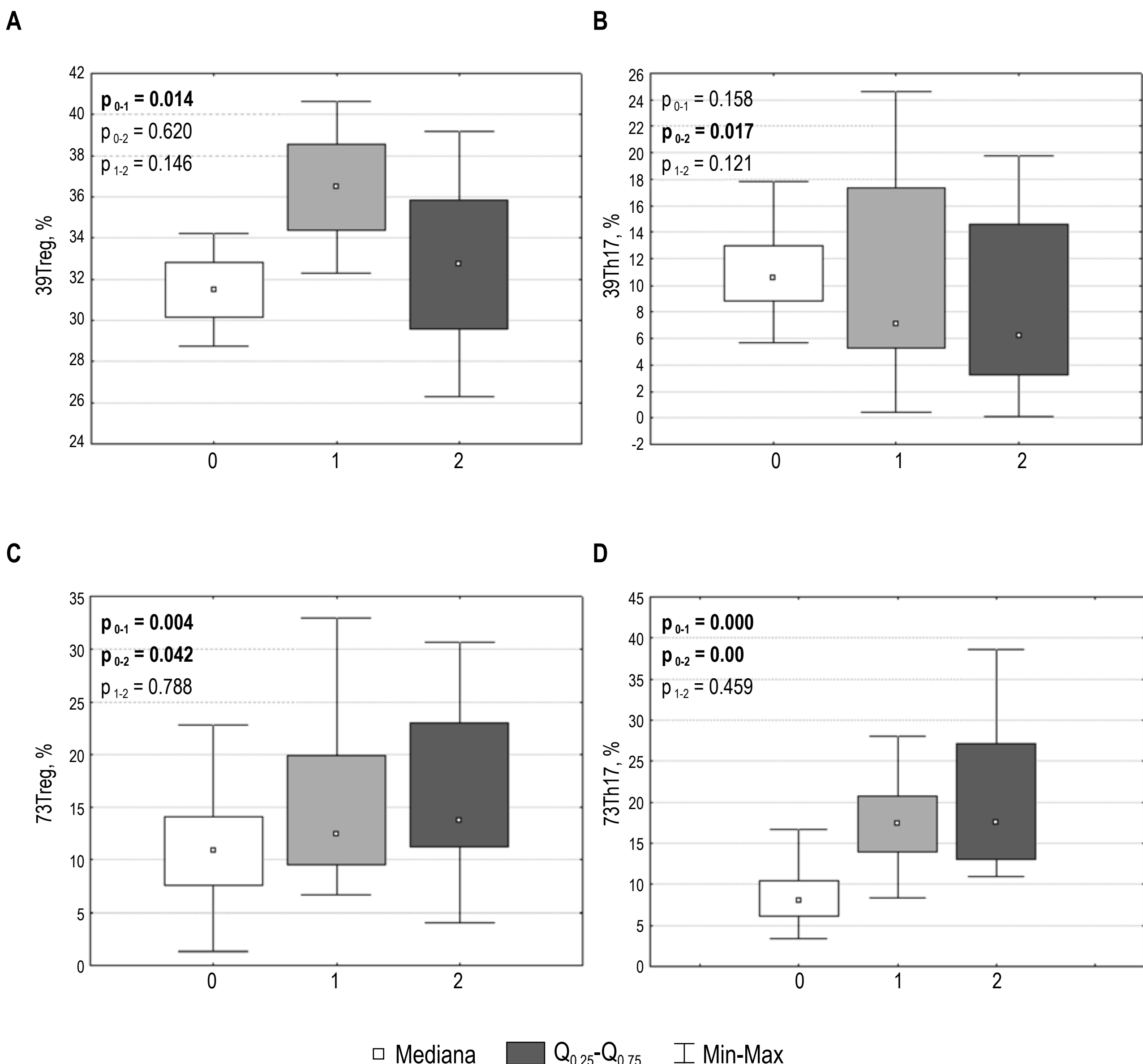


**Figure 1. Relative content of CD39 and CD73 in the populations of Th17 lymphocytes and Treg in patients with MS of the group of apparently healthy children**

Note. 0, group of apparently healthy children; 1, group of MS patients without active lesions; 2, group of MS patients with active lesions. p, reliability between groups of patients and conditionally healthy children. (A) % of Treg expressing CD39. (B) % of Th17 expressing CD39. (C) % of Treg expressing CD73. (D) % of Th17 expressing CD73.

active foci was shown (Figure 1A). The number of CD39+Th17 lymphocytes was significantly reduced in patients with MS with active lesions compared with apparently healthy children (Figure 1B). As for patients without active lesions, the number of Th17 expressing CD39+ was comparable to that of apparently healthy children. It is known that the Th17 population expressing CD39 ectonucleotidase (supTh17) has suppressor properties [6]. Thus, in children with MS in the group with active foci, a decrease in the content of supTh17 was revealed. For Thact, a significant decrease in the number of cells expressing CD39 was found in patients of both groups with MS, and in the group of patients with active foci, this decrease was more significant ($p_{0-1}$ = 0.000, $p_{0-2}$ = 0.000). At the same time, the expression of CD73 in the populations of Treg (Figure 1C), Thact ($p_{0-1}$ = 0.000, $p_{0-2}$ = 0.000) and Th17 (Figure 1D) was significantly higher in both groups of patients relative to the control group.

The greatest differences in the content of the main and small populations of lymphocytes in children with MS relative to conditionally healthy children were found in patients with active foci of demyelination according to MRI and consisted in an increase in the content of T lymphocytes, T helpers and a decrease in the content of NK cells. More significant differences were revealed when assessing small populations of lymphocytes, namely: a decrease in Treg in patients with active foci, while an increase in this regulatory population was detected in patients without active foci. In patients without active foci, in addition to an increase in the number of Treg, the content of cells expressing CD39 ectonucleotidase increased, and the amount of supTh17 was comparable with the group of conditionally healthy children. As for the increase in CD73 ectonucleotidase in Treg, Thact, and Th17 in patients with active foci, this can be regarded as a compensatory mechanism in response to a decrease in the regulatory populations of lymphocytes – Treg, NK cells, and supTh1.

## Conclusion

Thus, informative populations of lymphocytes (CD4+ cells, Treg, CD39+Treg, supTh17) have been identified, which can be used to monitor the condition of children with multiple sclerosis.

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# МЕТАЛЛОПРОТЕИНАЗА-9 И ЕЕ РОЛЬ В ПАТОГЕНЕЗЕ АЛЛЕРГИЧЕСКИХ ЗАБОЛЕВАНИЙ У ДЕТЕЙ

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**Резюме.** Металлопротеиназы (ММР) играют значимую роль в механизмах поддержания хронического воспаления и ремоделирования тканей. Изучение изменений концентрации этих ферментов в сыворотке крови у детей с аллергопатологией представляет большой практический и научный интерес.

Цель – изучить роль ММР-9 в патогенезе аллергических заболеваний у детей. 180 детей в возрасте от 1 года до 18 лет прошли комплексное клинико-лабораторное обследование. В это исследование были включены пациенты, страдающие бронхиальной астмой (ВА) (n = 54), атопическим дерматитом (AD) (n = 54) и сочетанием этих патологий (n = 72). Уровни ММР-9 в сыворотке крови определяли методом иммуноферментного анализа с использованием тест-систем Cloud-CloneCorp® (США). Результаты клинических, инструментальных и лабораторных исследований пациентов были соотнесены с данными ИФА-исследования уровня ММР-9 в сыворотке крови.

Анализ полученных данных показал, что среди пациентов с установленным диагнозом «БА» максимальная концентрация этого цитокина была зарегистрирована у детей со среднетяжелым течением заболевания. Проведенный корреляционный анализ показал наличие значимой корреляции между тяжестью БА и уровнем контроля над заболеванием (r = 0,63). Аналогичные данные были получены у пациентов с сочетанием БА и АД. У детей этой группы также наблюдалось значительное повышение уровня ММР-9 в сыворотке крови по сравнению со здоровыми пациентами (p = 0,015). Максимальные показатели были зарегистрированы у пациентов со среднетяжелым течением БА. Концентрация этой матриксной металлопротеиназы в сыворотке крови была несколько выше у детей с поливалентной сенсибилизацией, чем у пациентов с моноаллергической этиологией заболевания (p = 0,272). Показатели ММР-9 у пациентов только с кожными проявлениями атопии были достоверно выше, чем в контрольной группе (p = 0,025). При этом не было никаких различий в зависимости от пола. Было установлено, что максимальные значения ММР-9 были зарегистрированы среди детей с подростковой формой АД.

Полученные нами данные показали, что у всех обследованных нами пациентов наблюдалось значительное повышение уровня ММР-9 в сыворотке крови, что указывает на важную роль этого цитокина в патогенезе аллергических заболеваний у детей.

*Ключевые слова: металлопротеиназа-9, бронхиальная астма, атопический дерматит, диагностика, патогенез, дети*

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# METALLOPROTEINASE 9 AND ITS ROLE IN THE PATHOGENESIS OF ALLERGIC DISEASES IN CHILDREN

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**Abstract.** Metalloproteinases (MMP) play a significant role in the mechanisms of maintaining chronic inflammation and tissue remodeling. The study of concentration changes in these enzymes in the blood serum of children with allergopathology is of great practical and scientific interest.

Objective: to study the role of MMP-9 in the pathogenesis of allergic diseases in children. 180 children aged from 1 to 18 years passed a comprehensive clinical and laboratory examination. This study included patients suffering from bronchial asthma (BA) (n = 54), atopic dermatitis (AD) (n = 54) and combination of these pathologies (n = 72). Serum levels of MMP9 were determined by enzyme immunoassay using Cloud-CloneCorp® test systems (USA).

The analysis of the obtained data showed that among patients with the established diagnosis of BA, the maximum concentration of this cytokine was registered in children with a moderate course of the disease. The conducted correlation analysis showed the presence of a significant correlation between the severity of asthma and the level of control over the disease (r = 0.63). Similar data was obtained in patients with a combination of BA and AD. In children of this group, there was also a significant increase in serum MMP-9 compared with healthy patients (p = 0.015). The concentration of this matrix metalloproteinase in serum was slightly higher among children with polyvalent sensitization than in patients with monoallergic etiology of the disease (p = 0.272). The values of MMP-9 in patients with only skin manifestations of atopy were significantly higher than in the control group (p = 0.025).

The data we obtained showed that all the patients we examined had a significant increase in the level of MMP-9 in the blood serum, which indicates an important role of this cytokine in the pathogenesis of allergic diseases in children.

*Keywords: metalloproteinase 9, bronchial asthma, atopic dermatitis, diagnostic, pathogenesis, children*

## Introduction

Allergy is a specific immune reaction of the body to an allergen which is accompanied by the development of chronic inflammation and tissue damage. Unfortunately, allergic diseases belong to the group of the most common pathology of childhood and they are considered in modern society as a major medical and social problem [12]. The frequency of allergic diseases, according to different authors, varies widely, and atopic diseases such as asthma and atopic dermatitis are in the first place (they affect up to 20% of the population individually or in various combinations) in most industrialized countries.

Allergic diseases are chronically recurrent inflammatory diseases caused primarily by IgE-mediated allergic reactions and genetically associated with atopy [4]. However, the pathogenesis of allergic diseases is multifaceted and represents a rather complex system. In recent years, special attention has been paid to the study of non-inflammatory mechanisms underlying allergic diseases. It was found that metalloproteinases (MMP) play a significant role in the mechanisms of maintaining chronic inflammation and tissue remodeling [10]. For example, MMP-9 is involved in the processes of inflammation, repair and mobilization of matrix-related growth factors and cytokine processing. Substrates for MMP-9 include denatured type I collagen (gelatin), native collagens of types IV, V, VII, X and XI, fibrinogen, vitronectin, IL-1, and entactin which connects laminin and type IV collagen. Keratinocytes, monocytes, leukocytes, macrophages, and fibroblasts are sources of MMP-9. Basal levels of MMP-9 are usually low, it s expression can be induced by various cytokines/chemokines, including TNFα (tumor necrosis factor-alpha), and MMP-9 mainly secreted by inflammatory cells. The regulation of inflammation by gelatinases is carried out by cytokine/chemokine processing, since MMP-9 has a stimulating effect, and MMP-2 has an inhibitory effect on inflammation. The promoter region of MMP-9 has some functional binding sites of enhancers, such as NF-κB and AP-1 sites. These sites make MMP-9 capable of inducing Pro-inflammatory cytokines, especially TNFα, which is a key mediator in the pathogenesis and maintenance of chronic inflammation [1]. This gelatinase triggers a cascade of reactions of degradation of the extracellular matrix having the ability to destroy denatured collagen.

MMP-9 acts synergistically with other metalloproteinases in this case. This type of MMP can interact with various matrix proteins through its fibronectin-

like fragment being in the pericellular space. MMP-9 contributes to the creation of feedback between the state of the matrix and potential activation of MMP [1]. Therefore, the study of changes in the concentration of this enzyme in children with allergopathology represents the large practical and scientific interest.

**Objective:** to study the role of MMP-9 in the pathogenesis of allergic diseases in children.

## Materials and methods

180 children aged from 1 to 18 years were examined. This study included patients suffering from bronchial asthma (n = 54), atopic dermatitis (n = 54) and combination of these pathologies (n = 72). The average age of the examined patients was 11.44±4.67 years. The control group consisted of 56 children of the I and IIa health groups comparable in gender and age, without clinical manifestations of allergic diseases and positive allergy history.

The diagnosis of bronchial asthma (BA) was made on the basis of the National program "Bronchial asthma in children. Strategy of treatment and prevention" (2017) [13]. The diagnosis of atopic dermatitis (AD) was verified on the basis of the clinical recommendations "Atopic dermatitis in children" (2016) [4].

The criteria for inclusion in this study were: the presence of a confirmed diagnosis of BA, AD or a combination of these diseases, the absence of concomitant chronic pathology from other organs and systems, (age under 18 years), Russian nationality, the presence of signed patient (aged over 15 years) or parents (for children under 15 years) informed consent to conduct the study.

Exclusion criteria: the presence of previously established chronic and acute diseases of the bronchopulmonary system (tuberculosis, acute tracheobronchitis, pneumonia, etc.) or skin, the age of patients older than 18 years.

All children passed a comprehensive clinical and laboratory examination on the basis of the pediatric Department of the clinic of the Rostov State Medical University. Serum levels of MMP-9 were determined by enzyme immunoassay using Cloud-CloneCorp® test systems (USA). The results of clinical, instrumental and laboratory studies of patients were correlated with the data of the ELISA study of the level of MMP-9 in blood serum.

The study was conducted in compliance with all ethical standards set out in WAME (the World Association of Medical Editors) and approved by the Local Ethics Committee of Rostov State Medical University.

Statistical processing of results was performed by using Microsoft Office Excel 2003 and Statistica 12.0 for Windows software package.

## Results and discussion

Our study found that the values of MMP-9 in the examined patients with allergopathology in blood serum significantly exceeded the parameters set in the control group (Table 1).

The analysis of the obtained data showed that among patients with the established diagnosis of BA, the maximum concentration of this cytokine was registered in children with a moderate course of the disease 714.93 pg/mL (517.37-902.51), while with mild it was 569.0 pg/mL (377.79-825.29), and with severe – 2.89 pg/mL (121.35-362.91) (Figure 1).

The conducted correlation analysis showed the presence of a significant correlation between the severity of asthma and the level of control over the disease (r = 0.63). An inverse correlation was established between the concentration of MMP-9 and the severity of BA (r = -0.53).

Similar data was obtained in patients with a combination of BA and AD. Thus, in children of this group, there was also a significant increase in serum MMP-9 (596.285±82.169 pg/mL) compared with healthy patients (307.391±42.394 pg/mL) (p = 0.015). At the same time, the maximum indica-

**TABLE 1. MMP-9 INDICATORS IN THE BLOOD SERUM OF THE EXAMINED PATIENTS**

| Diagnosis | MMP-9 indicators in blood serum, ng/mL | p |
|---|---|---|
| **BA** | 489.20±59.52<br>431.76 (306.15-612.93) | $p_{1,4}$ = 0.023<br>$p_{2,4}$ = 0.042<br>$p_{3,4}$ = 0.019 |
| **AD** | 573.75±113.71<br>445.04 (266.43-790.06) | |
| **BA + AD** | 596.29±82.17<br>473.02 (339.33-687.26) | |
| **Control group** | 307.39±42.39<br>276.05 (160.33-397.02) | |

Note. $p_{1,4}$, significance of differences between BA and control; $p_{2,4}$, significance of differences between AD and control; $p_{3,4}$, significance of differences between BA + AD and control.

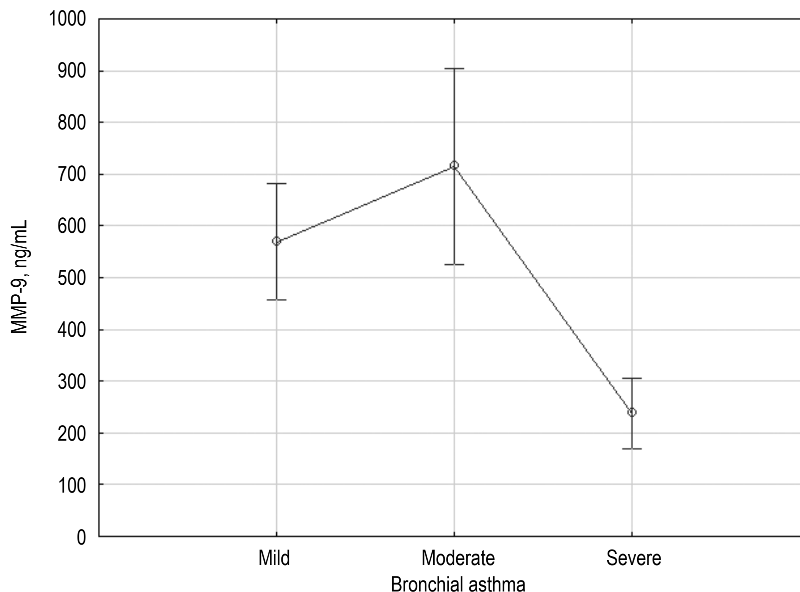


**Figure 1. Concentration of MMP-9 in blood serum depending on the severity of BA**

tors were registered in patients with moderate course of the disease (Table 2).

At the same time the MMP-9 values had no statistically significant differences between children with controlled (566.12 (424.94-687.26) pg/mL) and uncontrolled (376.55 (241.04-815.64) pg/mL) course of the disease ($p = 0.269$).

However, it is interesting to note that the concentration of this matrix metalloproteinase in serum was slightly higher (645.52±75.04 pg/mL) among children with polyvalent sensitization than in patients with monoallergic etiology of the disease (462.65±118.89 246.20 pg/mL) ($p = 0.272$).

The values of MMP-9 in patients with only skin manifestations of atopy were significantly higher (573.75±113.71 pg/mL) than in the control group (307.39±42.39 pg/mL) ($p = 0.025$). At the same time there were no differences depending on gender: the average values for girls were 538.09±119.64 or 385.24, while for boys – 680.73±324.17 pg/mL.

Analysis of indicators based on the severity of the disease showed that the patients in all three groups had a significant variation of MMP-9 indicators in blood serum. Meanwhile there were no significant differences in severity ($p \geq 0.05$).

However, there is a clear tendency to increase the concentration of MMP-9 in the blood serum of patients with changes in age-related forms of the disease. It was found that the maximum values of MMP-9 were registered among children with adolescent AD (1163.04±120.17 pg/mL), while with children the average values were 342.56±50.73, and with infants (924.71±124.74 pg/mL).

The results of our research are consistent with the data obtained by our foreign colleagues. Ko F.W. et al. conducted a study of the level of MMP-9 in patients with various degrees of BA severity and healthy patients. Scientists have found that uncontrolled moderate and severe asthma are associated with greater activity of proteolytic enzymes and, therefore,

**TABLE 2. DISTRIBUTION OF MMP-9 INDICATORS IN THE BLOOD SERUM OF PATIENTS WITH AD AND BA DEPENDING ON THE SEVERITY OF AD, M±Err**

| Severity of BA | MMP-9 indicators, pg/mL | p |
|---|---|---|
| **Mild BA** | 567.54±91.72<br>472.08 (424.32-598.46) | $p_{1,2} = 0.682$<br>$p_{1,3} = 0.042$<br>$p_{2,3} = 0.019$ |
| **Moderate BA** | 694.87±132.98<br>572.39 (413.77-776.06) | |
| **Severe BA** | 222.47±18.81<br>235.87 (185.33-246.20) | |

Note. $p_{1,2}$, significance of differences between mild and moderate BA; $p_{1,3}$, significance of differences between mild and severe BA; $p_{2,3}$, significance of differences between moderate and severe BA

despite a similar inflammation of the respiratory tract, this can play a significant role in the remodeling of the bronchi and accelerate the process of reducing lung function in these patients [10].

Maria P. Foschino Barbaro and her colleagues studied the concentration of MMP-9, pH, NO level in exhaled air, as well as inflammatory cells in sputum in patients suffering from BA. The results of the study showed a significant increase in the exhaled MMP-9 in patients compared to the control. It was noted that the maximum concentrations of MMP-9 were observed in patients with severe BA, compared with patients suffering from mild and moderate forms of the disease. An increase in MMP-9 was most frequently recorded in patients with neutrophilic inflammation of the respiratory tract. A correlation was also established between the exhaled MMP-9 and the percentage of neutrophils in sputum, FEV1, exhaled NO and PH. The obtained results indicate a significant role of MMP-9 in the pathogenesis of airway remodeling in asthma, and also suggest that monitoring MMP-9 in exhaled air can help not only to monitor the current airway remodeling, but also to recognize severe forms of BA in order to determine the appropriate choice of therapy in time [2]. This fact is confirmed by the research conducted by Grzela K. et al. [7, 9] it is important to note that an increase in the concentration of MMP-9 in exhaled air is accompanied by an increase in its concentration in the blood serum of patients with BA [3, 10].

A study conducted by the Bureau of Devillers A.C. and co-authors also found that MMP-9 plays an important role in maintaining allergic inflammation in AD. It has been shown that patients suffering from AD have a significant increase in the level of MMP-9 in the blood serum compared to the control group [5].

In the work of Harper J.I. and co-authors it was shown that MMP, and in particular MMP-9, represent an important potential component of AD pathology. Studies of washouts from AD-affected skin areas found that in samples obtained from patients, the activity of MMP is 10-24 times greater than in normal control skin ($p < 0.02$) and five times greater than in areas of unaffected AD skin taken from patients with this disease. A number of studies conducted by the author (gelatin cymography and analysis of the antibody array) revealed significant levels of MMP-9 in samples obtained in sick children, while lower levels of MMP-10 and tissue metalloproteinase inhibitors were observed, as well as low levels of MMP-1 (fibroblast collagenase), MMP-3 (stromelizin 1) and TIMP-4. The obtained research results once again prove the high significance of MMP in the pathogenesis of AD [8].

## Conclusion

The data we obtained showed that all the patients we examined had a significantly increase in the level of MMP-9 in the blood serum, which indicates an important role of this cytokine in the pathogenesis of allergic diseases in children.

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# РЕЗУЛЬТАТЫ ТЕРАПИИ ХРОНИЧЕСКОЙ КРАПИВНИЦЫ У ПАЦИЕНТОВ С IgE-ЗАВИСИМЫМ И IgE-НЕЗАВИСИМЫМ ПРОФИЛЕМ ЗАБОЛЕВАНИЯ

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**Резюме.** Главным механизмом возникновения крапивницы является дегрануляция тучных клеток. Доказано, что вне зависимости от пути активации клинические проявления не будут отличаться. По данным литературы до половины случаев хронической спонтанной крапивницы имеют аутоиммунный характер, могут сочетаться с аутоиммунной патологией щитовидной железы, СКВ и др. и имеют более тяжелое течение. В терапии традиционно используются антигистаминные препараты в стандартных или увеличенных дозировках. Однако часть пациентов не реагирует на проводимое лечение даже при кратном увеличении доз.

В терапии резистентной к традиционному лечению антигистаминными препаратами крапивницы рекомендовано применение генно-инженерной таргетной терапии препаратом Омализумаб. Целью исследования было определение профиля пациентов с хронической крапивницей (ХК) и сравнение эффективности лечения препаратом Омализумаб у пациентов с IgE-зависимой (1-я группа) и IgE-независимой (2-я группа) крапивницей. Обследован 81 пациент с хронической крапивницей (60 взрослых, 21 ребенок). Пациенты до начала терапии имели длительный стаж ХК: от 1 года до 20 лет. Все пациенты до начала таргетной терапии получали лечение антигистаминными препаратами в стандартных и кратно увеличенных дозах, однако контроля получено не было. Повышение уровня сывороточного IgE выявлено в 51,7% случаев у взрослых и 42% у детей. Сопутствующая сенсибилизация определялась у 48,3% взрослых, и 76,2% детей. У детей наиболее распространенной была пищевая, эпидермальная и пыльцевая сенсибилизация. У взрослых чаще встречалась пыльцевая и эпидермальная сенсибилизация. Уровень эозинофилии в 1-й группе был более выражен, чем во 2-й группе – 302,6 и 116,4 клеток/мкл (U = 61,5; p = 0,0097). Через 6 месяцев в 1-й группе отмечено улучшение балла симптомов (UCT) с 3,1 баллов ДИ (1,5-4,6) до 12,2 ДИ (10,8-13,7), (p = 0,0001). Во 2-й группе улучшение симптомов с 0,63 баллов ДИ (0,36-1,6), до 8,1 ДИ (5-11,2) через 6 месяцев. После 6 месяцев генно-инженерной биологической терапии (ГИБТ) полный контроль над симптомами ХК в 1 группе получен у 66,7% больных, частичный – у 33,7%. Во второй группе в 33,3% случаев положительных результатов лечения добиться не удалось. Таким образом, ГИБТ препаратом Омализумаб повышает контроль над течением ХК. Результаты лечения выше у пациентов с IgE-зависимым профилем заболевания.

*Ключевые слова: хроническая крапивница, омализумаб, IgE, биологическая терапия, эозинофилы, UCT-тест*

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# RESULTS OF THERAPY OF CHRONIC URTICARIA IN PATIENTS WITH IgE-DEPENDENT AND IgE-INDEPENDENT DISEASE PROFILE

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**Abstract.** The main mechanism for the occurrence of urticaria is the degranulation of mast cells. It has been proven that, regardless of the activation pathway, clinical manifestations will not differ. According to the literature, up to half of cases of chronic spontaneous urticaria are autoimmune in nature, can be combined with autoimmune thyroid disease, SLE, etc., and have a more severe course.

In therapy, antihistamines are traditionally used. However, some patients do not respond to the treatment, even with a multiple increase in doses. In the treatment of urticaria resistant to traditional antihistamines, the use of Omalizumab is recommended. The purpose of the study: to determine the profile of patients with chronic urticaria, as well as to evaluate the effectiveness of treatment with Omalizumab in patients with IgE-dependent and IgE-independent chronic urticaria.

Eight-one patients with chronic urticaria (60 adults, 21 children) were examined. Patients before the start of therapy had a long history of CU: from 1 to 20 years. Patients before the start of therapy were treated with antihistamines, but no control was obtained. An increase in the level of serum IgE was detected in 51.7% of cases in adults and 42% in children. Concomitant sensitization was determined in 48.3% of adults and 76.2% of children. In children, food, epidermal and pollen sensitization was the most common. Pollen and epidermal sensitization were more common in adults. The level of eosinophilia in the group with IgE-dependent was more pronounced than in other group ($p = 0.0097$). After 6 months, the group with IgE-dependent showed an improvement in the symptom score (UCT) from 3.1 CI (1.5-4.6) to 12.2 CI (10.8-13.7), ($p = 0.0001$). In other group, symptoms improved from 0.63 CI (0.36-1.6) to 8.1 CI (5-11.2) after 6 months (no control). After 6 months of genetically engineered biological therapy (GIBT), complete control over the symptoms of CU in group 1 was obtained in 66.7% of patients, partial – in 33.7%. In the second group, in 33.3% of cases, positive treatment results could not be achieved. Thus, genetically engineered biological therapy with Omalizumab increases the control over the course of CU. Treatment outcomes are higher in patients with an IgE-dependent disease profile.

*Keywords: chronic urticaria, omalizumab, IgE, biological therapy, eosinophils, UCT test*

## Introduction

Chronic spontaneous urticaria is a disease lasting more than 6 weeks, characterized by the appearance of blisters and/or angioedema, passing without a trace within 24 hours. Several pathways of mast cell activation have been proven. However, the mechanisms by which urticaria occurs are not fully understood. It has been shown that, regardless of the activation pathway, clinical manifestations in all types will be the same.

Mast cell degranulation may be associated with activation of high-affinity receptors (FcεRI) by immunoglobulin E or autoreactive anti-IgE or anti-FcεRI IgG, as well as autoreactive IgE [2, 6, 8].

Degranulation can also occur due to disruption of mast cell activation signaling mechanisms, for example, when exposed to complement components or unknown factors [3, 7].

According to the literature, up to half of cases of chronic spontaneous urticaria are autoimmune in nature, can be combined with autoimmune thyroid disease, SLE, etc., and have a more severe course.

In therapy, antihistamines are traditionally used in standard or increased dosages. However, some patients do not respond to the treatment, even with a multiple increase in doses [4].

According to current concepts, genetically engineered biological therapy (GIBT) Omalizumab therapy can be prescribed already on the second line [1]. And although this therapy is not disease-modifying, it is widely used to achieve disease control and improve the quality of life. The duration of therapy has not yet been determined, but should be at least 6 months. Early termination of therapy is not recommended, because there is a risk of losing patients with a late response. Even long-term therapy with Omalizumab does not eliminate the risk of relapse after treatment is stopped. The drug is characterized by a good safety profile and high efficacy.

**The purpose of the study:** to determine the profile of patients with chronic urticaria, as well as to evaluate the effectiveness of treatment with Omalizumab in patients with IgE-dependent and IgE-independent chronic urticaria.

## Materials and methods

A total of IgE level, peripheral blood eosinophil count, and association with comorbidities were assessed in 81 patients with CU. Of the patients who did not respond to antihistamine therapy at standard and elevated doses, 27 patients received biological therapy with Omalizumab, including 17 women and 10 men. The mean age of patients receiving GIBT was 45.4±12.6. Patients were divided into 2 groups: with IgE-dependent (gr 1; 18 patients) and IgE-independent urticaria (gr 2; 9 patients). All patients were treated with Omalizumab (as a lyophilisate for solution for subcutaneous administration) subcutaneously at a dosage of 300 mg once every four weeks. When dosing the drug, the initial level of IgE was taken into account. Efficacy was assessed after 3 and 6 months of therapy clinically and using the UCT questionnaire, where values of 16 points indicated complete control of the disease, more than 12 points – partial control, and values ≤ 11 – no control of urticaria [11].

Statistical data processing was carried out using generally accepted methods of variation statistics. Methods of nonparametric statistics were used, the Mann–Whitney U test (U), and the cross-tabulation method ($\chi^2$) were used. The critical value of the significance level was taken equal to 5%. The data obtained were processed using the application package AtteStat, version 10.5.1, Statistical formulas of the Microsoft Excel program, version 5.0.

## Results and discussion

Under observation were 81 people with chronic urticaria: 60 adults and 21 children. Among adults, in 34 people (56.7%), urticaria was combined with angioedema, among children, a combination with angioedema was observed in 8 people (38.1%).

In 51.7% of adult patients and 42% of children, an increase in the level of total IgE was not detected. The mean values of serum IgE in patients with elevated values of this indicator did not differ significantly in children and adults and amounted to 405.4 IU/mL CI (205-605.6) and 480.25 IU/mL CI (257.5-703), respectively (U = 144, p = 0.22).

Concomitant sensitization to one or more allergens was detected in 55.6% of patients: in adults in 48.3% of cases (29/60), in children in 76.2% (16/21). In children with CU, food, epidermal and pollen sensitization was most common: 38%, 38%, 33.3%, respectively. In adult patients with urticaria, pollen sensitization was most often noted – 33.3%, and epidermal sensitization – 18.3%.

All patients, before the start of GIBT, according to clinical guidelines, received treatment with antihistamines in standard and multiple-fold increased doses, however, no control was obtained against the background of this therapy. Patients before the start of therapy had a long history of CU: from 1 to 20 years. The average experience of urticaria in group 1 was 3.3 years, CI (2.2-4.4), in group 2 – 6.28, CI (0.13-12.43). The level of eosinophilia in group 1 was more pronounced and averaged 302.6 cells/μL CI (159-445), in group 2 – 116.4 cells/μL CI (65-179), (U = 61.5; p = 0.0097).

In group 1, at the beginning of therapy, the average symptom intensity score according to the UCT questionnaire was 3.1 points CI (1.5-4.6). There was a statistically significant increase in the mean symptom intensity score after 3 months to 10.6 CI (8.6-12.5), (U = 296.5, p = 0.0001), and after 6 months to 12.2 CI (10.8-13.7), (U = 353.5, p = 0.0001) – the disease acquired a well-controlled course. Complete control over the symptoms of urticaria was achieved in 66.7% of patients, partial control – in 33.3% of cases. In some patients who had not previously responded to GCS therapy, an improvement in sensitivity to this group of drugs was noted (Table 1).

In group 2, by the beginning of treatment, the intensity of symptoms according to UCT averaged 0.63 points CI (0.36-1.6), after 3 months of therapy 8.75 CI (6.92-10.6), and after 6 months 8.1 CI (5-11.2). In the group as a whole, the intensity of symptoms according to the UCT questionnaire increased already after 3 months of treatment (U = 64, p = 0.0007), however, in 33.3% of patients, the disease had an uncontrolled course after 6 months of therapy. Complete control over the symptoms of CU after 6 months of GIBT was obtained in 11.1% of cases, partial – in 55.6% of cases.

In a comparative analysis of the effectiveness of GIBT with Omalizumab, in general, in group 1, the positive effect of treatment (disease control) was more pronounced than in group 2 ($\chi^2$ = 7.4; p = 0.0245).

The high efficacy of Omalizumab in urticaria has been shown in numerous studies; it improves the condition of more than half of patients after 2 months. According to the literature, a more pronounced effect of Omalizumab therapy is observed in patients with clinically associated sensitization to

**TABLE 1. DYNAMICS OF THE INTENSITY OF SYMPTOMS OF URTICARIA AFTER 6 MONTHS OF THERAPY WITH OMALIZUMAB**

| | Group 1 | | | Group 2 | | |
|---|---|---|---|---|---|---|
| | before | after | p | before | after | p |
| UCT test | 3.1 points (1.5-4.6) | 12.2 (10.8-13.7) | p = 0.0001 | 0.63 points (0.36-1.60) | 8.1 (5.0-11.2) | p = 0.0040 |

allergens, confirmed by skin prick testing and/or serological methods, the level of eosinophilia more than 260 cells/μL, the duration of treatment with Omalizumab for more than 12 months, which is consistent with our results [5, 9].

## Conclusion

Thus, GIBT with Omalizumab increases the control over the course of CU. The results of treatment are higher in patients with an IgE-dependent disease profile: the presence of a higher level of eosinophilia, the presence of concomitant sensitization, confirmed by serology or skin tests.

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# ПРОФИЛИ ЭКСПРЕССИИ ГЕНОВ ФАКТОРОВ ВРОЖДЕННОГО ИММУНИТЕТА У ПАЦИЕНТОВ С АТОПИЧЕСКИМ ДЕРМАТИТОМ

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**Резюме.** Атопический дерматит – многофакторное генетически детерминированное воспалительное заболевание кожи, характеризующееся зудом, хроническим течением, возрастными особенностями локализации и морфологии очагов поражения. Патогенез атопического дерматита сложен и включает эпигенетические изменения, вовлеченные в геномную адаптацию, реакции иммунного ответа и дисфункцию эпителиального барьера, которые в совокупности запускают развитие этого заболевания. Целью данного исследования является определение уровня экспрессии генов *IL4*, *IL13*, *IL33*, *TLR2*, *TLR9* у пациентов с атопическим дерматитом.

Таргетные гены для дальнейшей оценки экспрессии были выбраны в соответствии с нашими предыдущими результатами полногеномного исследования метилирования ДНК. Нами были определены сигнальные пути с дифференциально метилированными генами, которые, скорее всего, имеют место в патогенезе атопического дерматита. Поэтому мы исследовали уровни экспрессии генов *IL4*, *IL13*, *IL33*, *TLR2*, *TLR9* в коже, мононуклеарных клетках периферической крови, а также в цельной крови с помощью ПЦР-РВ у 55 детей и 26 здоровых людей, а также у 50 пациентов старшего возраста. Статистический анализ проводился с использованием H-критерия Краскела–Уоллиса и U-критерия Манна–Уитни.

Анализ экспрессии генов показал, что в образцах кожи уровень экспрессия *TLR9* и *IL4* был в 12 раз ниже ($p < 0,0001$, $p < 0,0005$) в пораженной коже; а в случае *TLR2* – в 6 раз ($p < 0,01$); результаты для мононуклеарных клеток крови отличались, и уровни экспрессии для тех же цитокинов были значительно выше до лечения. Мы также обнаружили, что эти различия были сильно выражены в старшей возрастной группе (12-18 лет). Изучение экспрессии гена *IL33* в образцах цельной крови у

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взрослых пациентов показало, что его уровень достоверно выше у больных со среднетяжелой формой АтД. Кроме того, мы пришли к выводу, что локально в пораженной коже может доминировать воспалительный иммунный ответ; в мононуклеарных клетках, по-видимому, имеет место Th2-иммунный ответ.

Для понимания патогенеза атопического дерматита необходимо учитывать действие иммунологических факторов, а также связь между ними. Описанные гены и их белковые продукты могут являться потенциальными лекарственными мишенями, а также способствовать формированию тактики ведения пациентов с атопической патологией.

*Ключевые слова: атопический дерматит, цитокины, Toll-подобные рецепторы, метилирование, врожденный иммунитет, экспрессия генов*

# INNATE IMMUNE FACTOR GENE EXPRESSION PROFILES IN PATIENTS WITH ATOPIC DERMATITIS

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**Abstract.** Atopic dermatitis is a multifactorial genetically determined inflammatory skin disease characterized by itching, chronically relapsing dermatitis, age-related features of localization and morphology of lesions. The pathogenesis of atopic dermatitis is complex and includes epigenetic alterations, involved in the genomic adaptation, immune response reactions and dysfunction of the epithelial barrier that together trigger the development of atopic dermatitis. The aim of this study is to detect the expression level for *IL4*, *IL13*, *IL33*, *TLR2*, *TLR9* genes in the biological materials of atopic patients.

The targeted genes for further expression evaluation were selected according to our previous findings on genome-wide methylation study. We detected the cascades with the differentially methylated genes that are most likely to take place in atopic dermatitis. Thus, we investigated expression levels for the *IL4*, *IL13*, *IL33*, *TLR2*, *TLR9* genes in the skin, peripheral blood mononuclear cells and whole blood cells using RT-PCR on 55 pediatric patients and 26 healthy volunteers, and on 50 adult patients. Statistical analysis was performed with the use of Kruskal–Wallis H test and Mann–Whitney U test. Targeted expression analysis revealed that in the skin samples the expression of *TLR9* and *IL4* was 12 times significantly lower ($p < 0.0001$, $p < 0.0005$) in the lesional skin; and there was a 6-fold decrease in case of *TLR2* ($p < 0.01$). The results for blood mononuclear cells differed and expression levels for most of the assessed targets were significantly higher before treatment. We have also found out that those differences were strongly pronounced especially in an elder age group (12-18 y.o.). Studying the *IL33* gene expression in the whole blood samples of adults revealed that its level was significantly higher in case of patients with moderate form of AD. Besides, we concluded that locally in the affected skin inflammatory immune response may dominate; in the mononuclear cells Th2 immune response apparently takes place. New insights on immunological markers and links among them may shed a light on atopic dermatitis pathogenic mechanisms. The detected molecules could play role as potential therapeutic targets and form a management approach for patients with atopic dermatitis.

*Keywords: atopic dermatitis, cytokines, Toll-like receptors, methylation, innate immunity, gene expression*

## Introduction

Atopic dermatitis (AD) is a multifactorial genetically determined inflammatory skin disease characterized by itching, chronically relapsing dermatitis, age-related features of localization and morphology of lesions. Frequently the onset of AD occurs in childhood, and in some cases may become a chronic lifelong disease. The prevalence of the disease among the child population reaches 20%, among the adult population about 3% worldwide [7].

The pathogenesis of atopic dermatitis is complex and includes dysfunction of the epithelial barrier (gene mutations as well, e.g. *FLG* gene), epigenetic alterations, involved in the genomic adaptation, and immune response reactions. In most cases of AD, Th2 immune response dominates. However, depending on

the course of the disease, Th17 and Th22 responses may also take place [1]. In moderate-to-severe course of AD, an increase in the number of T and B cells is observed both at the systemic level in the blood cells and locally in the skin [3]. Depending on specific cytokines' presence and expression of their genes, it is possible to determine the exact phase of the AD pathogenesis.

Besides the cytokine release, some innate immune structures could play an important role as triggers of the AD development. AD is often complicated by recurrent bacterial or viral infection. In this case, pattern recognition receptors (PRRs) that recognize certain microbial molecules (known as PAMPs) become activated and trigger innate immune responses. Thus, gene polymorphisms of some PRRs (for example) TLRs are involved in the pathogenesis of various autoimmune and inflammatory diseases, including AD.

**The aim of this study** is to detect the expression level for *IL4*, *IL13*, *IL33*, *TLR2*, *TLR9* genes in the biological materials of atopic patients.

## Materials and methods

The study was conducted in accordance with the WMA Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects, 2013. The study was approved by the local ethics committee at the Mechnikov RIVS. All patients signed a voluntary informed consent to participate in the study.

Fifty-five pediatric patients from 6 to 18 years of age and 50 adult patients from 18 to 60 years old with the verified diagnosis of atopic dermatitis were enrolled in the study. According to the SCORAD score we divided patients into two groups: moderate, SCORAD 25-50 ($n = 38$ children; $n = 26$ adults) and severe, SCORAD $\geq 50$ ($n = 17$ children; $n = 24$ adults) forms. Biological materials such as biopsies of the affected skin and blood samples were taken from patients in the Medical Research Center for Children's Health; 26 healthy volunteers formed a control group. Whole blood samples were taken from adult patients in the Clinic for Skin and Venereal Diseases named after V. A. Rakhmanov.

The targeted genes for further expression evaluation were selected according to our previous findings on genome-wide methylation study. After the enrichment analysis we got canonical signaling pathways, including IRAK-TRAF6-NFκB cascade that may be triggered by different types of TLRs and influence the production of cytokines; and the IgE-dependent cascade that also have an effect on cytokine profile.

RNA was extracted from PMBCs of patients and healthy donors using Extract RNA reagent as per the manufacturer (Evrogen, RF). Total RNA from whole blood samples from adult patients was extracted using the AmpliPRIME RIBO-sorb kit (NextBio, Russia), rRT-PCR was performed using the SYBR Green Syntol kit (Syntol, RF), and oligonucleotide primers for *TLR2*, *TLR9*, *IL4*, *IL13*, *IL33*, and *ACTB* genes were synthesized by Syntol (Syntol, RF). The reaction was carried out under the following conditions: 1 cycle at 95 °C for 5 min; 40 cycles at 95 °C for 15 s and 60 °C (or 58 °C) for 50 s; melting. Beta-actin was used as the reference gene for the analysis of target genes. The $2^{(-\ c(t))}$ method was used for analysis of the obtained data. Statistical analysis was performed with the use of Kruskal-Wallis H test and Mann–Whitney U test.

## Results and discussion

The first set of questions aimed to *IL4* and *IL13* cytokine expression profiles in the PBMCs. All results considered to be significant at the $p \leq 0.05$ levels. It must be mentioned that all significant differences were found in case of adolescents (12-18 years of age). Figure 1 provides an example of the experimental data on *IL4* gene expression. There was a significant increase of *IL4* relative expression level (median for elder group of pediatric patients = 1717.3, median for control group = 0.4, $p < 0.0001$; median for patients of 6-12 y.o. = 29.4). Similar results were obtained regarding *IL13* gene: median for adolescent group = 380.0, median for control group = 0.3, $p < 0.0001$; median for younger group of patients was 3.5).

Further analysis revealed the expression levels for *TLR2* and *TLR9* genes in the PBMCs. Comparing the data for patients of childhood years and healthy controls again did not show any significant results. Figure 2 shows an example of the results on *TLR9* gene expression. It can be seen from the graph that there was an increase of this gene expression in comparison with control group ($p < 0.0001$). Medians for 6-12 age group, 12-18 age group, and healthy donors were equal 82.7, 1478.6, and 1.3 respectively. Similar situation is for *TLR2* gene: medians for children = 33.0, adolescents = 10226.3, and healthy donors = 8.3, $p < 0.0001$.

Changes in *IL33* gene expression in the whole blood samples were assessed in the adult group of patients. As can be seen from the Figure 3, patients with moderate form of AD reported significantly higher expression level of *IL33* than patients with severe form. Median for the moderate AD group equals 57.7 and for severe AD group = 4.4 ($p < 0.01$).

Our study showed differences between groups of atopic patients and healthy donors concerning the expression profile of *IL4, IL13* cytokines and pattern-recognition receptors *TLR2* and *TLR9* in the blood mononuclear cells. Increased levels of all suggested molecules may be an evidence of Th2 immune response enhancement in the acute phase of the dermatitis, which is, in fact, an acknowledged

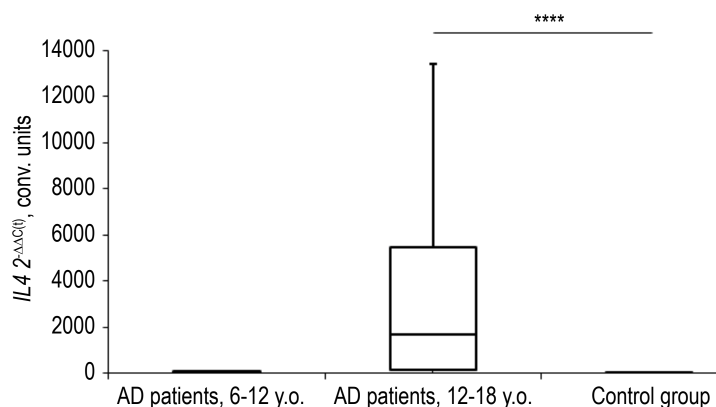


**Figure 1. *IL4* gene expression level in the PBMCs of pediatric ad patients compared to the control group**

Note. ****, $p < 0.0001$.

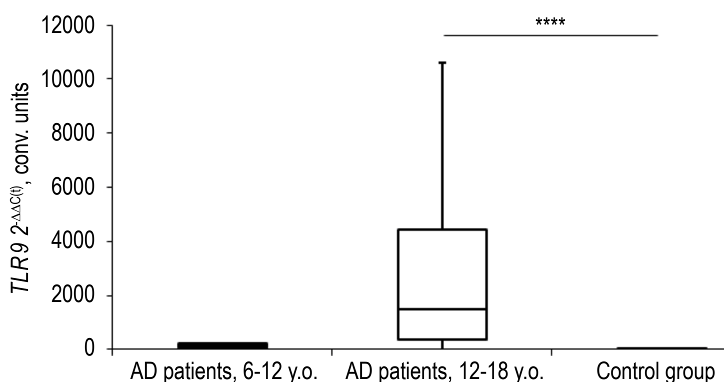


**Figure 2. *TLR9* gene expression level in the PBMCs of pediatric ad patients compared to the control group**

Note. ****, $p < 0.0001$.

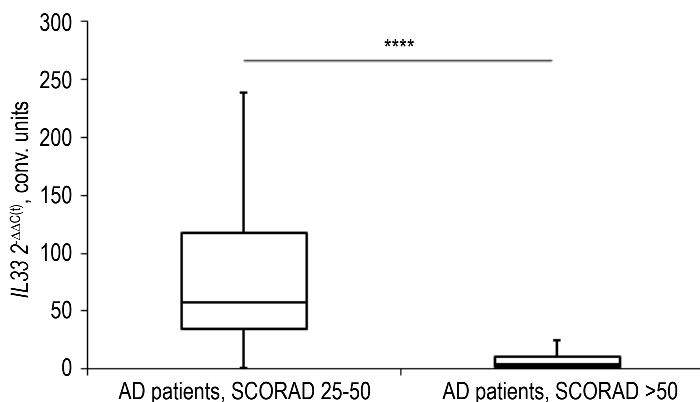


**Figure 3. *IL33* gene expression level in the whole blood samples of adult ad patients**

Note. ****, $p < 0.0001$.

classic model of the systemic AD pathogenesis. Nevertheless, our previous studies showed that at the same time there might be a difference at the local level (in the skin), where the inflammation takes place and anti-inflammatory mechanisms prevail [2]. Targeted expression analysis revealed that in the skin samples the expression of *TLR9* and *IL4* was 12 times significantly lower ($p < 0.0001$, $p < 0.0005$) in the lesional skin; and there was a 6-fold decrease in case of *TLR2* ($p < 0.01$). Such differences may occur due to different factors, epigenetic alterations in particular.

The findings on *IL33* expression level suggested that in case of moderate form of AD in adults, where the chronic form of the disease often takes place, this cytokine may prevail on systemic level. Presumably, in case of severe form *IL33* may be released locally, in the affected skin, so its systemic effect reduces. Thus, further studies are required.

There is much research that emphasis the importance of *IL4* and *IL13* cytokines in the pathogenesis and management of atopic dermatitis. These cytokines are proven targets for treatment of moderate-to-severe forms of AD with a systemic upregulation of the mentioned interleukins. Dupilumab is the targeted biologic agent approved in many countries that inhibits the *IL4*-receptor α, the shared subunit of *IL4* and *IL13* [4]. Due to the mechanism of this medical agent, it is important to prescribe it not only regarding the symptoms and severity of AD, but also the inadequate levels of Th2 type cytokines (e.g., via blood or serum analyses).

IL-33 belongs to the IL-1 inflammatory cytokine family. IL-33 is one of the inflammatory cytokines associated with innate immunity. It can activate group 2 innate lymphoid cells without antigen stimulation to induce type 2 cytokines. There is evidence that it may be produced in keratinocytes of the lesional skin and even become a trigger for itch-scratch cycle [5]. But there is lack of studies explaining its systemic role in the AD pathogenesis.

*TLR2* is a PRR family member, which is also known to be expressed on immune cells. *TLR2* is able to recognize variety of microbial components and form a link between innate and adaptive immunity. Iwamoto et al. suggested that in situ analysis of isolated Langerhans cells and inflammatory dendritic epidermal cells there was a decreased level of *TLR2* expression and this could partly contribute to the immune deviation in AD [6]. In another study conducted by Yangyang et al., an excessive chemokine mRNA expression (CCL5, CCL8, CCL13, CCL18, and CCL22) in PBMCs was shown to be induced by *TLR2* activation, which was associated with the AD development [9]. All in all, our findings agree with the studies as due to different biological material (local tissue or systemic level cells) the results may differ.

*TLR9* is identified as a CpG DNA sensing receptor expressed in professional innate immune cells such as dendritic cells and macrophages. It was proved that polymorphisms of the *TLR9* gene in the promoter region with significantly increased activity were associated with the development of AD. In a study by Moriwaki et al., it was shown that *S. aureus* in AD was captured by keratinocyte lysosomes, which led to the secretion of the proinflammatory cytokine IL-1α via *TLR9* [8]. Our study did not show any activation of this receptor locally in the skin, although its higher expression in the blood may contribute to inflammation maintaining.

## Conclusion

New insights on immunological markers and links among them may shed a light on atopic dermatitis pathogenic mechanisms. The detected molecules could play role as potential therapeutic targets and form a management approach for patients with atopic dermatitis.

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# РОЛЬ ПОЛИМОРФНЫХ МАРКЕРОВ В ГЕНАХ *TLR2, TLR4* И *TLR9* В РИСКЕ РАЗВИТИЯ АТОПИЧЕСКОГО ДЕРМАТИТА

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**Резюме.** Атопический дерматит – распространенное хроническое воспалительное заболевание кожи, ассоциированное со значительным снижением качества жизни. Считается, что атопический дерматит развивается у лиц с генетической предрасположенностью к атопии под воздействием факторов окружающей среды, иммунной дисрегуляции и изменением микробиома кожи. Толл-подобные рецепторы (TLR) являются неотъемлемой частью врожденной иммунной системы и участвуют в распознавании патоген-ассоциированных молекулярных паттернов. Целью исследования было оценить генетический риск развития атопического дерматита на основании изучения полиморфных маркеров в генах *TLR2, TLR4* и *TLR9*. В исследование были включены 100 пациентов с атопическим дерматитом средней (n = 56) и тяжелой (n = 44) степенью тяжести. Возраст варьировал от 18 до 65 лет. В группу контроля были включены 72 добровольца старше 18 лет, не имеющих в анамнезе каких-либо аллергических заболеваний кожи. В ходе исследования были проанализированы следующие маркеры: rs5743708 в гене *TLR2*, rs4986791 в гене *TLR4* и rs352140 в гене *TLR9*. Исследование генетических маркеров SNP rs5743708 в гене *TLR2* и rs4986791 в гене *TLR4* не выявило статистически значимых различий в распределении аллелей и генотипов. Изучение полиморфного маркера rs352140 в гене *TLR9* показало статистически достоверное различие между группой пациентов с атопическим дерматитом средней степени тяжести и контрольной выборкой. Частота встречаемости гомозиготы GG в группе с атопическим дерматитом составила 0,169, в то время как в группе контроля – 0,329 ($p < 0,05$; OR = 0,42; 95% CI = 0,18-0,97). На сегодняшний день изучение влияния полиморфных генетических локусов на риск развития различных заболеваний представляет особый интерес. Однако среди многочисленных

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исследований, направленных на поиск SNP-маркеров для диагностики повышенного риска развития заболеваний, встречаются лишь единичные публикации об ассоциации полиморфных маркеров в генах TLRs с атопическим дерматитом. Выбранные нами полиморфные маркеры rs5743708 в гене *TLR4* и rs4986791 в гене *TLR9* были изучены впервые при атопическом дерматите. В заключение, по результатам исследования был выявлен один полиморфный маркер, ассоциированный с риском развития атопического дерматита. Так, было показано, что гомозигота GG генетического маркера SNP rs352140 *TLR9* может быть предиктором относительно риска развития атопического средней степени тяжести. Таким образом, полученные данные могут быть использованы при оценке риска развития атопического дерматита у здоровых лиц с отягощенным семейным анамнезом.

*Ключевые слова: атопический дерматит, TLR, SNP, маркер, атопические заболевания, врожденный иммунитет*

# ASSOCIATION OF SINGLE NUCLEOTIDE POLYMORPHISMS OF *TLR2, TLR4* AND *TLR9* WITH ATOPIC DERMATITIS

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**Abstract.** Toll-like receptors (TLRs) are the most studied among all Pattern Recognition Receptors, the main function of which is to initiate innate immune response by recognizing pathogen-associated molecular patterns of various microorganisms on the skin surface. TLR-mediated recognition plays an important role in linking innate and adaptive immunity that ultimately leads to the production of key cytokines, chemokines and antimicrobial peptides. Today, there is growing interest in research on single nucleotide polymorphisms (SNPs) in *TLR* genes and its influence on susceptibility to inflammatory disease, including atopic dermatitis. The aim of the research was to study the association of the rs5743708 gene polymorphism in the *TLR2* gene, the rs4986791 gene polymorphism in the *TLR4* gene and the rs352140 gene polymorphism in the *TLR9* gene with the risk of developing severe cases of AD. A total of 100 patients with AD were included in the study (38 male and 62 female). The age range was from 18 to 65 years old. All participants were divided into 2 groups according to the SCORAD index (SCORing Atopic Dermatitis). The control group included 72 volunteers over 18 years old. The results of our study showed a statistically significant difference between the moderate AD group and healthy controls in the rs352140 gene polymorphism in the *TLR9* gene (Figure 1). The frequency of the GG genotype of SNP rs352140 in *TLR9* was 0.169 in the AD group versus 0.329 in the control group ($p < 0.05$; OR = 0.42; 95% CI = 0.18-0.97).

In conclusion, the results of our study showed that the *TLR9* rs352140 gene polymorphism may be linked to an increased risk of atopic dermatitis. Moreover, it was found that the GG genotype of SNP rs352140 in *TLR9* can be used as a predictor of the risk of developing moderate AD.

*Keywords: atopic dermatitis, TLR, SNP, marker, atopic diseases, innate immunity*

## Introduction

Atopic dermatitis (AD) is a common chronic inflammatory skin disease associated with the significantly decreased quality of life. The first signs of AD usually appear in infancy, which may precede the development of other allergic diseases, including asthma, allergic rhinitis or food allergy [2, 8]. Clinical presentations are diverse and may vary over time depending on age and the course of disease. Generally, the acute phase of AD is characterized by erythematous skin lesions and edema, whereas chronic stage features lichenification, generalized xerosis and

post-inflammatory hyperpigmentation. Although the pathophysiology of AD is still not completely understood, it is regarded as a complex interaction between immune dysregulation, altered skin microbiome and environmental factors in individuals with a positive family history of atopy [2, 4].

Skin microbiome research has highlighted its role in the development and exacerbation of AD. The composition of microbial communities in AD patients is usually characterized by loss of microbial diversity and predominance of *Staphylococcus aureus* (*S. aureus*) in both lesion and non-lesional skin [5]. That in turn induces altered immune responses by maintaining type 2 inflammation due to the elaboration of a wide range of staphylococcal enterotoxins (SEs) and other virulence factors by *S. aureus* strains [10]. Thus, these data suggest that the disturbed microbial composition and overgrowth of *S. aureus* are significantly involved in the clinical manifestation and pathogenesis of AD flares.

Toll-like receptors (TLRs) are the most studied among all Pattern Recognition Receptors (PRRs), the main function of which is to initiate innate immune response by recognizing pathogen-associated molecular patterns (PAMPs) of various microorganisms on the skin surface, such as lipopolysaccharide of Gram-negative bacteria, peptidoglycan and lipoteichoic acid of Gram-positive bacteria. TLR-mediated recognition plays an important role in linking innate and adaptive immunity that ultimately leads to the production of key cytokines, chemokines and antimicrobial peptides. These TLRs are expressed by both immune cells and non-immune cells, including keratinocytes. To date, there are 10 members of the TLR family (*TLR1-TLR10*) identified in humans, which are further divided based on their localization into extracellular TLRs (*TLR1,TLR 2, TLR4, TLR5, TLR6, TLR10*) and intracellular TLRs (*TLR3, TLR7, TLR8, TLR9*) [9, 10]. The latter recognize nucleic acids of viruses, bacteria as well as damaged host cells. Conversely, extracellular TLRs can detect a broad spectrum of structures of Gram-positive bacteria and also components of damaged host cells. In addition, *TLR4* is implicated in the recognition of lipopolysaccharide of Gram-negative bacteria. *TLR5* binds bacterial flagellin found in many Gram-negative and Gram-positive bacteria. *TLR2* and *TLR4* are known for their ability to bind endogenous ligands such as heat shock protein and hyaluronic acid [10, 11].

There is growing interest in research on single nucleotide polymorphisms (SNPs) in *TLR* genes and its influence on susceptibility to inflammatory disease. For instance, certain polymorphisms were associated with impaired *TLR2* and *TLR4* function in individuals with AD, which may contribute to the prevalence of Th2-mediated immune responses [1, 7].

**The aim of the research** was to study the association of the rs5743708 gene polymorphism in the *TLR2* gene, the rs4986791 gene polymorphism in the *TLR4* gene and the rs352140 gene polymorphism in the *TLR9* gene with the risk of developing severe cases of AD.

## Materials and methods

A total of 100 patients with AD were included in the study (38 male and 62 female). All patients were admitted in the clinic of skin and venereal diseases named after V.A. Rakhmanov (Sechenov University). The age range was from 18 to 65 years old. The diagnosis of AD was established by using Hanifin and Rajka's criteria [3]. All participants were divided into 2 groups according to the SCORAD index (SCORing Atopic Dermatitis) results: moderate (n = 56) and severe (n = 44) [6]. The control group included 72 volunteers over 18 years old without atopic dermatitis and other allergic diseases. Peripheral blood samples were collected into EDTA tubes (VACUETTE® TUBE 4 ml K3E K3EDTA, Greiner Bio-One). Total RNA was extracted using the "AmpliPRIME RIBO-sorb" kit (Amplisens, Russia). The following markers were analyzed by PCR-based techniques using "SYBR Green I RT-PCR" kit (Syntol, Russia) and DT Prime 5 Real-Time PCR device (DNA-Technology, Russia): *TLR2* rs5743708, *TLR4* rs4986791 and *TLR9* rs352140. Statistical analysis was performed by using the $\chi^2$ test. Results were considered statistically significant if a p-value was less than 0.05 ($p < 0.05$).

## Results and discussion

Genotyping of the rs5743708 gene polymorphism in the *TLR2* and rs4986791 gene polymorphism in the *TLR4* did not reveal any statistically significant differences in allele frequency and genotype distribution. However, the results of our study showed a statistically significant difference between the moderate AD group and healthy controls in the rs352140 gene polymorphism in the *TLR9* gene (Figure 1). The frequency of the GG genotype of SNP rs352140 in *TLR9* was 0.169 in the AD group versus 0.329 in the control group ($p < 0.05$; OR = 0.42; 95%CI = 0.18-0.97).

Although studies focused on SNP analysis have been of particular interest in recent decades, the data on potential associations between TLR polymorphisms and AD is limited to a few publications. To our knowledge, the investigated SNPs rs5743708 in the *TLR4* gene and rs4986791 in the *TLR9* gene were studied for the first time in patients with atopic dermatitis.

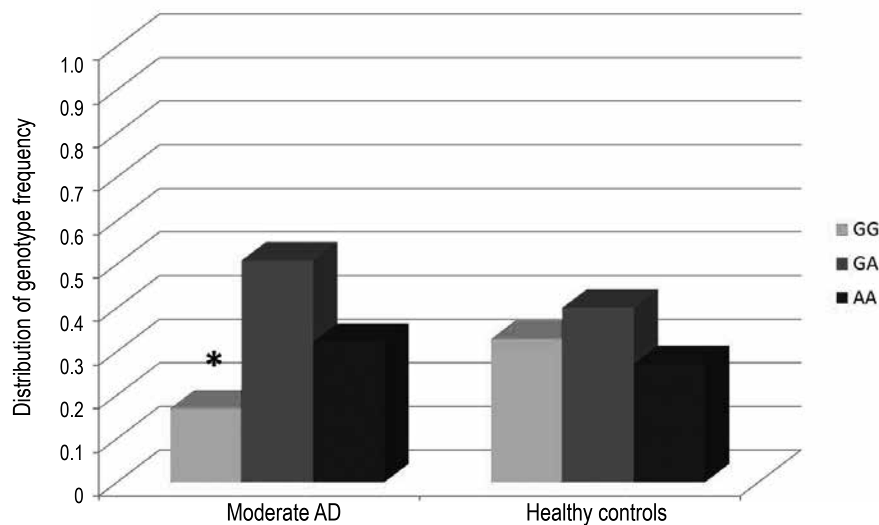


**Figure 1. Distribution of TLR9 SNP rs352140 genotypes in patients with moderate atopic dermatitis compared to healthy controls**

Note. *, $p < 0.05$.

According to a meta-analysis conducted by Zhang Y. et al. (2019), carriage of *TLR2* SNP rs5743708 GA genotype revealed a significantly higher risk for developing AD in the Caucasian race [12]. Nevertheless, the results of our study found no difference between patients with AD and healthy subjects.

It is notable that the scientific literature contains scarce data related to the analysis of *TLR4* gene polymorphisms and the susceptibility of AD. Zhao J. et al. (2017) conducted a meta-analysis that included more than 340 articles. It has been reported that rs4986791 in *TLR4* was significantly associated with the risk of developing asthma [13]. However, no data on AD was found.

According to the literature, the most common *TLR9* polymorphisms are rs5743836, rs187084, rs352140, and rs2066807. In particular, SNPs rs5743836 and rs187084 in the *TLR9* gene have been previously studied in patients with AD, but no significant association with AD was found [14]. And since there was previously detected a significant association of the rs352140 SNP in *TLR9* with regard to other diseases, we chose this particular SNP [15].

## Conclusion

In conclusion, the results of our study showed that the *TLR9* rs352140 gene polymorphism may be linked to an increased risk of atopic dermatitis. Moreover, it was found that the GG genotype of SNP rs352140 in *TLR9* can be used as a predictor of the risk of developing moderate AD.

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# ИЗМЕНЕНИЯ СУБПОПУЛЯЦИОННОГО СОСТАВА Т-ХЕЛПЕРОВ 17 ТИПА И ЦИТОКИНОВОГО ПРОФИЛЯ В ЗАВИСИМОСТИ ОТ КЛИНИЧЕСКОЙ КАРТИНЫ САРКОИДОЗА

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**Резюме.** При саркоидозе происходит гиперактивация клеток иммунной системы, а продуцируемые ими цитокины играют важную роль в патогенезе заболевания. Клеточно-опосредованные реакции являются основными в иммунопатогенезе саркоидоза. В их развитии участвуют субпопуляции Т-хелперов (Th), в том числе Th17-типа и регулирующих их цитокинов. Были исследованы образцы плазмы крови больных саркоидозом (n = 123), 18% с острым и 82% с хроническим течением. Контрольная группа – образцы, полученные от практически здоровых лиц (n = 43). Определялось содержание субпопуляций лимфоцитов методом проточной цитофлуориметрии и концентраций цитокинов (пг/мл) методом мультиплексного анализа по технологии xMAP (Luminex). Содержание «классических» Th17 достоверно снижено в образцах больных острым саркоидозом относительно хронического: 28,3% против 33,3%, p = 0,046. Уровень «дважды-позитивных» Th17 (DP Th17) был достоверно повышен в образцах крови больных с хроническим и острым саркоидозом относительно группы контроля: 31,7% и 34,2% против 26,2%, p < 0,001; содержание «не классических» Th17.1 оказалось достоверно снижено у больных хроническим течением относительно условно здоровых лиц: 27,9% и 35,9%, p < 0,001. Анализ клинико-лабораторной значимости определения DP Th17 среди CD45RA-негативных клеток Th эффекторной памяти выявил, что при остром саркоидозе относительно группы условно здоровых лиц: чувствительность – 82%; специфичность – 71%; при хроническом: 67% и 56%

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соответственно. У больных саркоидозом относительно условно здоровых лиц отмечалось достоверно значимое повышение уровней цитокинов IL-12 (p70) – 1,3 против 0,56, p = 0,028; IL-17A – 1,5 против 0,43, p < 0,001; IFNγ – 4,1 против 1,1, p < 0,001; TNFα – 21,7 против 6,7 пг/мл, p < 0,001. Таким образом, выявление субпопуляций CCR6$^+$Th17 и DP Th17, а также уровни синтезируемых такими клетками цитокинов, являются важными в диагностике саркоидоза при его разном течении: показана прямая корреляционная зависимость между уровнем активности ангиотензин-превращающего фермента и относительным содержанием DP Th17-клеток памяти; у больных с прогрессирующим течением в сравнении с регрессирующим течением заболевания было достоверно снижено абсолютное содержание всех CD45RA$^-$Th17-клеток памяти и клеток центральной памяти; у больных с экстрапульмональными проявлениями саркоидоза отмечалось достоверно повышенное относительное содержание DP Th17 CD45RA$^-$ и DP Th17 эффекторной памяти; при хроническом саркоидозе были достоверно повышены концентрации IL-17A, IFNγ, IL-12 и обнаружена положительная корреляция между уровнем IFNγ и активностью ангиотензин-превращающего фермента.

*Ключевые слова: саркоидоз, Т-хелперы, дифференцировка Т-хелперов, Т-хелперы 17 типа, проточная цитометрия, цитокины*

# SARCOIDOSIS CLINICAL PICTURE GOVERNS ALTERATIONS IN TYPE 17 T HELPER CELL SUBSET COMPOSITION AND CYTOKINE PROFILE

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**Abstract.** Immune cell hyperactivation along with cytokines they overproduce plays an important role in sarcoidosis and related disease pathogenesis. A central place in the immunopathogenesis of sarcoidosis is held by diverse cell-mediated reactions governed by T helper (Th) cell populations including Th17 subsets and relevant signature cytokines. We studied peripheral blood plasma samples of the patients with sarcoidosis (n = 123): 18% with acute and 82% with chronic course. The control group – samples from healthy volunteers (n = 43). T cell subset composition was assessed by flow cytometry. Cytokine concentrations (pg/mL) were measured by multiplex analysis using xMAP technology (Luminex). The level of "classical" Th17 turned out to be significantly reduced in acute *vs* chronic sarcoidosis: 28.3% *vs* 33.3% (p = 0.046). The level of "double-positive" Th17 (DP Th17) was significantly increased in chronic and acute *vs* control group: 31.7% and 34.2% *vs* 26.2% (p < 0.001 in both cases), without differences patient inter-group; "non-classical" Th17.1 were shown to have significantly reduced level only in chronic *vs* healthy subjects: 27.9% and 35.9% (p < 0.001). Clinical and laboratory diagnostic characteristics for blood DP Th17 levels in CD45RA-negative Th effector memory cells in sarcoidosis: in acute sarcoidosis *vs* healthy subjects, they were characterized by sensitivity – 82%; specificity – 71%, whereas in chronic: 67% and 56%, respectively. In patients with sarcoidosis *vs* healthy subjects were found to have significantly increased level of IL-12 (p70) – 1.3 *vs* 0.56, p = 0.028; IL-17A – 1.5 *vs* 0.43, p < 0.001; IFNγ – 4.1 *vs* 1.1, p < 0.001; TNFα – 21.7 *vs* 6.7, p < 0.001. Thus, CCR6$^+$ Th17 and DP Th17 subsets and relevant signature cytokines are important in diagnostics of sarcoidosis of varying clinical course: a direct correlation was shown between the level of angiotensin-converting enzyme activity and percentage of memory DP Th17; disease progression *vs* regression had significantly reduced absolute number of total CD45RA$^-$ memory and CM Th17; extrapulmonary manifestations had a significantly increased percentage of DP Th17 CD45RA$^-$ and EM DP Th17; in chronic sarcoidosis are significantly increased concentration of IL-17A, IFNγ, IL-12 and positively correlation between IFNγ and the activity of angiotensin-converting enzyme.

*Keywords: sarcoidosis, CD4$^+$ T cells, Th cell differentiation, Th17 cell subsets, flow cytometry, cytokines*

## Introduction

Sarcoidosis is a multisystem inflammatory granulomatous disease of unknown etiology characterized by developing highly organized immune cell aggregates (granulomas) lacking signs of necrosis in the affected organs, most commonly found in the respiratory organs [12, 14, 16]. Sarcoid granulomas are predominantly localized in the bronchopulmonary lymph nodes as well as directly in the lung tissue, less commonly being observed in other anatomical sites. Extrapulmonary or systemic sarcoidosis manifestations are presented due to damage to the skin, eyes, joints, heart, nervous system and some other organs [4, 14, 16].

Immune cell hyperactivation along with cytokines and chemokines they overproduce plays an important role in developing sarcoid granuloma and related disease pathogenesis [13, 16]. According to current concepts, a central place in the immunopathogenesis of sarcoidosis is held by diverse cell-mediated reactions including T helper cell subsets (Th): Th1, Th17 types, as well as relatively recently described “non-classical” or “plastic” Th17 subpopulations (Th1/Th17 and Th17.1), expressing surface chemokine receptors and capable of simultaneously producing several signature cytokines [5, 13, 16, 18]. It was shown that various Th17 subsets and relevant cytokines (IL-17A, IL-22, IFNγ) also take part in granuloma formation. Currently, special attention is paid to the role of Th17 cells in the immunopathogenesis, course and outcome of sarcoidosis, which are detected in the center and along the periphery of sarcoid granulomas [3, 4, 12, 13, 16].

Alterations in cytokine profile and the effects related to IFNγ, IL-12 and TNFα may result in transition of Th1/Th17 cells to plastic IFNγ-producing Th17, called “non-classical” Th17 [8, 17, 18]. Th17 cells include IFNγ/IL-17A-producing double positive (Th1/Th17 cells) and IFNγ single positive cell subsets defined on the basis of surface expression of chemokine receptors: Th17.1 cells [3, 13] co-expressing CCR6 (Th17 lineage) and CXCR3 (Th1 lineage) [17].

While investigating immune-related pathogenesis of sarcoidosis it is important to assess cytokine and chemokine profile ensuring directed migration of immune cells from the circulation to the focus of inflammation followed by sarcoid granuloma formation [1, 2, 3, 11, 13, 14]. Moreover, both cytokines and diverse immune cell types largely account for resolution or outcomes of granulomatous process in sarcoidosis, from resorption of granulomas in spontaneous remission to developing pulmonary fibrosis in unfavorable scenario, are also largely determined by the participation of various cells and cytokines [1, 2, 4, 12, 13].

Various studies in the field often report contradictory data, therefore underlying a need to search for and identify most diagnostically significant and informative markers (T cell subset composition and cytokine profile) aligned with disease clinical signs and course. Thus, **the aim of the study** was to assess type 17 T helper cell subset composition and some cytokine levels in accordance to clinical course of sarcoidosis.

## Materials and methods

Patients with sarcoidosis (n = 123) were enrolled to the study, of which 18% (22/123) with acute (“Löfgren’s syndrome”) and 82% (101/123) with chronic disease course (“non-Löfgren’s syndrome”) were examined at the Research Institute of Interstitial and Orphan Lung Diseases, Pavlov First Saint Petersburg State Medical University, Ministry of Health of Russia. Sarcoidosis in all patients examined was verified as first-onset disease, without therapeutic interventions applied at time of examination.

Informed consent to participate in the study was obtained from all patients. Studies were conducted in accordance with the Declaration of Helsinki of the World Association “Ethical principles for conducting scientific medical research involving humans” amended in 2013, “Rules of Clinical Practice in the Russian Federation”, approved by the Order of the Ministry of Health of the Russian Federation dated of 19.06.2003 No. 266 and the “Rules of Good Clinical Practice in the Russian Federation”, approved by the Order of the Ministry of Health of the Russian Federation dated 01.04.2016 No. 200n.

Patients with sarcoidosis were enrolled to main group: 49 males, 74 females, aged 20 to 67 years. Diagnosis in all patients was established based on comprehensive clinical and radiological (including chest computed tomography) study. The diagnosis of sarcoidosis was verified by histological examination after biopsy of bronchopulmonary tissue and/or mediastinal lymph nodes was collected in 67% (82/123) patients as well as according to clinical and radiological data in 33% (41/123) patients. In the control group, there were analyzed biological samples from 43 apparently healthy subjects lacking any clinical manifestations including respiratory diseases (18 males, 25 females, aged 20 to 61 years), age- and sex-matched to sarcoidosis patients.

According to the recommendations of the American Thoracic Society (ATS), the European Respiratory Society (ERS), and the World Association of Sarcoidosis and Other Granulomatosis (WASOG) [6], radiological classification was used to characterize patient groups. Patients were examined by using generally accepted methods (analyzed complaints, history of disease, life, occupational anamnesis, physical examination, palpation, percussion, auscultation, clinical blood count, general urinalysis, biochemical blood and urine tests for assessing disease course and activity). All patients underwent chest multislice computed tomography (MSCT), a comprehensive functional examination of external respiration

(CFEER), echodopplercardiography (EchodopplerCG) based on calculated systolic pressure in the pulmonary artery (SPPA, mm Hg), abdominal ultrasound examination, videothoracoscopy (VTS), if necessary, was performed with a biopsy of the lung tissue or mediastinal lymph node, fibrobronchoscopy (FBS) with endobronchial biopsy of the bronchial mucosa or transbronchial biopsy of the lung tissue, followed by histological examination in laboratories and departments of the Pavlov First Saint Petersburg State Medical University.

Based on the aforementioned data, patients were stratified as follows: group with pulmonary sarcoidosis without systemic disease manifestations (44%, 39/88) and group with respiratory sarcoidosis along with extrapulmonary manifestations (56%, 49/88).

Level of blood serum angiotensin-converting enzyme (ACE) was used to assess disease activity. Units of measurement – units of activity: ACE Unit (= 1 IU/mL). Reference values for ACE activity for subjects over 18 – 20-70 ACE Units. Accordingly, for patients with sarcoidosis elevated ACE activity level was set above 70 ACE Units.

Clinical disease course was assessed 3 months, six months and one year after establishing a verified diagnosis, in 38 patients enrolled to the study, based on the chest CT scan data, functional parameters and detected extrapulmonary manifestations. A retrospective analysis of immunological parameters was carried out depending on the clinical picture of the disease one year later by taking into account: presence or absence of spontaneous regression; severity of extrapulmonary manifestations; severity of pulmonary fibrosis.

There were investigated venous blood samples collected by puncture of peripheral vein into K3EDTA-containing vacuum tubes. T cell subset composition in peripheral blood samples was assessed by flow cytometry using a Navios diagnostic cytometer (Beckman Coulter, Inc., USA) equipped with three lasers with emittance at 405, 488, and 638 nm wavelengths. Data processing was carried out using Navios Software v. 1.2, Kaluza™ v. 2.0 (Beckman Coulter, USA). Various combinations of direct monoclonal antibodies were applied for immunophenotyping. The data on lymphocyte level are presented as follows: % – percentage out of total number of lymphocytes or examined lymphocyte subset; absolute (cell/μL, number of cells in 1 μL of peripheral blood) number of lymphocytes.

In the study, 100 μL of peripheral blood samples were stained with monoclonal antibodies in accordance with the manufacturer's recommendations; we have described the details previously [7]. Erythrocytes were removed by using VersaLyse lyse (Cat. No. A09777) adding 975 μL sample with *ex tempere* prepared 25 μL of IOTest 3 Fixative Solution (Cat. No. A07800). After incubation, the samples were washed once from unbound antibodies using phosphate buffered saline, by centrifugation for 7 minutes at 330g. The supernatant was removed, and cell pellet was resuspended in 200 μL of same solution (pH 7.2-7.4) containing 2% neutral paraformaldehyde (Cat. No. HT5011, Sigma-Aldrich, USA).

The following antibody cocktail was used to analyze peripheral blood Th cell subset composition: antibodies against CD3 (clone UCHT1) and CD4 (clone 13B8.2); Th cells were detected as $CD3^+CD4^+$ lymphocytes. In order to identify individual Th cell subsets at various differentiation stages, antibodies against surface CD45RA (clone 2H4LDH11LDB9 (2H4)) and CD62L (clone DREG56) were used. "Naive" Th cells bearing the $CD45RA^+CD62L^+$ phenotype were not used for further studies due to the lack of expressed surface chemokine receptors. CD45RA-positive terminally differentiated effector memory T cells (TEMRA) bearing $CD45RA^+CD62L^-$ phenotype were also excluded from further analysis due to almost full lack of such cell population in the peripheral blood in apparently healthy donors.

Memory Th cells were subdivided based on CD62L and CD45RA surface expression into central (CM) and effector (EM) memory T helper cells with $CD45RA^-CD62L^+$ and $CD45RA^-CD62L^-$ phenotypes, respectively. The expression levels of the following chemokine receptors were analyzed on Th cell subsets by using monoclonal antibodies: CD45RA-FITC, CD62L-PE, CXCR5- PerCP/Cy5.5, CCR6-PE/Cy7, CXCR3-APC, CD3-APC/Cy7, CD4-PacBlue, CCR4-BV510. We used antibodies against CD3, CD4, CD45RA and CD62L, respectively (Beckman Coulter, USA). Antibodies against CCR4, CCR6, CXCR3 and CXCR5, respectively (BioLegend, USA) were also used.

Polarized Th17 cell subset composition was analyzed by immunophenotyping as follows: four major Th17 cell subsets were identified based on expression patterns of surface chemokine receptors CXCR3 and CCR4, and according to the spectrum of functional properties in total pool of $CCR6^+$ cells of all CD45RA-negative, CM Th, and EM Th cells [15]. According to the phenotyping strategy, peripheral blood cells were divided into the following subsets: "classical" Th17 cells with $CCR4^+CXCR3^-$ phenotype, "double-positive" $CCR4^+CXCR3^+$ (DP Th17), "non-classical" $CCR4^-CXCR3^+$ (Th17.1) and "double-positive" $CCR4^-CXCR3^+$ (Th17.1), "double-negative" $CCR4^-CXCR3^-$ (DN Th17).

Blood plasma cytokine levels (pg/mL) were analyzed by using commercially available Milliplex MAP kits (Millipore, USA) along with magnetic microspheres (Milliplex Mag, USA), according to manufacturer's instructions. Data registration and analysis were carried out using Luminex MAGPIX instrument (Luminex, USA).

The data obtained were statistically processed by using software Statistica 8.0 (StatSoft, USA) and GraphPad Prism 5.00 for Windows (GraphPad Prism Software Inc., USA). For this, there were used standard

nonparametric methods of statistical processing. Quantitative data were presented as median (Me) and interquartile range ($Q_{0.25}$-$Q_{0.75}$). Non-parametric Mann–Whitney test, non-parametric Spearman rank correlation method and coefficient (r) calculation were applied. To determine a diagnostic relevance of the data, ROC analysis (receiver-operating-characteristic ROC curve) was used. The area under curve (AUC) of the operating characteristic, magnitude of the optimal point (criterion) of separation, sensitivity and specificity level were determined. Hypotheses were considered as statistically different with significance level set at $p < 0.05$.

## Results and discussion

During the study there were analyzed parameters between main comparison groups: patients with acute onset of sarcoidosis – group 1, patients with chronic sarcoidosis – group 2, and control group – group 3.

It was found that CCR6-positive Th17 cell level was altered in EM Th cell subset composition. In particular, peripheral blood samples from patients with acute *vs* chronic sarcoidosis and control group were shown to have significantly increased absolute number of CCR6-positive Th17 cells: 73 cells/μL (60-150) *vs* 57 (38-88) and 59 cells/μL (46-75), respectively, at $p_{1-2} = 0.011$ and $p_{1-3} = 0.032$. However, no changes in percentage and absolute number of CM Th17 cells were observed (Table 1).

While assessing polarized Th17 cell subset composition in peripheral blood samples from patients with sarcoidosis, it was found that Th17 cell level was significantly altered in total CD45RA-negative memory T cell pool.

The level of "classical" Th17 cells turned out to be significantly reduced in acute *vs* chronic sarcoidosis: 28.3% (23.6-35.4) *vs* 33.3% (26.5-40.4) (p = 0.046). In addition, the level of DP Th17 cells was significantly increased in chronic and acute sarcoidosis *vs* control group: 31.7% (26.2-38.9) and 34.2% (29.1-42.7) *vs* 26.2% (22.6-28.3) (p < 0.001 in both cases), without differences between patient groups. Regarding "non-classical" Th17.1 cells, it was found that their level was significantly reduced only in chronic sarcoidosis *vs* healthy subjects: 27.9% (23.0-33.9) and 35.9% (26.5-41.3) (p < 0.001). A significant decline in level of DN Th17 cells was revealed in chronic and acute sarcoidosis *vs* control group: 4.7% (3.2-6.1) and 4.2% (2.7-5.3) *vs* 5.9% (4.4-7.7) (p = 0.002).

The data comparing the level of such Th17 cell subsets in CD45RA-negative CM and EM Th17 cells are shown in Figure 1 and Figure 2, respectively. Along with that, $CCR6^+$ CM Th cell subset level was significantly reduced for "non-classical" Th17.1 and DN Th17 cells in chronic sarcoidosis compared to control group: 22.6% (18.6-28.1) *vs* 27.5 % (21.8-34.3) (p < 0.001) and 5.6% (3.9-7.1) *vs* 6.7% (4.8-8.9) (p = 0.004), respectively. The level of CCR4-expressing DP Th17 cells in chronic sarcoidosis was significantly higher than in control group: 30.9% (26.2-37.4) *vs* 27.2% (23.6-31.6) (p = 0.002), whereas in acute disease it was noted to increase as compared to healthy subjects (32.3% (28.5-37.4) *vs* 27.2% (23.6-31.6), p = 0.003) along with decreased DN Th17 cell levels (5.3% (3.2-7.2) *vs* 6.7% (4.8-8.9) (p = 0.017).

While analyzing magnitude of CCR6-positive EM Th cells capable of exiting from the circulation and migrating to peripheral tissues, similar changes were observed. Patients with chronic and acute sarcoidosis *vs* control group were noted to have significantly reduced EM level of "non-classical" Th17.1 cells: 36.2% (30.3-44.9) and 40.3% (30.6-46.9) *vs* 45.9% (38.3-54.7) (p < 0.001 and p = 0.017, respectively). Moreover, patients with both chronic and acute onset sarcoidosis had a significantly increased DP Th17 cell level compared to control group: 30.9% (24.4-

**TABLE 1. PERCENTAGE AND ABSOLUTE NUMBER OF PERIPHERAL BLOOD CENTRAL MEMORY (CM) AND EFFECTOR MEMORY (EM) Th17 CELL SUBSET LEVEL IN ACUTE AND CHRONIC SARCOIDOSIS COMPARED TO CONTROL SUBJECTS, Me ($Q_{0.25}$-$Q_{0.75}$)**

| Th cell subset | | Group 1 (n = 22) | Group 2 (n = 101) | Group 3 (n = 43) | p |
|---|---|---|---|---|---|
| **Th17 CM*** | % | 37.1 (32.8-41.3) | 39.3 (33.4-46.3) | 36.8 (31.6-43.3) | Th17 CM* |
| | cells/μL | 84 (58-125) | 83 (58-1110) | 96 (76-143) | $p_{1-2} = 0.656$<br>$p_{1-3} = 0.343$<br>$p_{2-3} = 0.074$ |
| **Th17 EM**** | % | 57.5 (44.9-67.6) | 58.6 (44.3-69.4) | 51.3 (40.9-60.1) | Th17 EM** |
| | cells/μL | 73 (60-150) | 57 (38-88) | 59 (46-75) | $p_{1-2} = 0.011$<br>$p_{1-3} = 0.032$<br>$p_{2-3} = 0.583$ |

Note. Significant differences according to the Mann–Whitney U test: $p_{1-2}$, differences between groups of patients with acute and chronic onset sarcoidosis; $p_{1-3}$, differences between groups of patients with acute onset sarcoidosis and apparently healthy subjects; $p_{2-3}$, differences between groups of patients with chronic onset sarcoidosis and apparently healthy subjects (control group). *, level within total CM Th cell population. **, level within total EM Th cell population.

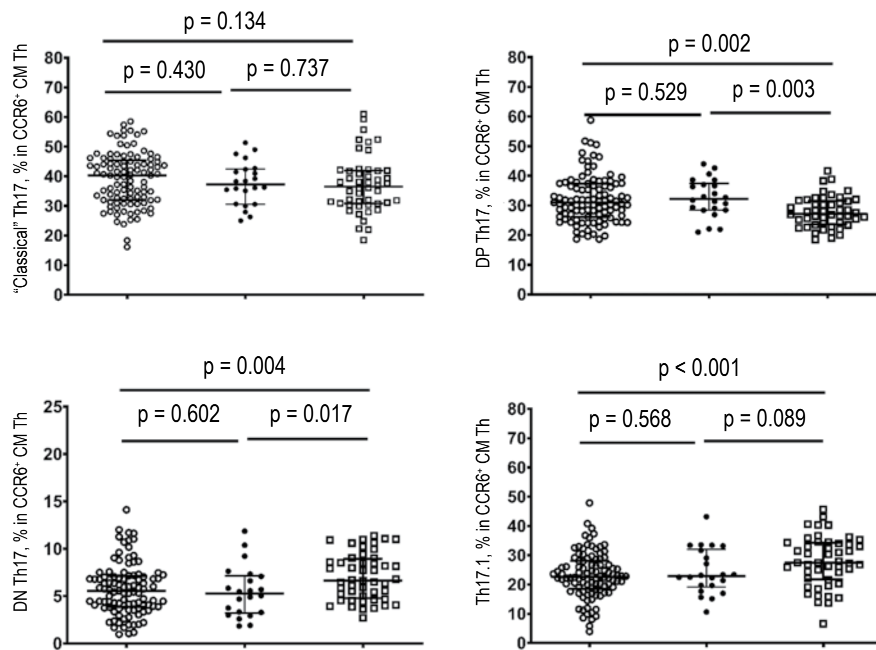


**Figure 1. Major Th17 cell subset distribution within total CCR6⁺ CD45RA⁻CD62L⁺ central memory (CM) Th cells in chronic (n = 101) and acute (n = 22) sarcoidosis as well as control subjects (n = 43)**

Note. White circles, patients with chronic onset sarcoidosis; black circles, patients with acute onset sarcoidosis; white squares, group of apparently healthy donors. Results are presented as median and interquartile range (Me ($Q_{0.25}$-$Q_{0.75}$)). Differences between groups are presented based on nonparametric Mann–Whitney test. Phenotypes of major Th17 cell subsets are as follows: "classical" Th17 – $CCR6^+CCR4^+CXCR3^-$, "double-positive" DP Th17 – $CCR6^+CCR4^+CXCR3^+$, "non-classical" or Th17.1 – $CCR6^+CCR4^-CXCR3^+$, "double-negative" DN Th17 – $CCR6^+CCR4^-CXCR3^-$.

40.9) and 33.4% (27.1-52.9) *vs* 24.7% (20.2-27.5), respectively (p < 0.001).

To determine a relevance for data on percentage of peripheral blood DP Th17 cells in the examined patients, we analyzed operating characteristic curves (ROC analysis) by plotting representative ROC curves and calculating AUC. It was found that levels of DP Th17 among total CD45RA-negative memory cells in acute onset sarcoidosis *vs* control group were characterized with AUC = 0.812 (p < 0.001). Applying a separation criterion > 29%, the sensitivity was 82%, with specificity reaching 81%. In contrast, chronic sarcoidosis *vs* control group was associated with AUC = 0.727 (p < 0.001), separation criterion > 27%, sensitivity and specificity of 72% and 63%, respectively. The data on assessing DP Th17 cell level among CD45RA-negative EM Th cells in acute sarcoidosis *vs* healthy subjects were characterized by AUC = 0.807, p < 0.001, separation criterion > 27%, sensitivity – 82%; specificity – 71%, whereas in chronic disease: AUC = 0.709, p < 0.001, separation criterion > 25%, sensitivity – 67%; specificity – 56%.

A relation between level of peripheral blood Th17 cell subsets and ACE as a generally accepted laboratory parameter of disease activity was assessed. A correlation analysis revealed a direct relationship between ACE activity level and percentage of DP Th17 cells in total CD45RA-negative memory cells, DP Th17 CM and EM memory cells: r = 0.422, p = 0.001; r = 0.330, p = 0.012 and r = 0.410, p = 0.002, respectively. It was shown that chronic onset sarcoidosis was associated with positive correlations between level of ACE activity and percentage of DP Th17 CD45RA-negative, DP Th17 CM and DP Th17 EM memory cells: r = 0.498, p < 0.001; r = 0.366, p = 0.012, and r = 0.512, p < 0.001, respectively.

Previously, we reported on cytokine magnitude and highest relevance in patients with sarcoidosis aligned with the characteristics of the disease course [9, 10]. In particular, along with the quantitative characterization of plastic Th17 cells, there were analyzed levels of crucial cytokines they produce in patients with varying clinical course of sarcoidosis.

Blood plasma samples from patients with sarcoidosis *vs* healthy subjects were found to have significantly increased level of cytokines IL-12 (p70) – 1.3 pg/mL (0.56-2.0) *vs* 0.56 pg/mL (0.23-1.3), p = 0.028; IL-17A/CTLA8 – 1.5 pg/mL (0.44-3.3) *vs* 0.43 pg/mL (0.15-1.2), p < 0.001; IFNγ – 4.1 pg/mL (2.7-6.9) *vs* 1.1 pg/mL (0.29-2.3), p < 0.001; TNFα – 21.7 pg/mL (12.6-30.3) *vs* 6.7 pg/mL (3.4-10.6), (p < 0.001). In contrast, no difference was observed for IL-1 and IL-12 (p40) in patients *vs* control subjects: 3.0 pg/mL (1.2-10.6) *vs* 7.5 pg/mL (0.6-14.7), p = 0.391 and 11.9 pg/mL (3.9-28.4) *vs* 12.8 pg/mL (0.36-17.2), (p = 0.633), respectively.

While assessing patients with chronic *vs* acute sarcoidosis, it was shown that level of cytokines IL-17A/CTLA8 was significantly increased (1.9 pg/mL (0.67-3.9) *vs* 0.67 pg/mL (0.42-1.9), p = 0.018),

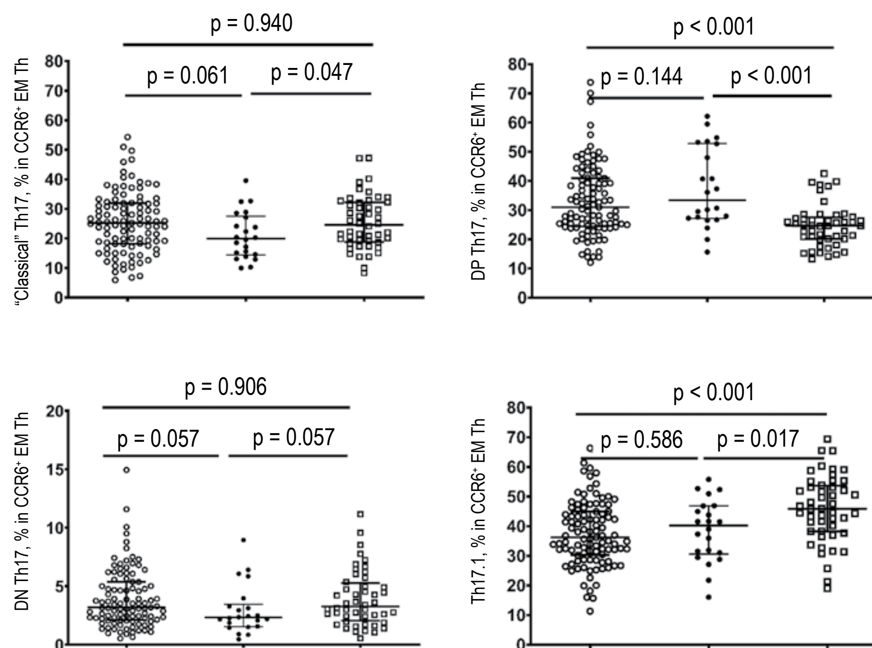


**Figure 2. Major Th17 cell subset distribution within total CCR6$^+$ CD45RA$^-$CD62L$^-$ effector memory (EM) Th cells in chronic (n = 101) and acute (n = 22) sarcoidosis as well as control subjects (n = 43)**

Note. As for Figure 1.

IFNγ (5.1 pg/mL (2.9-8.1) *vs* 2.7 pg/mL (2.5-4.6), p = 0.027). At the same time, IL-17A/CTLA8 were also significantly increased in chronic sarcoidosis *vs* healthy subjects: 1.9 pg/mL (0.67-3.9) and 0.43 pg/mL (0.15-1.2), at p < 0.001). The concentration of cytokine IL-12 (p70) was significantly increased only in chronic sarcoidosis *vs* control group comprising 1.3 pg/mL (0.56-2.0) *vs* 0.56 pg/mL (0.23-1.3), p = 0.037). While comparing cytokine levels based on clinical course of sarcoidosis, a positive correlation was found between level of IFNγ (pg/mL) and ACE activity (r = 0.349; p = 0.032).

Retrospectively analyzed alterations in type 17 T helper cell composition in patients with respiratory sarcoidosis aligned with changes in clinical picture during a one-year follow-up allowed to find that 31.6% (12/38) of the examined patients with a newly diagnosed disease receiving no immunosuppressive therapy had spontaneous regression of clinical and radiological signs as well as normalized functional parameters. In contrast, 68.4% of subjected (26/38 patients) receiving immunosuppressive therapy were characterized by signs of disease progression (deteriorating pulmonary changes based on chest organ CT scan data, functional parameters, and documented extrapulmonary manifestations).

Flow cytometry analysis assessing level of polarized peripheral blood Th17 subset composition in sarcoidosis aligned with clinical disease course allowed to find the following alterations: disease progression *vs* regression was associated with significantly reduced absolute number of total CD45RA$^-$Th17 and CD45RA$^-$CM T cells: 111 cells/μL (63-148) *vs* 180 cells/μL (138-315), p = 0.009 and 56 cells/μL (37-88) *vs* 110 cells/μL (66-157) (p = 0.006), respectively.

Patients with disease progression *vs* regression were found to have significantly reduced absolute number of both CD45RA$^-$Th17 and CD45RA$^-$CM cells: 111 cells/μL (63-148) *vs* 180 cells/μL (138-315), p = 0.009 and 56 cells/μL (37-88) *vs* 110 cells/μL (66-157), (p = 0.006), respectively. In case of extrapulmonary manifestations in sarcoidosis, a significantly increased percentage of DP Th17 CD45RA$^-$ and DP Th17 EM cells was observed: 38.3% (29.6-41.6) *vs* 29.4% (23.2-34.6), (p = 0.037) and 43.6% (25.4-48.9) *vs* 26.9% (21.9-30.8), (p = 0.018), respectively.

Here, we present data regarding a role for peripheral blood plastic Th17 cells in various types of clinical course of sarcoidosis. Our data suggest an important pathogenetic role played by plastic Th17 cells, not only in triggering granulomatous inflammation in sarcoidosis, but also in kinetics of granuloma formation potentially resulting in distinct outcomes of the pathological process (spontaneous disease remission in acute course and progression with formation of pulmonary fibrotic in chronic course). While comparing the patterns described here with available publications, it turned out that the data on peripheral blood Th17 cell subset composition in sarcoidosis were contradictory.

Some studies suggest about increased level of CCR6$^+$ effector Th (CD45RA$^-$CD45R0$^+$) cells in patients with sarcoidosis compared with control group, whereas others evidence that number of peripheral blood IL-17A-producing T cells is markedly reduced compared to healthy subjects [8, 12, 13,

16]. At the same time, numerous studies are noted showing increased Th17-produced cytokine and chemokine level in sarcoidosis, e.g., IL-6, IL-17, IL-22, IFNγ and CCL20 [12, 13, 18]. At the same time, bronchoalveolar lavage fluid (BALF) along with granulomatous tissue samples are not only characterized by elevated cytokine level, but also contain higher number of cells involved in cytokine production [13].

Th17 cells are detected in granulomas of patients with sarcoidosis, both during active and recurrent disease course [13, 18]. Apparently, Th1/Th17 cells play a central role in developing pulmonary inflammation in sarcoidosis, because their increased level is observed in BALF and peripheral blood samples [3, 4, 8].

A study by Broos et al. showed that the mediastinal lymph nodes of patients with sarcoidosis *vs* control group contained elevated number of $CCR6^+$ Th17, including classical Th17 and Th17.1 cells [3]. In addition, transitional $CCR6^+CXCR3^+CCR4^+$Th Th17/Th17.1 or DP $CCR6^+$ Th cell level was increased [3, 13].

While analyzing our data, we found significantly increased level of peripheral blood CM and EM Th17 memory cells providing a deeper insight into understanding granuloma formation, because among all Th17 subsets, it is DP Th17 cells that are characterized by higher potential to migrate to peripheral anatomical sites due to the high level of expressed adhesion molecules and chemokine receptors [3, 12, 18].

Many studies have been attempting to establish a role for Th17 cell subsets in developing granulomas. It was shown that cytokines IL-1 , IL-6 and IL-23, necessary for polarization towards Th17 cells, or IL-12 responsible for naive Th0-to-Th1 cell differentiation, the majority of DP Th17 cells, in *in vitro* settings, acquired the phenotype and properties of non-classical Th17 cells [3, 4, 8, 12, 18]. It is the “non-classical” Th17 or Th17.1 cell subsets, which seem to represent the major IFNγ producers in developing granulomas [17, 18]. It should be noted that BALF from patients with sarcoidosis was noted in many studies to have increased levels of ligands for surface Th17.1 cell chemokine receptors CXCR3 (e.g., CXCL10 [1]) and CCR6 (CCL20 [4]).

It may be assumed that compared with other Th17 cell subsets, this cell type is able to migrate more efficiently along the chemokine gradient to be selectively accumulated in the focus of inflammation, which is confirmed by the data on predominantly detected Th17.1 cells in the foci of granuloma formation and BALF in sarcoidosis [3].

Apart from this, it was also observed that Th17 cell subset composition was altered providing, with most valuable relevance found for level of memory DP Th17 cells, correlations with ACE activity level as well as their increased level in respiratory sarcoidosis with extrapulmonary manifestations.

It has been uncovered that cytokines such as IL-1 , IL-6, IL-12, IL-17, IL-23, TNFα, and IFNγ are involved in cell differentiation and granuloma formation. Moreover, the roles of IL-2, IL-4, IL-5, IL-6, IL-10, and IL-13, as well as a number of anti-inflammatory cytokines (IL-1RA, IL-10), are of importance in the pathogenesis of sarcoidosis [3, 11, 12, 13, 14, 18]. While assessing the cytokine profile, we showed that patients with chronic sarcoidosis had significantly increased level of cytokines IL-17A/CTLA8, IFNγ, and IL-12 (p70).

Aligning the level of the peripheral blood pro-inflammatory cytokine IFNγ with varying types of sarcoidosis allowed to find that first diagnosed respiratory sarcoidosis prior to immunosuppressive therapy was associated with a direct correlation between IFNγ and ACE level. Such parameters mirror disease activity. It is known that IFNγ is a key mediator in developing granulomatous inflammation. Current data indicate that not only Th1 cells, but also various plastic Th17 cell types, and to a greater extent, Th17.1 cells, are capable of producing IFNγ [1, 3, 13, 18]. The study by Arger et al. revealed the role for IFNγ in developing sarcoid granulomas coupled to importance of identifying plastic Th17 cell types. Other studies show a correlation between the number of Tbet-positive Th17.1 and severity of manifestations in systemic sarcoidosis as well as the level of IFNγ-dependent chemokines: CXCL9, CXCL10, and CXCL11 [1].

## Conclusion

Thus, our study allowed to find that peripheral blood plastic CCR6-positive particularly DP Th17 cell subsets as well as level of cytokines they produce driving their differentiation in sarcoidosis are important in diagnostics of sarcoidosis with varying clinical course:

– a direct correlation was shown between the level of angiotensin-converting enzyme activity and percentage of memory DP Th17 cells;

– patients with disease progression *vs* regression had significantly reduced absolute number of total CD45RA-negative memory and CM Th17 cells;

– patients with extrapulmonary manifestations of sarcoidosis had a significantly increased percentage of DP Th17 $CD45RA^-$ and effector memory DP Th17 cells;

– patients with chronic sarcoidosis were shown to have significantly increased concentration of blood plasma cytokines IL-17A/CTLA8, IFNγ, IL-12 (p70) paralleled with positively correlated level between IFNγ (pg/mL) and the activity of angiotensin-converting enzyme.

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# ВЗАИМОСВЯЗЬ СИСТЕМНОЙ ВОСПАЛИТЕЛЬНОЙ РЕАКЦИИ И ГИПЕРКОАГУЛЯЦИИ У ПАЦИЕНТОВ С ИММУНОВОСПАЛИТЕЛЬНЫМИ РЕВМАТИЧЕСКИМИ ЗАБОЛЕВАНИЯМИ

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**Резюме.** Взаимосвязь процессов коагуляции и воспаления обеспечивает защиту организма от потенциально опасных биологических агентов. Однако гипервоспаление влечет за собой повышение прокоагуляционного потенциала, а активация факторов гемостаза поддерживает воспалительный процесс. Этот процесс получил название «иммунотромбоз» или «тромбовоспаление». Гиперцитокинемию можно трактовать как проявление системной воспалительной реакции (СВР), которая является атрибутным феноменом типового патологического процесса – системного воспаления. Иммуновоспалительные ревматические заболевания (ИВРЗ) являются идеальной моделью для изучения взаимосвязи процессов коагуляции и воспаления на системном уровне. Изучение тромбовоспалительных механизмов является актуальной проблемой современной медицины, поскольку в перспективе поможет улучшить терапию заболеваний, в патогенезе которых тромбовоспаление играет существенную роль.

Цель работы – провести сравнительный анализ выраженности системной воспалительной реакции у пациентов с иммуновоспалительными ревматическими заболеваниями (ИВРЗ) с наличием и отсутствием проявлений гиперкоагуляции.

Для достижения поставленной цели был проведен сравнительный анализ провоспалительных маркеров (IL-6, IL-8, IL-10, TNFα, sIL-2R, CRP, ECP, β2-микроглобулин) в крови пациентов с ИВРЗ (системной красной волчанкой, ревматоидным артритом, реактивным артритом, анкилозирующим спондилитом, псориатическим артритом, ревматической болезнью сердца). На основании определяемых биомаркеров воспаления по оригинальной авторской методике оценивали также интегральный показатель выраженности СВР – уровень реактивности (RL). По наличию повышенного уровня D-димера (> 500 нг/мл) выборка была разделена на 2 группы: с наличием признаков гиперкоагуляции (n = 56) и без признаков гиперкоагуляции (n = 119). Группу контроля составили доноры крови (n = 50).

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Результаты исследования показали, что у пациентов с ИВРЗ, независимо от гемостатического потенциала крови, развивается выраженная СВР. Пациенты с признаками гиперкоагуляции характеризовались более высокими значениями большинства провоспалительных молекулярных маркеров (наибольшие отклонения выявлены в отношении уровня IL-6), а также повышенным интегральным уровнем СВР, что свидетельствует о тесной взаимосвязи процессов коагуляции и воспаления на системном уровне. И, напротив, с возрастанием выраженности СВР (оцененной с помощью интегрального показателя – УР) увеличивается вероятность развития гиперкоагуляции. Таким образом, наблюдается переход количественно более выраженных факторов на иной качественный уровень развития патологического процесса.

Патогенез иммуновоспалительных ревматических заболеваний характеризуется развитием системной воспалительной реакции (гиперцитокинемией, острофазным ответом, внутрисосудистой активацией лейкоцитов), выраженность которой тесно связана с внутрисосудистым микротромбообразованием.

*Ключевые слова: системная воспалительная реакция, гиперкоагуляция, тромбовоспаление, иммунотромбоз, ревматические заболевания, цитокины*

# RELATIONSHIP BETWEEN SYSTEMIC INFLAMMATORY RESPONSE AND HYPERCOAGULATION IN PATIENTS WITH IMMUNO-INFLAMMATORY RHEUMATIC DISEASES

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**Abstract.** The relationship between the processes of coagulation and inflammation protects the organism from potentially dangerous biological agents. However, hyperinflammation leads to an increase in the procoagulation potential, and activation of hemostasis factors maintains the inflammatory process. This phenomenon is called “immunothrombosis” or “ thromboinflammation”. The study of thromboinflammatory mechanisms is an actual problem of modern medicine, because in the future it will help to improve the therapy of diseases, in the pathogenesis of which thromboinflammation plays a significant role. The aim: to carry out a comparative analysis of the severity of the systemic inflammatory response in patients with immuno-inflammatory rheumatic diseases depending on the manifestations of hypercoagulation.

To achieve the aim, a comparative analysis of proinflammatory markers (IL-6, IL-8, IL-10, TNFα, sIL-2R, CRP, ECP, β2-microglobulin) in the blood of patients with immune-inflammatory rheumatic diseases (systemic lupus erythematosus, rheumatoid arthritis, reactive arthritis, ankylosing spondylitis, psoriatic arthritis, rheumatic heart disease) was performed. Based on these inflammatory markers according to the authors’ original methodology, the integral index of systemic inflammatory response (SIR) – Reactivity Level (RL) – was calculated. The cohort was divided into 2 groups: with the presence of signs of hypercoagulation and without signs of hypercoagulation according to the presence of elevated D-dimer level (> 500 ng/mL). Control group – healthy blood donors.

The results of the study showed that SIR develops in patients with immuno-inflammatory rheumatic diseases regardless of the blood hemostatic potential. Patients with signs of hypercoagulation were characterized by higher values of most proinflammatory molecular markers, as well as increased integral level of SIR, which indicates a strong relationship between coagulation processes and inflammation at the systemic level. In addition, the probability of hypercoagulation increases with increasing severity of SIR (assessed by means of the integral index – RL). Thus, there is a transition of quantitatively more pronounced signs to a new qualitative level of pathological process development.

The pathogenesis of immuno-inflammatory rheumatic diseases is characterized by the development of SIR (hypercytokinemia, acute phase response, intravascular leukocyte activation), the severity of which is closely related to intravascular microthrombosis.

*Keywords: systemic inflammatory response, hypercoagulation, thromboinflammation, immuno-thrombosis, rheumatic diseases, cytokines*

The reported study was funded by the Government contract of the Institute of Immunology and Physiology (122020900136-4).

## Introduction

The relationship between the processes of coagulation and inflammation protects the organism from potentially dangerous biological agents. However, uncontrolled mutual activation of the hemostasis and inflammation mechanisms causes the development of pathological process, recently named "immunothrombosis", or "thromboinflammation" [4, 9]. Molecular studies have allowed to explain the complex relationship between certain factors of the platelet-vascular and coagulation hemostasis, inflammation and immunity [3, 5, 11]. Cytokines play an important role in intercellular interactions, including those in thromboinflammation. Hypercytokinemia and especially "cytokine storm" can be interpreted as a manifestation of systemic inflammatory response (SIR), which is an attributive phenomenon of a general pathological process – systemic inflammation [14]. Immuno-inflammatory rheumatic diseases (RD) is an optimal model to study the relationship between coagulation and inflammation processes at the systemic level.

**The aim of the present work** was to carry out a comparative analysis of the severity of the systemic inflammatory response in patients with immuno-inflammatory rheumatic diseases depending on the manifestations of hypercoagulation.

## Materials and methods

A retrospective study included 175 patients with rheumatic diseases, including systemic lupus erythematosus (n = 49), rheumatoid arthritis (n = 42), reactive arthritis (n = 30), ankylosing spondylitis (n = 27), psoriatic arthritis (n = 12), chronic rheumatic valvular heart disease (n = 15). The levels of a key molecular marker of hypercoagulation, D-dimer, as well as levels of inflammatory mediators such as C-reactive protein (CRP) and cytokines (IL-6, IL-8, TNFα, IL-10) were measured in blood plasma in all patients. On the basis of these inflammatory markers according to the authors' original methodology, the integral index of SIR – Reactivity Level – RL (min 0 – max 5) was calculated [14]. Additional markers of SIR, indicating intravascular activation of various leukocyte subpopulations, were also measured in some patients: levels of eosinophilic cationic protein (n = 96), soluble receptor to IL-2 – sIL-2R (n = 91), and β2-microglobulin (n = 108). The study was performed using a closed system for immunochemiluminometric assay, Immulite (Siemens Medical Solutions Diagnostics, USA).

The cohort was divided into 2 groups: with the presence of signs of hypercoagulation (n = 56) and without signs of hypercoagulation (n = 119) according to the presence of elevated D-dimer level (> 500 ng/mL). The control group was healthy blood donors aged 18-55 years (n = 50).

Statistical analyses were performed using Statistica 12.0 program (Stat Soft, Inc., USA). The descriptive statistics are presented by their main characteristics: m (mean value) ± SD (standard deviation). Comparisons between the groups were performed using the Mann–Whitney test. All the results were considered statistically significant if the p-value was < 0.05.

## Results and discussion

In both study groups, the levels of all proinflammatory mediators (IL-6, IL-8, TNFα, CRP, ECP, β2-microglobulin, sIL-2R) and RL values were statistically significantly higher than those in the control group, which indicates the development of significant SIR in patients with rheumatic diseases, regardless of the blood hemostatic potential (Table 1). The IL-10 concentration did not exceed the analyzer detection level (5 pg/mL) in 88.6% of the total patient sample and in 100% of the control subjects, so no basic statistical characteristics were calculated for this index. Thus, SIR, manifested as intravascular leukocyte activation, hypercytokinemia and acute phase response, plays a significant role in the pathogenesis of immuno-inflammatory rheumatic diseases.

A comparative analysis demonstrated that patients with elevated D-dimer levels were characterized by significantly higher proinflammatory markers (except for ECP levels) compared to patients without signs of hypercoagulation (Table 1). The greatest differences (more than 6-fold) were found for IL-6. The results obtained are in agreement with the data of some authors indicating a significant correlation of proinflammatory cytokines with hypercoagulability indices in rheumatic diseases [2, 12].

Since SIR is a multi-factorial process, it was important to assess its severity in the studied subgroups not only by individual markers, but also using integral indices. Our suggested integral RL was also significantly (p < 0.05) higher in patients with hypercoagulation (Table 1). An analysis of the distribution of patients by RL from 0 (no SIR) to 5 (hyperergic variant of SIR) showed the following. Increased hemostatic potential in rheumatic diseases is most often associated with R = 1-2 (SIR, which is most typical for Low-grade systemic inflammation and classical inflammation); RL = 0, which indicates the absence of significant SIR, was found in this group only in single cases. In contrast, patients without signs of hypercoagulation are most characterized by RL = 0-1. It is noteworthy that unusual for a chronic process RL = 5 (increase of proinflammatory cytokines in the blood by thousands

**TABLE 1. VALUES OF PROINFLAMMATORY MARKERS IN THE STUDIED GROUPS**

| Marker | | Patients with hypercoagulation | Patients without hypercoagulation | Control group |
|---|---|---|---|---|
| **CRP, mg/dL** | | 2.32±2.66 | 0.97±1.25* | 0.26±0.24 |
| **IL-6, pg/mL** | | 373.50±1762.95 | 59.75±347.82* | 2.02±0.45 |
| **IL-8, pg/mL** | | 1326.72±4895.19 | 266.96±926.30* | 5.58±1.56 |
| **TNFα, pg/mL** | | 53.12±116.97 | 53.31±179.69* | 4.33±1.03 |
| **ECP, ng/mL** | | 11.37±12.74 | 6.76±10.69 | 3.87±1.61 |
| **sIL-2R, U/mL** | | 1598.5±1900.1 | 696.0±738.7* | 315.6±101.2 |
| **β2-microglobulin, ng/mL** | | 2707.7±1319.1 | 2198.5±835.3* | 1508.4±232.1 |
| **RL, point** | | 2.07±1.20 | 1.08±1.27* | 0 |
| **RL, %** | **RL = 0** | 5.4 | 43.7 | 100 |
| | **RL = 1** | 32.1 | 28.6 | 0 |
| | **RL = 2** | 30.4 | 12.6 | 0 |
| | **RL = 3** | 16.1 | 6.7 | 0 |
| | **RL = 4** | 14.3 | 7.6 | 0 |
| | **RL = 5** | 1.7 | 0.8 | 0 |

Note. The control group was statistically significant different from both studied groups for all indicators ($p < 0.05$); *, $p < 0.05$ between the groups of patients with and without signs of hypercoagulation.

and tens of thousands of times) were observed in two patients with systemic lupus erythematosus.

The ranking of the total sample by RL showed that, in general, the increase in the severity of SIR was associated with an increase in the rate of D-dimer detection (Figure 1).

Also of interest was a comparative assessment of commonly used clinical criteria of diseases activity depending on the manifestation of signs of hypercoagulation. This analysis was performed in the groups of patients with rheumatoid arthritis (DAS28 scale) and systemic lupus erythematosus (SLEDAI scale). In this case, statistically relevant differences were found only in the rheumatoid arthritis group (DAS28: 5.92±1.11 points in the group with D-dimer and 4.65±1.45 points in patients without D-dimer). In patients with SLE with and without signs of hypercoagulation, the SLEDAI index did not differ significantly and was 23.2±12.1 and 19.8±10.5, respectively. This phenomenon can be explained by the fact that the clinical scales mainly focus on

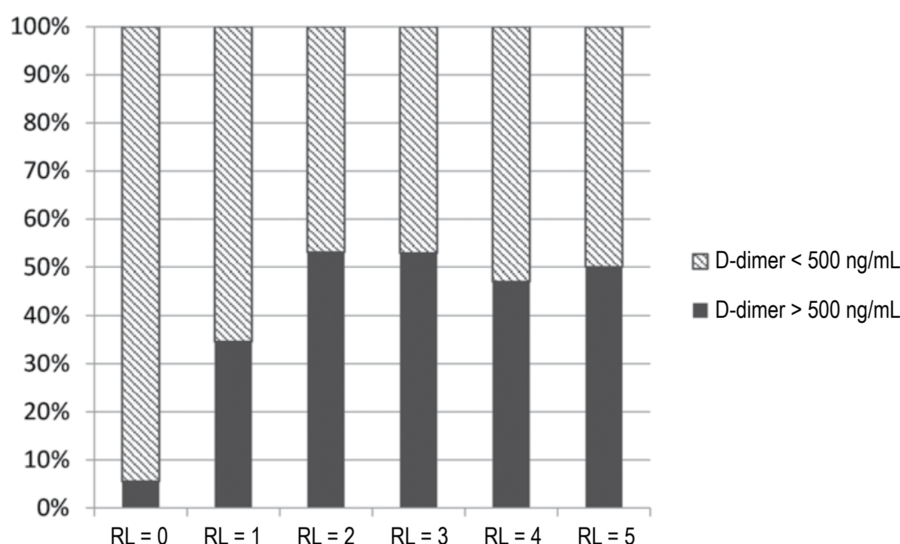


**Figure 1. Rates of D-dimer detection at different severities of SIR**

specific clinical manifestations of diseases, but not on the systemic pathogenetic pattern of these disorders.

Thus, the detected increase in separate molecular and integral markers of SIR in patients with increased procoagulation potential indicates a strong relationship between these processes in patients with rheumatic diseases. The impact of damage factors (in particular, immune complexes, cytotoxic antibodies, molecules from own damaged cells, etc.) as well as proinflammatory triggers (primarily cytokines) leads to microcirculatory disorders. These are based on such processes as endothelial glycocalyx degradation and endothelium activation with increased vascular permeability, interstitial edema, exposure of endotheliocyte cell membranes expressing receptors to proinflammatory and platelet-derived factors, and increased procoagulant potential. Increased hemostatic potential is associated with the production of tissue factor, as well as with the inhibition of anticoagulant pathway and suppression of fibrinolysis (in particular, by increasing the production of Plasminogen activator inhibitor-1 – PAI-1).

It is known that some proinflammatory cytokines (IL-6, TNFα, TGF-β) significantly increase PAI-1 synthesis [7]. Thus, hyperinflammation leads to a shift of hemostatic balance towards its increase, and activation of vascular and platelet hemostasis, production of soluble coagulation factors promote maintenance of the inflammatory process. Thus, there is formed a self-sustaining vicious pathogenetic circle of pathological process, which becomes independent of the damage factors at a certain stage of development. This process, called “thromboinflammation” or “immunothrombosis” is considered today as a universal pathogenetic mechanism of many acute and chronic diseases [1, 5, 8, 10, 11]. The results of the present study showed that the risk of hypercoagulation increases with increasing severity of SIR. Thus, there is a transition of quantitatively more pronounced signs to a new qualitative level of pathological process development.

It is also noteworthy that unlike acute conditions in which pro-inflammatory remodeling of the microcirculation leads to the development of critical complications, including acute disseminated intravascular coagulation and multiple organ dysfunction, microcirculatory changes in chronic diseases are latent. Probably, in the latter case an adequate long-term anti-inflammatory therapy plays a role, as well as “inclusion” of the feedback mechanisms, including inhibition of the system of transcription factors (for example, increase in the expression of mRNA of suppressor of cytokine signaling 1 (SOCS1), suppressing the activity of JAK) [6], negative control of the expression of receptors to proinflammatory mediators on the target cells, and increased levels of soluble forms of receptors in the blood circulation [13].

## Conclusion

The pathogenesis of immuno-inflammatory rheumatic diseases is characterized by the development of SIR (hypercytokinemia, acute phase response, intravascular leukocyte activation), the severity of which is closely related to intravascular microthrombosis. At the same time, the levels of individual proinflammatory mediators and the severity of systemic inflammatory response in general are higher in patients with hypercytokinemia, and, on the other hand, the more intensive the impact of proinflammatory mediators, the higher the procoagulation potential of blood.

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# СРАВНИТЕЛЬНОЕ ИССЛЕДОВАНИЕ АКТИВАЦИИ ЭНДОГЕННОГО РЕТРОВИРУСА ЧЕЛОВЕКА *HERV-E λ 4-1* ПРИ АУТОИММУННОЙ ПАТОЛОГИИ

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**Резюме.** Учитывая наличие у эндогенных ретровирусов человека иммуномодулирующих свойств – (1) способности к активации врожденного иммунного ответа нуклеиновыми кислотами HERVs; (2) антигенности молекулы протеина оболочки транскрипционно-компетентных эндогенных ретровирусов, вызывающей поликлональную активацию лимфоцитов; (3) отсутствие экспрессии HERVs и продукции протеинов в тимусе во время формирования иммунной толерантности, что позволяет рассматривать эти вирусы как аутоантигены или неоантигены, представлялось актуальным исследовать ассоциацию репликационно-компетентного эндогенного ретровируса человека *HREV-E λ 4-1* с течением ряда аутоиммунных заболеваний – рассеянного склероза, ревматоидного артрита и системной красной волчанки. Целью настоящей работы было сравнительное исследование частоты активации эндогенного ретровируса человека *HERV-E λ 4-1* в мононуклеарных клетках крови при рассеянном склерозе, ревматоидном артрите, системной красной волчанке, а также хронических непрогрессирующих заболеваниях нервной системы и дегенеративно-дистрофическом заболевании костно-мышечной системы. Мононуклеарные клетки периферической крови выделяли при помощи центрифугирования венозной крови на градиенте плотности фиколла 1,078 г/см$^3$. Экспрессию гена *envelope HERV-E λ 4-1* выявляли методом обратно-транскриптазной полимеразной цепной реакции. Было обнаружено, что частота экспрессии гена *envelope HERV-E λ 4-1* при хронических непрогрессирующих заболеваниях нервной системы, также, как и при дегенеративно-дистрофическом заболевании суставов сопоставима с частотой экспрессии у условно-здоровых лиц. Однако частота экспрессии гена *envelope HERV-E λ 4-1* при аутоиммунных заболеваниях значительно превышала таковую и у условно-здоровых, и при невоспалительных заболеваниях. Максимальные значения частоты экспрессии отмечались при активном рассеянном склерозе, значительно превышающие показатели при системной красной волчанке и ревматоидном артрите в стадии обострения. Причем частота экспрессии в состоянии ремиссии рассеянного склероза была значительно ниже показателя при ремиттирующем течении в стадии обострения, а также при прогредиентном течении. Оценка частоты экспрессии гена *envelope HERV-E λ 4-1* при различных степенях тяжести рассеянного склероза выявила ее максимальные показатели при степени тяжести III и IV-V, как при ремиттирующем, так и при прогредиентном течении

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рассеянного склероза. Таким образом, активация эндогенного ретровируса человека *HERV-E λ 4-1* ассоциирована с течением аутоиммунных заболеваний – рассеянного склероза, ревматоидного артрита, системной красной волчанки и положительно коррелирует с активностью и степенью тяжести рассеянного склероза.

*Ключевые слова: эндогенный ретровирус HERV-E λ 4-1, экспрессия, рассеянный склероз, ревматоидный артрит, системная красная волчанка, мононуклеарные клетки крови*

# A COMPARATIVE STUDY OF HUMAN ENDOGENOUS RETROVIRUS *HERV-E* λ *4-1* ACTIVATION IN AUTOIMMUNE PATHOLOGY

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**Abstract.** Considering the presence of immunomodulatory properties of human endogenous retroviruses, namely (i) the ability to activate the innate immune response by HERVs nucleic acids; (ii) the antigenicity of transcriptionally competent endogenous retroviruses envelope protein molecule, which causes polyclonal activation of lymphocytes; (iii) the absence of HERVs expression and protein production in the thymus during the immune tolerance formation, which allows us to consider these proteins as autoantigens or neoantigens, it seemed relevant to investigate the association of replication-competent human endogenous retrovirus *HERV-E λ 4-1* with course of some of autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis and systemic lupus erythematosus. The aim of this work was a comparative study of the human endogenous retrovirus *HERV-E λ 4-1* activation frequency in blood mononuclear cells in multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, as well as in chronic nervous system non-progressive diseases and the degenerative-dystrophic disease of the musculoskeletal system. The peripheral blood mononuclear cells were isolated by the venous blood centrifugation on Ficoll density gradient of 1.078 g/cm$^3$. Expression of the *HERV-E λ 4-1 envelope* gene was detected by reverse transcriptase polymerase chain reaction. It was found that the *HERV-E λ 4-1 envelope* gene expression frequency in the chronic non-progressive diseases of nervous system, as well as in degenerative-dystrophic joint disease, is comparable to the expression frequency in conditionally healthy individuals. However, the *HERV-E λ 4-1 envelope* gene expression frequency in autoimmune diseases significantly exceeded that in conditionally healthy individuals and in non-inflammatory diseases. The maximum values of expression frequency were observed in active multiple sclerosis, significantly higher than in systemic lupus erythematosus and rheumatoid arthritis in the acute stage. Moreover, the expression frequency in the remission stage of multiple sclerosis was significantly lower than in the acute stage of the relapsing-remitted course, as well as in the progredient course. Estimation of *HERV-E λ 4-1 envelope* gene expression frequency at different severity levels of multiple sclerosis revealed its maximum rates at III and IV-V severity levels, both in relapsing-remitting and progressive course of multiple sclerosis. Thus, activation of the human endogenous retrovirus *HERV-E λ 4-1* is associated with the course of autoimmune diseases, namely multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus; it positively correlates with the activity and severity of multiple sclerosis.

*Keywords: endogenous retrovirus HERV-E λ 4-1, expression, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, blood mononuclear cells*

## Introduction

The ability of human endogenous retroviruses (HERVs) to regulate immune response has made it possible to consider these genome elements as potentially involved in autoimmune disease development. Altered expression of HERVs is also considered to be as one of autoimmune disorders triggers. It was confirmed by the presence of increased levels of proviral RNA and antibodies to some of HERVs proteins in the sera of many autoimmune diseases patients, namely multiple sclerosis (MS), systemic lupus erythematosus (SLE), type 1 diabetes mellitus, rheumatoid arthritis (RA) [3, 5, 8, 10]. Thus,

the spectrum of HERVs associated with autoimmune diseases and the mechanisms of its direct involvement in diseases pathogenesis are still far from being fully understood.

Among the classical mechanisms of virus-driven autoimmunity, such as molecular mimicry and epitope spreading, special attention is given to the role of HERVs nucleic acids in direct activation of innate immunity and in the epigenetic modulation of interferon status. HERVs mechanism of action cannot be fully explained either by de novo insertional mutagenesis, or by the viral particle's formation. It has been suggested that potential HERVs pathogenicity may be realized through the presence of their proviral DNA in the genome, acting as a regulatory sequence that changes the neighboring and distant genes expression. Moreover, HERVs proviral DNA may be considered as a binding site for transcription factors. Therefore, HERVs potential effects will be limited to some genomic window around the primary insertion site of the provirus [1, 7]. However, there is evidence which supports a more global HERVs mechanism of action.

Some HERVs, in particular *HREV-E λ 4-1*, are able to encode an envelope protein, and its presence in a number of autoimmune diseases has been identified. In addition, it was suggested that HERVs envelope proteins mechanisms of action are based on antigenicity of their molecule, that possibly causing lymphocyte's polyclonal activation, ie. they function as "superantigens". Moreover, proteins encoded by HERVs, which are a part of human genome, should be considered as autoantigens or neoantigens, since they are not expressing in the thymus during the immune tolerance formation [6, 7, 12]. It was shown that the sequence similarity between *HERV-W* envelope proteins and myelin can induce an immune response in MS [11].

Retroviral nucleic acids and viral proteins can be perceived by various pattern recognition receptors, such as Toll-like (TLR) or NOD-like receptors [14], which leads to the induction of autoimmunity [15]. A direct interaction between some HERVs and TLR proteins has been shown. For example, the envelope protein of *HERV-W* binds to TLR4 and CD14 and stimulates the pro-inflammatory cytokines production, including IL-1β, IL-6, and TNFα [9]. Although the majority of HERVs sequences are nonfunctional, some of HERVs loci are coding and can be activated when exposed to some external and internal environmental factors [4].

**The aim of this work** was a comparative study of the human endogenous retrovirus *HERV-E λ 4-1* activation frequency in blood mononuclear cells of patients with multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, as well as with chronic non-progressive diseases of the nervous system and with degenerative-dystrophic disease of the musculoskeletal system.

## Materials and methods

Examination of patients and collection of the material was carried out in the Clinic of Research Institute of Fundamental and Clinical Immunology, the Research Institute of Clinical and Experimental Lymphology, Branch of the Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences and the Novosibirsk's NIITO named after. Ya. L. Tsivyan.

The study included analysis of:

– 96 healthy volunteers, 45 men and 51 women with an average age 38.0 (24.0-46.0) years;

– 205 unrelated patients with a diagnosis of MS (G35) established in accordance with the McDonald criteria (2010, 2017) and confirmed by magnetic resonance imaging. Forty-five people were in the stage of disease remission (21 men and 24 women, with an average age 36.0 (29.0-43.0) years), and 160 people – in the stage of MS exacerbation (76 men and 84 women with an average age 38.0 (30.0-43.0) years), with a disease duration of 2-18 years, an average age of the disease onset in both groups of 25.0 (23.5-31.5) years, corresponding to the inclusion/exclusion criteria and signed a voluntary informed consent;

– 26 patients with an established diagnosis of systemic lupus erythematosus (SLE) (M32) in the acute stage (12 men and 14 women with an average age 33.0 (27.0-42.0)) years);

– 53 patients with rheumatoid arthritis (RA) (M05), in the acute stage (25 men and 28 women with an average age (34 (28-46) years)

– patients with chronic non-progressive organic diseases of the nervous system (CND) with static, motor, mental and speech disorders, including 16 patients (8 men and 8 women with an average age 36 (18-33) years) with children's cerebral palsy (CCP) (G80), and 20 patients (12 men and 8 women with an average age 34 (27-43)) years) with long-term consequences of spinal injury, in some cases in combination with craniocerebral injury (T91.3)

– 24 patients with deforming osteoarthritis (DOA) (M16-M17) (14 men and 10 women with an average age 46 (38-53) years).

The control groups were formed from healthy volunteers, the patients with relapsing-remitting type of MS in remission stage, patients with CCP, long-term consequences of spinal injury and DOA. The study groups were formed from the patients with autoimmune diseases in acute stage, namely MS, SLE, and RA.

The study protocol was developed in accordance with the Helsinki Declaration of the World Medical Association "Ethical principles for conducting scientific medical research involving humans" as

**TABLE 1. COMPARATIVE FREQUENCY OF *HERV-E λ 4-1 ENVELOPE* GENE EXPRESSION IN BLOOD MONONUCLEAR CELLS OF AUTOIMMUNE DISEASE PATIENTS**

| Patient group | Expression of *HREV-E λ 4-1 envelope* gene, persons | Expression of *HREV-E λ 4-1 envelope* gene, persons, % |
|---|---|---|
| **Conditionally healthy, n = 96** | 3 | 3.13 |
| **Children's cerebral palsy, n = 16** | 1 | 6.25 |
| **Spinal injury, n = 20** | 2 | 10.0 |
| **Deforming osteoarthritis, n = 24** | 1 | 4.17 |
| **Multiple sclerosis, remission, n = 45** | 11 | 24.4** |
| **Systemic lupus erythematosus, n = 26** | 8 | 30.77* |
| **Rheumatoid arthritis, n = 53** | 22 | 41.5* |
| **Multiple sclerosis, relapsing course, exacerbation, n = 82** | 51 | 62.2* *** |
| **Severity I-II, n = 29** | 17 | 58.6* |
| **Severity III, n = 31** | 20 | 64.5* *** |
| **Severity IV-V, n = 22** | 18 | 81.8* *** |
| **Multiple sclerosis, progressive course, n = 78** | 58 | 74.4* *** |
| **Severity I-II, n = 27** | 17 | 62.9* |
| **Severity III, n = 28** | 22 | 81.5* *** |
| **Severity IV-V, n = 23** | 20 | 86.9* *** |

Note. Statistical significance of differences (F – Fisher's test): *, p < 0.05 between control groups and study groups; **, p < 0.05 between control groups; ***, p < 0.05 between study groups.

amended in 2013 and the "Rules of Good Clinical Practice", approved by the Russian Federation Ministry of Health Order No. 200n dated April 1, 2016. This work was approved by local ethical committees.

Peripheral blood mononuclear cells (PBMCs) were isolated by venous blood centrifugation on a Ficoll density gradient of 1.078 g/cm$^3$ (Lymphocyte separation medium, MP Biomedicals, LLC, Eschwege, Germany) at a ratio of 3:1, at 1500 rpm within 45 min. Cells collected from the interphase were washed thrice with 199 medium and precipitated by centrifugation. Isolation of total RNA was carried out by the phenol extraction method, using the test system VectoRNA – extraction (Vector-Best, Novosibirsk). The obtained DNA amplification was carried out in a programmable thermocycler "Tertsik", (DNA-technology, Moscow), using pairs of oligonucleotide primers to the human endogenous retrovirus *HERV-E λ 4-1 envelope* gene, homologous to the conservative regions of antiparallel DNA chains. The resulting cDNA fragments were analyzed by electrophoresis in 2% agarose gel with the addition of 0.00001% ethidium bromide (VectoDNA-EF, Vector-Best, Russia). The resulting cDNA segment was detected as a discrete band after electrophoretic separation of cDNA molecules. Samples with a visible cDNA band in the gel corresponding to the expected amplicon size were considered as positive.

Statistical data processing was carried out using the software package "Statistica 10.0" (StatSoft, USA). Comparison of gene expression frequencies between the studied samples was carried out using the Fisher's exact F-test. The statistical significance level was taken at $p < 0.05$. The sample was checked for normal distribution using the Kolmogorov-Smirnov test. To study the correlation relationships, the method of the Spearman rank correlation coefficient calculating was used.

## Results and discussion

The results of the frequency of *HERV-E λ 4-1 envelope* gene expression study in PBMC of patients with MS, SLE and RA, compared with patients with CND and DOA, are presented in Table 1.

It was found that the *HERV-E λ 4-1 envelope* gene expression frequency in PBMC of patients

with chronic non-progressive diseases of the nervous system, as well as with DOA, is comparable to the expression frequency in PBMC of healthy individuals, but less than in MS in remission stage. The frequency of the *HERV-E λ 4-1 envelope* gene expression in autoimmune diseases (MS, SLE, RA) significantly exceeded parameters in the control groups. The maximum of expression frequency observed in active MS, exceeding those in SLE and RA in the acute stage. Moreover, the expression frequency in the stage of MS remission was significantly lower than its rate in the relapsing course in the acute stage and in the progredient course of MS. Estimation of the *HERV-E λ 4-1 envelope* gene expression frequency in various MS severity levels revealed its highest values in severity III and IV-V, both in relapsing-remitting and progressive MS. The frequency of the *HERV-E λ 4-1 envelope* gene expression positively correlated with MS activity, as well as with its severity ($r = 0.75$ and $r = 0.78$, respectively).

Thus, the human endogenous retrovirus *HERV-E λ 4-1* activation is associated with the course of autoimmune diseases, namely MS, RA and SLE. Expression of the *HERV-E λ 4-1 envelope* gene positively correlates with the MS activity and severity.

Our data on human endogenous retrovirus *HERV-E λ 4-1* activation in autoimmune diseases are consistent with the results of the study, indicating an increase of mRNA *HERV-E* clone *4-1* expression in $CD4^+T$ cells in patients with SLE, which positively correlated with the diseases activity. Among the possible mechanisms of *HREV-E λ 4-1* involvement in the pathogenesis of these diseases, activation of the $Ca^{2+}$/calcineurin (CaN)/NFAT1 and E2/ER-α signaling pathways and hypomethylation of the 5'LTR DNA of the *HERV-E 4-1* clone have been described. Clone *HERV-E 4-1* also has been implicated in the disease pathogenesis through the microRNA MiR P-302d/methyl-CpG-binding domain protein 2 (MBD2) expression, DNA hypomethylation, and IL-17 signaling pathway via its 3'LTR [13].

In addition, HERVs are known to interfere with various processes related to the nervous system development and functioning: human sodium-dependent type 1 neutral amino acid transporters (hASCT1) and hASCT2 have been recognized as cellular receptors for *HERV-W* Env. Both receptors play a role in the glutamatergic transmission regulation in the brain. Also, the *HERV-W envelope* gene overexpression activated the $Ca^{2+}$-activated $K^+$ conduction channel in human neuroblastoma cells through the cAMP response element (CREB), which suggests a significant role of the HERV in the regulation of neuronal activity in the nervous system diseases. Activation of HERV transcription (the *HERV-W envelope* gene expression) regulated the brain-derived neurotrophic factor (BDNF) expression, neurotrophic tyrosine kinase receptor type 2 (NTRK2), and dopamine D3 receptor (DRD3) genes [2]. Such mechanisms may be involved in formation of pathological process in MS and other autoimmune diseases involving *HERV-E λ 4-1.*

## Conclusion

Thus, the involvement of human endogenous retrovirus *HERV-E λ 4-1* in the course of autoimmune diseases, especially multiple sclerosis, opens new perspectives for the development of pathogenetically determined therapy.

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# СОДЕРЖАНИЕ СУБПОПУЛЯЦИЙ CD4⁺T-КЛЕТОК В ПРОГНОЗЕ ЭФФЕКТИВНОСТИ БИОЛОГИЧЕСКОЙ ТЕРАПИИ ПСОРИАЗА У ДЕТЕЙ

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**Резюме.** Псориаз – хроническое воспалительное заболевание кожи, характеризующееся повышенной пролиферацией эпидермальных клеток, нарушением кератинизации и воспалительной реакцией в дерме, обусловленной активацией Т-лимфоцитов и синтезом провоспалительных цитокинов. Патофизиология псориаза также связана со снижением противовоспалительных функций иммуносупрессивных клеток. В последнее время все чаще встречаются случаи развития резистентности к проводимой терапии биопрепаратами в детском возрасте, требующие отмены препарата или его замены. Цель исследования – оценить содержание субпопуляций Т-хелперов в прогнозе эффективности биологической терапии у детей с псориазом. Иммунофенотипирование популяций Т-хелперов периферической крови проводили у 110 детей с вульгарным псориазом до назначения биологической терапии, на 16-й и 52-й неделе лечения. Возраст детей составил от 6 до 18 лет. Тяжесть псориаза и эффективность терапии оценивали по индексу PASI, который изменялся от 0 до 68 баллов. Методом проточной цитометрии определяли содержание Т-хелперов, регуляторных Т-клеток (Treg), активированных Т-хелперов (Thact) и Th17-лимфоцитов. В группе пациентов с хорошим эффектом биологической терапии получено значимое снижение PASI, как на 16-й неделе терапии ($p = 0,000$), так и к году лечения ($p = 0,017$). У детей с псориазом, не зависимо от длительности и эффективности биопрепаратов, был увеличен процент Thact относительно нормативных значений. В группе 1 до назначения биологической терапии был увеличен процент Thact ($p = 0,005$) и Th17 ($p = 0,001$). Анализ

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динамики содержания популяций Т-хелперов в течение года биологической терапии у детей с разной эффективностью лечения показал, что наиболее значимые изменения выявлены в содержании Th17 и Treg, а также их отношения Th17/Treg. ROC-анализ показал, что при отклонении Th17 выше 53%, Thact выше 181% и Th17/Treg выше 2,6 до назначения биопрепаратов в 75% случаев можно ожидать недостаточную эффективность терапии к году. К окончанию индукционного курса при отклонении Th17 выше 102% и отношении Th17/Treg выше 2,6 вероятность неэффективного лечения составляет уже 82%. В исследовании показана информативность оценки Thact до назначения биологической терапии, динамика Th17 к концу индукционного курса и Treg после 16 недель терапии в прогнозе эффективности ГИБП у детей с псориазом.

*Ключевые слова: дети, псориаз, биопрепараты, Т-хелперы, Th17, Treg*

# CONTENT OF CD4$^{+}$T CELL SUBPOPULATIONS IN PREDICTING THE EFFICACY OF BIOLOGICAL THERAPY FOR PSORIASIS IN CHILDREN

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**Abstract.** Psoriasis is a chronic inflammatory skin disease characterized by increased proliferation of epidermal cells, impaired keratinization and an inflammatory reaction in dermis caused by activation of T lymphocytes and synthesis of pro-inflammatory cytokines. The pathophysiology of psoriasis is also associated with a decrease in anti-inflammatory functions of immunosuppressive cells. Recently, there are more cases of development of resistance to ongoing therapy with biologics in children, requiring cancellation of drug or its replacement. The aim of the study was to evaluate the content of T helper subpopulations in prognosis of effectiveness of biologics in children with psoriasis. Immunophenotyping of T helper populations was performed in 110 children with psoriasis vulgaris before appointment of biologics, at 16 and 52 weeks. Age of children ranged from 6 to 18 years. Severity of psoriasis and effectiveness of therapy were assessed by index PASI, which varied 0-68. Content of Tregs, Thact and Th17 was determined by flow cytometry. In group with a sufficient effect of biologics, a decrease in PASI was obtained, both at week 16 of therapy ($p = 0.000$) and by year of treatment, $p = 0.017$. In children with psoriasis, regardless of duration and effectiveness of biologics, percentage of Thact was increased relative to normal values. In group 1 before prescription of biologics was increased percentage of Thact ($p = 0.005$) and Th17 ($p = 0.001$). Analysis of dynamics of content of small populations of T helper during 1 year of use of biologics in children with different efficacy of therapy showed that significant changes were found in content of Th17 and Treg, as well as their Th17/Treg. ROC analysis showed that when Th17 deviation was above 53%, Thact above 181% and Th17/Treg above 2.6 before biologics were prescribed, insufficient efficacy of therapy could be expected in 75% of cases by year. By the end of induction course, with a Th17 deviation above 102% and a Th17/Treg above 2.6, probability of ineffective treatment was already 82%. The study shows the informative value of assessment of Thact before appointment of biologics, dynamics of Th17 by the end of induction course and Treg after 16 weeks of therapy in prognosis of effectiveness of biologics in children with psoriasis.

*Keywords: children, psoriasis, biologics, T helpers, Th17, Treg*

## Introduction

Psoriasis is a systemic chronic inflammatory disease with an immunogenetic basis [3, 11]. Adaptive immune cells in psoriasis are activated by aberrant signaling of innate immune cells, and subsequently release inflammatory mediators that enhance psoriatic manifestations on the skin. The cellular link of the immune system is predominantly involved in the development and maintenance of inflammation in psoriasis [3, 14]. Clinically, psoriasis is characterized by well-defined red, scaly plaques, and histologically by increased proliferation of keratinocytes, dense skin inflammatory infiltrates and angiogenesis [7, 14].

A special role in psoriasis is assigned to Th17-lymphocytes (Th17) and cytotoxic T lymphocytes ($CD8^+$), since they largely produce cytokines of the interleukin (IL)-17 family, thereby stimulating the proliferation of keratinocytes [9, 13, 14]. It has been shown that in psoriasis, Th17 enhances the immune response of Th1 cells, mainly due to the production of IL-17A, which is responsible for the recruitment of neutrophils, activation of innate immunity cells, enhancement of B-cell functions and the release of pro-inflammatory cytokines [7, 9]. A high percentage of Th17 cells have been found in the circulating and affected skin of psoriasis patients and a direct correlation has been established between the number of Th17 cells and the PASI (Psoriasis Area and Severity Index) [8, 9, 13].

The pathophysiology of psoriasis is not only related to the activation of pro-inflammatory reactions, but also to a decrease in the anti-inflammatory functions of immunosuppressive cells [10, 11]. It has been shown that regulatory T cells (Treg), regulatory B cells and myeloid-derived suppressor cells (MDSCs) do not perform their classical homeostatic functions in psoriasis [8, 10, 11]. It is known that Tregs are able to suppress the activation and proliferation of effector cells through the production of TGF-β and IL-10 [5, 12]. An increase in Th17 content and a decrease in the proportion of Treg in the peripheral blood of patients with psoriasis lead to an imbalance of Th17/Treg, which correlates with the severity of the disease [7, 8, 9, 13].

Over the past decade, the use of biologics in children with psoriasis has shown its effectiveness, persistent long-term remission and significantly improved the quality of life of patients [2, 15]. The targeting effect of genetically engineered biological drugs (GEBD) is based on a blockade of the main pro-inflammatory cytokines of disease pathogenesis, such as tumor necrosis factor-alpha (TNFα), IL-17, IL-12 and IL-23 [2, 15]. However, in recent years, there are more and more cases of resistance to biologic therapy in children, requiring withdrawal or replacement of the drug [1, 4, 6]. Factors in the loss of response to GEBD therapy in patients with psoriasis include a high body mass index, a burdened family history and the production of antibodies to biologics [1, 2, 4, 6]. Nevertheless, there is a lack of information and guidelines on the tactics of switching biologics, and a complete lack of clear laboratory markers for predicting the efficacy of GEBD in children with psoriasis.

**The aim of the study** was to evaluate the content of $CD4^+$ cell subpopulations (Thact, Th17, Treg) in predicting the effectiveness of biologics in children with psoriasis.

## Materials and methods

In 110 children with psoriasis vulgaris, peripheral blood T helper subpopulations were examined before biological therapy, at weeks 16 and 52 of therapy with adalimumab, etanercept and ustekinumab. The effectiveness of therapy was assessed by achieving "PASI 75" or more by the year of therapy: group 1 included children with insufficient effect of biologics ("IE", less than PASI 75, n = 52), group 2 – children with good effect of biologics ("PASI 75" and more, n = 58). The age of the examined children ranged from 6 to 18 years. The children of groups 1 and 2 did not differ in age: 12.3 (7.8-16.4) years versus 12.5 (8.8-15.3) years, p = 0.821. Inclusion criteria in the study: the age of children from 6-18 years old, established diagnosis of psoriasis vulgaris, compliance with the frequency and dose of administration GEBD. Exclusion criteria: other forms of psoriasis in children, age over 18, inability to obtain a blood sample. The severity of psoriasis was assessed by the PASI, which varied from 0 to 68 (Me 14.0 (9.0-19.9)). The study complied with the ethical principles of the Declaration of Helsinki (WMA Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects, 2013) and was approved by the local ethical committee National Medical Research Center for Children's Health of the Russian Ministry of Health (protocol No. 2 of 14.02.2020).

Immunophenotyping of peripheral blood lymphocyte populations was performed by flow cytometry using monoclonal antibodies from the "Beckman Coulter" (USA). Sample preparation involved incubating 100 μL of whole blood with 10 μL of fluorochrome-labelled monoclonal antibodies for 20 minutes in the dark. For lysis of erythrocytes was used BD FACS™ Lysing Solution (BD Biosciences, USA), with incubation time in the dark at room temperature not exceeding 10-12 minutes. The samples obtained were recorded on flow cytometer the Novocyte (ACEA Biosciences, USA). The following populations were determined by the sequential gating method: $CD3^+CD4^+$ (T helpers), $CD4^+CD127^{low}CD25^{high}$ (Treg), $CD4^+CD25^+CD127^{high}$ (activated T helpers – Thact) and $CD3^+CD4^+CD161^+$ (Th17). Statistical analysis was performed using Statistica 10.0 (StatSoft,

USA) and ROC analysis using SPSS 16.0 (SPSS: An IBM Company, USA). Descriptive statistics of the number of cells are presented in the form of a median (lower – upper quartiles) – Me ($Q_{0.25}$-$Q_{0.75}$). The non-parametric Mann–Whitney test considered differences between independent groups; differences were considered significant at $p < 0.05$. The study included children of different ages; in this regard, in order to assess changes in the lymphocyte population, the deviation of individual indicators from the age norm level was calculated according to the formula:

$X_n = (X_{min}\text{-}X) / 0{,}01*(X_{max} - X_{min})$,

$X_n$, value of the individual index normalized to the age norm; X, value of the studied index; $X_{max}$, upper limit of the age norm; $X_{min}$, lower limit of the age norm. The range of age norm was taken as 100%.

## Results and discussion

Analysis of the efficacy of biologics in children with psoriasis, as measured by index the PASI, showed a significant and significant reduction in disease severity in the group of patients with good effect of GEBD (PASI 75), both at 16 weeks of therapy (PASI decreased from 20.1 (14-31) to 11.3 (7-15), p = 0.000), and by year of treatment – PASI decreased to 6.1 (1.5-9.9), p = 0.017. However, in the group of children with insufficient effect of biological therapy (group 1), the decrease in the PASI index was less pronounced and by the year of treatment of biologics PASI exceeded 10 points (16 weeks – 16.2 (15-21), 52 weeks of GEBD – 10.9 (4.9-22). It is worth noting that the groups of children with different efficacy of biologics before the appointment of therapy according to the PASI index did not differ (p = 0.631), but by the 16th week of therapy, a significant difference was revealed between the groups according to PASI (p = 0.000), which persisted by the year of treatment (p = 0.002).

An assessment of the content lymphocyte population showed that children with psoriasis, irrespective of the duration and effectiveness of therapy GEBD, had an increased percentage of Thact relative to the normal values (over 100; Table 1). In patients of group 1, the percentage of Thact (p = 0.005) and Th17 (p = 0.001) was significantly increased before the appointment of therapy, relative to the indicators of group 2 (Table 1, Figure 1).

At the end of the biological therapy induction course (16 weeks), children with psoriasis in group 1, as before the start of therapy, had a higher Th17 content than in group 2 (Table 1, Figure 1). The assessment of content Th17 by the year of biological therapy treatment in patients with insufficient effect also showed a significant increase in the content of this population relative to the indicators of group 2 (p = 0.003; Figure 1, Table 1).

Analysis of content Treg in children with psoriasis showed that regardless of the effectiveness of biological therapy both before treatment and at the end of

**TABLE 1. CONTENT OF Thact, Th17, Treg POPULATIONS IN CHILDREN WITH PSORIASIS AT DIFFERENT EFFECTIVENESS OF BIOLOGICAL THERAPY (% DEVIATION FROM AGE-SPECIFIC NORM)**

| Population | Duration of therapy, week | Group 1 Insufficient effect (IE, n = 52) | Group 2 PASI 75 (n = 58) | p |
|---|---|---|---|---|
| Thact, % CD4 | 0 | 225.2 (183.3-264.1) | 164.3 (114.3-235.9) | 0.005 |
| | 16 | 181.3 (149.1-211.7) | 202.3 (101.6-269.0) | 0.956 |
| | 52 | 198 (125.0-246.9) | 156.1 (79.7-169.0) | 0.279 |
| Treg, % CD4 | 0 | 31.8 (-12-82) | 70.8 (4.5-136.0) | 0.116 |
| | 16 | 66.2 (7.58-125.00) | 67.5 (48.0-131.8) | 0.359 |
| | 52 | -18.4 (-68.2-19.6) | 90.1 (16.0-159.1) | 0.008 |
| Th17, % CD4 | 0 | 73.5 (51.5-135.9) | 25.2 (-5.9-54.4) | 0.001 |
| | 16 | 161.1 (109.1-177.2) | 33.7 (-5.6-83.8) | 0.000 |
| | 52 | 117.4 (72-215) | 41.7 (-1.5-56.5) | 0.003 |

Note. p, differences between independent groups by Mann–Whitney test; p < 0.05.

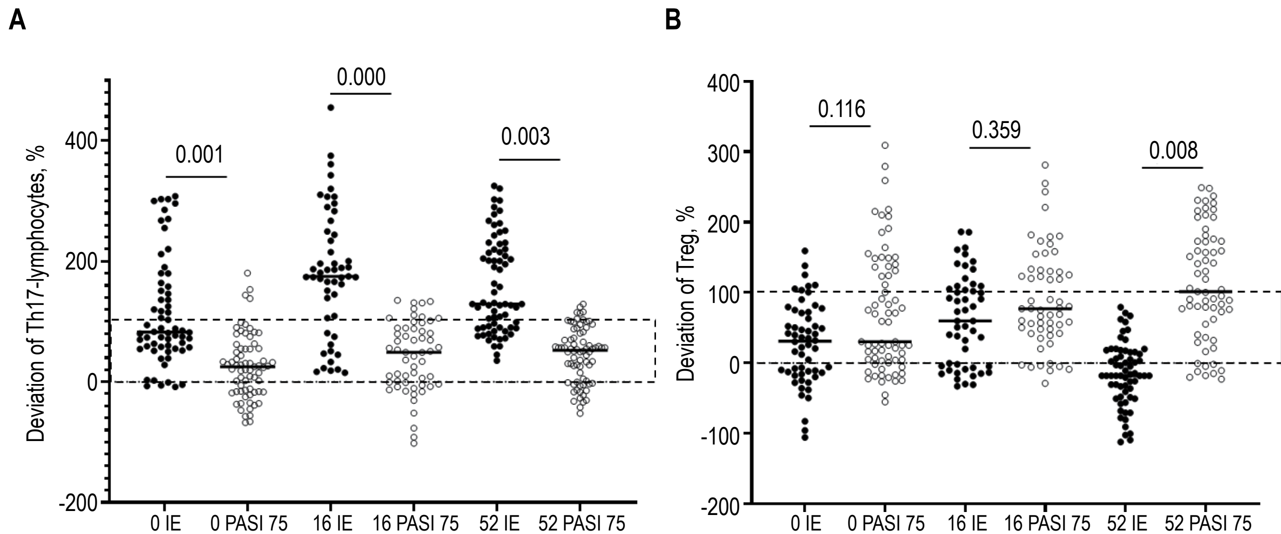


**Figure 1. Deviation of the relative numbers of Th17 (A) and Treg (B) with insufficient effect (IE) and achievement of PASI 75 in children with psoriasis before, at 16 and 52 weeks of biological therapy**

Note. The range of age norm was taken as 100%.

the induction course (16 weeks), the percentage of Treg in the groups was in the range of normal values (Figure 1, Table 1). However, by the year of treatment with biologics in group 1, a significant decrease in the percentage of Treg (p = 0.008) was obtained relative to the indicators of group 2 (p = 0.008), while in the group with insufficient effect of biologics, the percentage of Treg was lower than the normal values (Figure 1).

Assessment of the dynamics of T helper populations in children with psoriasis with insufficient effect of biological therapy revealed a significant increase in Th17 by 16 weeks of therapy ($p_{0-16} = 0.046$), which persisted to a year of therapy and was higher than normal values. Analysis of the dynamics of content Treg in this group showed a significant decrease in the population by year of therapy ($p_{0-52} = 0.047$, $p_{16-52} = 0.006$).

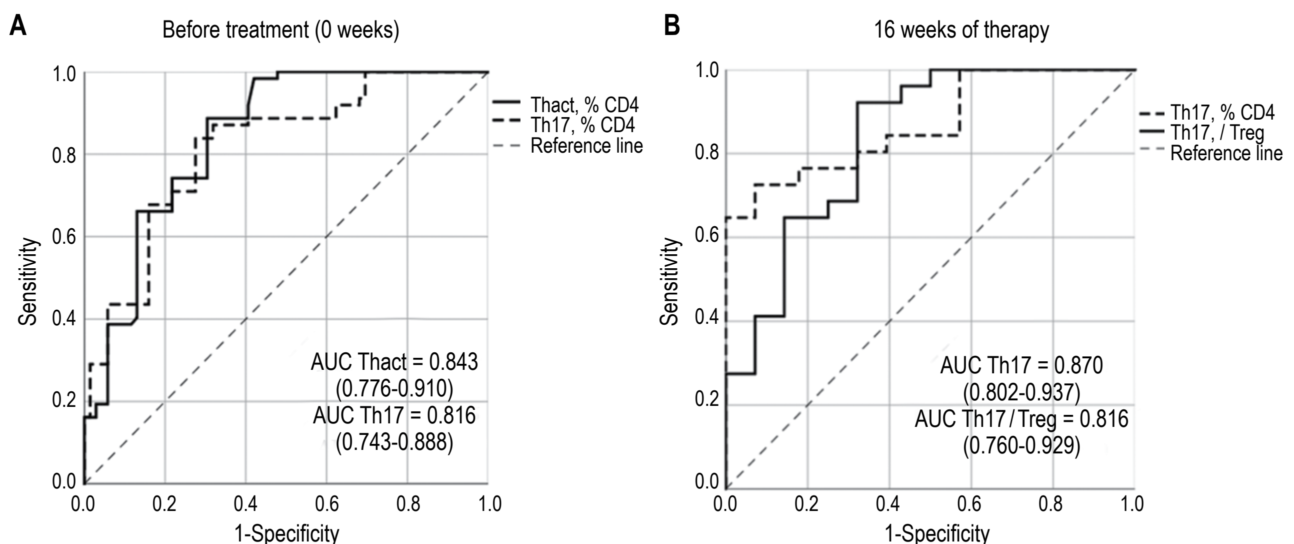


**Figure 2. ROC-analysis of deviation of Th17, Thact in predicting efficacy of biological therapy in children with psoriasis before prescription (A) and Th17, ratio Th17/Treg at 16 weeks of biological therapy (B)**

Note. AUC, area under curve.

Analysis of the Th17/Treg ratio in children with psoriasis at different effectiveness of biological therapy showed that the Th17/Treg ratio was significantly increased in the group of children with insufficient effect of biologics before therapy and during the year relative to group 2 (week 0: 3.0 (2.6-3.6) *vs* 1.6 (1.2-2.5), p = 0.001; week 52: 3.9 (2.6-4.3) *vs* 1.7 (1.2-2.3), p = 0.000).

The performed ROC analysis showed an excellent separating model for the "PASI 75 – insufficient effect" states for Th17, Thact and Th17/Treg ratio before the appointment of biological therapy and for Th17, Th17/Treg at 16 week of biologics (AUC > 0.8; Figure 2). The calculation of threshold values of indicators with the coincidence of sensitivity (Se) and specificity (Sp) showed that with a deviation of Th17 above 53%, Thact above 181% and Th17/Treg above 2.6 before the appointment of biologics in 75% of cases, insufficient effectiveness of biological therapy can be expected by the year. ROC analysis of the immunological parameters at 16 weeks showed that with a Th17 deviation above 102% and a Th17/Treg ratio above 2.6, there is already an 82% chance of ineffective treatment (Figure 2).

## Conclusion

Thus, this analysis allowed us to identify the most informative $CD4^{+}$ cell counts in predicting and evaluating the effectiveness of biologics in children with psoriasis. Analysis of the dynamics of small populations T helper during year of biologics in children with different efficacy of therapy showed that the most significant changes were detected in the content of Th17 and Treg populations and their ratio (Th17/Treg), which is consistent with the results in adult patients with psoriasis [5, 7, 8, 13]. The study demonstrates the information value of content Thact estimation before biological therapy, the dynamics of Th17 by the end of induction course and the dynamics of content Treg after 16 weeks of biological therapy in prognosis of biologics efficacy in children with psoriasis.

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# МИЕЛОИДНЫЕ СУПРЕССОРНЫЕ КЛЕТКИ В КАЧЕСТВЕ БИОМАРКЕРОВ ЭФФЕКТИВНОСТИ ТЕРАПИИ НОВЫМИ БИОЛОГИЧЕСКИМИ ПРЕПАРАТАМИ У БОЛЬНЫХ АКСИАЛЬНЫМ СПОНДИЛОАРТРИТОМ

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**Резюме.** Большое значение в патогенезе аксиального спондилоартрита (АксСп) отводится клеткам врожденного иммунитета, в том числе клеткам миелоидного ряда – супрессорным клеткам миелоидного происхождения (МС). МС представляют гетерогенную популяцию незрелых клеток, способных подавлять реакции врожденного и приобретенного иммунитета с наиболее выраженной супрессорной активностью в отношении Т-клеток. Терапия генно-инженерными биологическими препаратами позволяет снизить клинико-лабораторную активность заболевания у больных АксСп, однако их эффективность широко варьирует у разных пациентов. Настоящее исследование направлено на изучение различных субпопуляций МС и их супрессорного потенциала при АксСп, в зависимости от ответа на терапию генно-инженерными биологическими препаратами. В исследование были включены пациенты с АксСп с продолжительностью заболевания 16,5 лет (медиана); HLA-B27 (+) статус был выявлен в 79% случаев. Все пациенты в течение как минимум последних 12 недель получали биологическую терапию, в том числе ингибиторы TNF (этанерцепт, цертолизумаб пэгол, адалимумаб или голимумаб) или ингибиторы IL-17 (секукинумаб, иксекизумаб или нетакимаб). Относительное содержание гранулоцитарных МС (Г-МС, $Lin^-HLA\text{-}DR^-CD33^+CD66b^+$), моноцитарных МС (М-МС, $HLA\text{-}DR^{low/-}CD14^+$), МС ранних стадий дифференцировки (Р-МС, $Lin^-HLA\text{-}DR^-CD33^+CD66b^-$), а также внутриклеточную экспрессию аргиназы-1 оценивали методом проточной цитометрии. Пациенты со стабильным ответом на биологическую терапию значимо не отличались от здоровых доноров

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по содержанию всех трех популяций МС в периферической крови. Пациенты с АксСп, не отвечающие на терапию, демонстрировали повышенное относительное и абсолютное количество Р-МС по сравнению со здоровыми донорами ($p_U$ = 0,01 и $p_U$ = 0,02 соответственно) и пациентами со стабильным ответом ($p_U$ = 0,03 и $p_U$ = 0,07 соответственно). При этом повышенное содержание Р-МС в случае отсутствия ответа на терапию ассоциировалось с показателями активности – СОЭ ($R_s$ = 0,821; p = 0,023), СРБ ($R_s$ = 0,714 p = 0,07) и $ASDAS_{СРБ}$ ($R_s$ = 0,829; p = 0,042). В группе пациентов со стабильным ответом корреляционной зависимости между активностью заболевания и содержанием МС не обнаружено. Для оценки супрессорного потенциала МС была проанализирована экспрессия внутриклеточной молекулы аргиназа-1, которая участвует в ингибировании Т-клеточного ответа. Пациенты со стабильным ответом характеризовались повышенной экспрессией аргиназы-1 в Р-МС ($p_U$ = 0,02). При отсутствии ответа на терапию значимых изменений в экспрессии Arg-1 не выявлено, однако доля Arg-1-экспрессирующих Г-МС находилась в прямой сопряженности с индексами воспаления $ASDAS_{СОЭ}$ ($R_s$ = 0,857; p = 0,014) и BASDAI ($R_s$ = 0,785; p = 0,036). Таким образом, Р-МС, а также экспрессия супрессорной молекулы Arg-1 в МС могут служить биомаркерами эффективности ответа на проводимую терапию генно-инженерными биологическими препаратами, а также выступать в роли потенциальных маркеров-кандидатов с точки зрения раннего предиктора ответа на проводимую терапию.

*Ключевые слова: миелоидные супрессоры, аксиальный спондилоартрит, биологическая терапия, аргиназа-1, ингибитор TNF, ингибитор IL-17*

# MYELOID-DERIVED SUPPRESSOR CELLS AS BIOMARKERS OF THE EFFECTIVENESS OF THERAPY WITH NEW BIOLOGICAL AGENTS IN AXIAL SPONDYLOARTHRITIS

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**Abstract.** Innate immune cells, including myeloid cells – myeloid derived suppressor cells (MDSCs) – are supposed to play an important role in the pathogenesis of axial spondyloarthritis (AxSp). Myeloid derived suppressor cells represent a heterogeneous population of immature cells capable of suppressing innate and adaptive immune responses with the most pronounced suppressor activity against T cells. Biological disease-modifying antirheumatic drugs (bDMARDs) can reduce the clinical and laboratory disease activity, but their effectiveness varies widely in different patients with AxSp. The present study is aimed at studying MDSCs subpopulations and their suppressive function depending on the response to bDMARD therapy in AxSp. The study included AxSp patients with a disease duration of 16.5 years (median); HLA-B27 (+) status was detected in 79% of cases. All patients received bDMARDs at least the past 12 weeks, including TNF inhibitors (etanercept, certolizumab pegol, adalimumab, or golimumab) or IL-17 inhibitors (secukinumab, ixekizumab, or netakimab). Percentage of granulocytic MDSCs (G-MDSCs, $Lin^-HLA\text{-}DR^-CD33^+CD66b^+$), monocytic MDSCs (M-MDSCs, $HLA\text{-}DR^{low/-}CD14^+$), MDSCs of early stage differentiation (E-MDSCs, $Lin^-HLA\text{-}DR^-CD33^+CD66b^-$), as well as intracellular expression of arginase-1 was assessed by flow cytometry. Frequency of circulating MDSC subpopulations of patients with a stable response to bDMARDs (responders) did not differ significantly compared to healthy donors. Patients not responding to bDMARDs therapy showed increased relative and absolute number of E-MDSCs compared to healthy donors ($p_U$ = 0.01 and $p_U$ = 0.02, respectively) and the responders ($p_U$ = 0.03 and $p_U$ = 0.07, respectively). Increased percentage of E-MDSCs was positively correlated to disease activity – ESR ($R_s$ = 0.821; p = 0.023), CRP ($R_s$ = 0.714; p = 0.07) and $ASDAS_{CRP}$ ($R_s$ = 0.829; p = 0.042) in the non-responder group. Responder patients exhibited no correlation between disease activity and circulating MDSCs. The suppressor potential of MDSCs was analyzed by the

intracellular expression of arginase-1 molecule which is involved in the inhibition of T cell response. Patients with the stable response were characterized by increased expression of arginase-1 in E-MDSCs compared to donors ($p_U = 0.02$). Non-responders did not demonstrate significant changes in Arg-1 expression, however, the percentage of arginase-1-expressing G-MDSCs was positively correlated to indexes $ASDAS_{ESR}$ ($R_s = 0.857$; $p = 0.014$) and BASDAI ($R_s = 0.785$; $p = 0.036$). Thus, E-MDSCs as well as arginase-1 expression in MDSCs may serve as biomarkers of effectiveness bDMARD therapy, and act as potential candidate predictors of response to therapy in AxSp.

*Keywords: myeloid-derived suppressor cells, axial spondyloarthritis, biological therapy, arginase-1, TNF inhibitor, IL-17 inhibitor*

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## Introduction

Axial spondyloarthritis (AxSp) is a chronic inflammatory disease of the axial skeleton with frequent involvement of entheses and peripheral joints, as well as other organs and systems, in the pathological process. The leading role in the development and progression of AxSp is assigned to pro-inflammatory cytokines – IFNγ, TNFα, IL-1 and IL-6 [12]. Advances in understanding the immunopathogenesis of AxSp have served as the basis for the development of new therapies aimed at neutralizing the activity of these pro-inflammatory cytokines [2, 12]. The appointment of biological disease-modifying antirheumatic drugs (bDMARDs) allows to quickly reduce the clinical and laboratory disease activity, improve quality of life and slow radiographic AxSp progression [2, 7]. Despite the high efficiency, it has been reported failure of TNFα or IL-17 inhibitors in a significant percentage of AxSp patients [1, 4, 10]. The use of new biomarkers that could be considered predictors of good response/non-response to bDMARD therapy would effectively control the disease and reduce the economic burden of biological therapy.

As AxSp is believed to be more of an autoinflammatory than an autoimmune disease, innate immunity cells, in particular myeloid cells – myeloid derived suppressor cells (MDSCs) – play an important role in its pathogenesis. MDSCs represent a heterogeneous immature cell population of myeloid origin, capable of suppressing the innate and adoptive response with the most pronounced suppressor activity against T cells [3]. In humans, MDSCs includes granulocytic (polymorphonuclear), monocytic MDSCs, and MDSCs of early stage of differentiation (G-MDSCs, M-MDSCs, and E-MDSCs, respectively), which differ in morphological features, expression of membrane markers, and mechanisms of their suppressor activity. However, data on the functional activity of MDSCs in autoimmune and autoinflammatory diseases are controversial, including reports of intact suppressive activity of MDSCs, diminished or its complete absence [5, 6, 8, 11, 13].

**The present study was aimed** to analyzed circulating MDSC subpopulations and their suppressor function depending on response to bDMARDs in AxSp patients.

## Materials and methods

The study included 28 patients with AxSp (19 men and 9 women aged 19 to 56 years) and 36 age-matched healthy donors. Informed consent was obtained from all patients and donors according to the Declaration of Helsinki. All patients met the modified New York criteria for diagnosis of AxSp and/or Assessment of Spondyloarthritis International Society criteria for AxSp. The disease duration was 16.5 years (median). Twenty-two patients (79%) were HLA-B27-positive. All patients received bDMARDs at least for the past 12 weeks. Nineteen out of 28 patients received TNF inhibitors (etanercept, certolizumab pegol, adalimumab, or golimumab); nine out of 28 patients received IL-17 inhibitors (secukinumab, ixekizumab, or netakimab).

Disease activity was determined according to $ASDAS_{ESR}$ and/or $ASDAS_{CRP}$ indexes. Most patients (20/28, 71%) demonstrated low/moderate AxSp activity (ASDAS index < 2.1). In 29% of cases (8/28) high/very high AxSp activity was determined (ASDAS index ≥ 2.1). Additionally, the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) and Bath Ankylosing Spondylitis Functional Index (BASFI) were used to evaluate the activity and severity of functional limitations, respectively.

The frequencies of G-MDSCs ($Lin^-HLA\text{-}DR^-CD33^+CD66b^+$), M-MDSCs ($CD14^+HLA\text{-}DR^{low/-}$), and E-MDSCs ($Lin^-HLA\text{-}DR^-CD33^+CD66b^-$) was assessed by flow cytometry using anti-Lineage Cocktail 1 (CD3, CD14, CD16, CD19, CD20, CD56; FITC, BD Biosciences, USA), anti-CD14 (FITC, BD Biosciences), anti-CD33 (PerCP-Cy5.5, BD Biosciences), anti-CD66b (APC, BioLegend, USA), anti-HLA-DR (FITC, APC-Cy7, PerCP, BD Biosciences) monoclonal antibodies. The number of MDSC subpopulations are presented as a percentage of mononuclear cells (MNCs). The absolute number of MDSCs was calculated using data on the percentage

**TABLE 1. MYELOID-DERIVED SUPPRESSOR CELL SUBPOPULATIONS DEPENDING ON THE PATIENT RESPONSE TO bDMARD THERAPY IN AxSp**

| MDSCs | Donors (n = 21-36) | AxSp patients | |
|---|---|---|---|
| | | Responders (n = 17-18) | Non-responders (n = 7-10) |
| G-MDSCs (%) | 0.05 (0.04-0.07) | 0.08 (0.02-0.16) | 0.13 (0.04-0.24) |
| E-MDSCs (%) | 0.82 (0.662-1.340) | 1.1 (0.62-1.58) | 1.9* # (1.0-2.6) |
| M-MDSCs (%) | 1.7 (1.2-2.4) | 2.1 (1.57-3.00) | 1.6 (1.2-2.7) |
| MNCs (× $10^9$/L) | 2.5 (2.1-2.7) | 2.9* (2.6-3.4) | 2.8* (2.5-3.2) |
| G-MDSCs (× $10^6$ cells/mL) | 1.4 (1.1-2.0) | 2.8 (0.7-5.2) | 2.6 (0.9-5.1) |
| E-MDSCs (× $10^6$ cells/mL) | 25.4 (13.0-31.4) | 32.2 (17.3-44.8) | 47.9* (29.3-58.0) |
| M-MDSCs (× $10^6$ cells/mL) | 46.3 (28.2-71.6) | 56.6 (42.4-95.5) | 50.6 (24.7-86.8) |

Note. Relative (%) and absolute (× $10^6$ cells/ml) numbers of granulocytic (G-MDSCs), early-stage MDSCs (E-MDSCs) and monocytic (M-MDSCs) MDSCs in peripheral blood samples are shown as median and interquartile range (IQR) in the donor group and the responders and non-responders to biological disease-modifying antirheumatic drugs; *, $p_U$ value < 0.05 compared to donors; #, $p_U$ value < 0.05 compared to responders.

**TABLE 2. ARGINASE-1 EXPRESSION IN MYELOID-DERIVED SUPPRESSOR CELL SUBPOPULATIONS DEPENDING ON THE PATIENT RESPONSE TO bDMARD THERAPY IN AxSp**

| Arg-1⁺ cells (%) | Donors (n = 16-32) | AxSp patients | |
|---|---|---|---|
| | | Responders (n = 8-13) | Non-responders (n = 7) |
| Arg-1⁺G-MDSCs | 70.0 (43.0-89.4) | 86.3 (71.3-96.6) | 75.0 (29.0-87.2) |
| Arg-1⁺E-MDSCs | 16 (5.8-31.0) | 44.3* (33.9-50.7) | 19.0 (16.0-36.0) |
| Arg-1⁺M-MDSCs | 19.8 (11.4-38.8) | 14.2 (6.5-26.7) | 7.0 (4.5-21.3) |

Note. Data are presented as median and interquartile range (Me ($Q_{0.25}$-$Q_{0.75}$) of the percentage of arginase-1-positive cells among granulocytic (G-MDSCs), monocytic (M-MDSCs) and early-stage MDSCs (E-MDSCs) in peripheral blood of donors and AxSp patients with different response to biological disease-modifying antirheumatic drugs; *, $p_U$ value < 0.05 compared to donors.

of MDSC subpopulations and the absolute number of MNCs (lymphocytes + monocytes; blood test).

To assess the intracellular expression of arginase-1 (Arg-1), MNCs were stained with fluorochrome-conjugated monoclonal antibodies to G-MDSCs, M-MDSCs, E-MDSCs according to the standard method for determining surface antigens. Then, cells were permeabilized using Fixation/permeabilization solution kit (BD Cytofix/Cytoperm™, USA) according to the manufacturer s instructions and incubated with PE- or APC-conjugated anti-Arg-1 (BD PharMingen, USA) monoclonal antibodies. The relative amount of MDSCs expressing Arg-1 was assessed among $Lin^-HLA\text{-}DR^-CD33^+CD66b^+$, $Lin^-HLA\text{-}DR^-CD33^+CD66b^-$ and $CD14^+HLA\text{-}DR^{low/-}$ cells. In each experiment, isotype-matched control monoclonal antibodies were used to determine non-specific background staining.

Statistical data processing was performed using the Statistica 6.0 (StatSoft) and GraphPad Prism 8 software package. Data are presented as median (Me) and interquartile range ($Q_{0.25}$-$Q_{0.75}$). To identify significant differences, the nonparametric Mann–Whitney U-test was used for independent samples. Correlation analysis was performed using Spearman's rank correlation ($R_s$). Differences were considered statistically significant at p value < 0.05.

## Results and discussion

In patients with a stable response to bDMARDs (during the last 12 weeks of therapy; responders), including 13 patients receiving TNF inhibitors and 5 patients receiving IL-17 inhibitors, the frequency of circulating G-MDSCs, M-MDSCs and E-MDSCs was not significantly different from healthy donors (Table 1). Patients who did not respond to bDMARDs (according to the criteria for the absence of effectiveness; non-responders), including 6 patients receiving TNF inhibitors and 4 patients with IL-17 inhibitors, showed an almost two-fold increase in the relative and absolute number of E-MDSCs compa-

red with healthy donors ($p_U$ = 0.01 and $p_U$ = 0.02, respectively). Besides, non-responders were characterized by a significantly increased percentage ($p_U$ = 0.03) and tendency to higher absolute number of E-MDSCs ($p_U$ = 0.07) compared with the responder group.

In the non-responder group, the increased frequency of E-MDSCs was associated with inflammation activity. The frequency of E-MDSCs was revealed to have a high correlation with ESR ($R_s$ = 0.821; p = 0.023), CRP ($R_s$ = 0.714; p = 0.07) and $ASDAS_{CRP}$ ($R_s$ = 0.829; p = 0.042). Absolute E-MDSC count was correlated as a trend with ESR ($R_s$ = 0.607; p = 0.15) and CRP ($R_s$ = 0.607; p = 0.15). In the group of patients with the stable response to bDMARDs, no correlation between AxSp activity and MDSCs was found.

To assess the suppressor potential of MDSCs in AxSp, intracellular expression of Arg-1 molecule (Table 2) involved in the arginine depletion in the extracellular space and inhibiting the T cell activation was analyzed. Non-responders demonstrated a tendency to reduced expression of Arg-1 in M-MDSCs ($p_U$ = 0.12), whereas the responder group had no difference with the donor group. Responders to bDMARDs were characterized by more than 2.5-fold increased expression of Arg-1 in E-MDSCs ($p_U$ = 0.02). Non-responders did not demonstrate significant changes in Arg-1-expressing E-MDSCs.

In the responder group, Arg-1 expression in G-MDSCs directly correlated with the BASDAI index ($R_s$ = 0.667; p = 0.07), and in E-MDSCs it inversely associated with the BASDAI index ($R_s$ = -0.691; p = 0.057). In the non-responder group, the percentage of Arg-1-expressing G-MDSCs was positively correlated to indexes $ASDAS_{ESR}$ ($R_s$ = 0.857; p = 0.014) and BASDAI ($R_s$ = 0.785; p = 0.036).

Thus, the present study has shown that the ineffectiveness of bDMARD therapy in AxSp patients was associated with the increased number of E-MDSCs, as well as the trend towards the decrease in the Arg-1-expressing M-MDSCs. The achievement of stable response to therapy with bDMARDs was associated with the similar Arg-1 expression in M-MDSCs and significantly increased expression of Arg-1 in E-MDSCs comparable with the donors.

Previously, we have shown that patients with AxSp were characterized by an increased number of G-MDSCs and M-MDSCs both in the first line of therapy and in bDMARD therapy [9]. However, we did not take into account the responsiveness of patients to bDMARDs. The data obtained in the present study have demonstrated no differences in the number of MDSC subpopulations between the donors and patients with the stable response to bDMARDs. The inhibition of TNF or IL-17, as the key cytokines in maintaining chronic inflammation, may be also accompanied by blocking the differentiation of myeloid progenitors into immature myeloid cells. Accumulation of E-MDSCs in the non-responders to TNF or IL-17 inhibitors seems to be due to the involvement of other factors in AxSp pathogenesis, which determine resistance to bDMARDs.

Therapy with bDMARDs may lead to changes in Arg-1 expression in MDSCs. The responder group have demonstrated significantly increased expression of Arg-1 in E-MDSCs, which may be important in terms of the regulation of inflammation. The decrease in expression of Arg-1 in M-MDSCs detected at high / very high AxSp activity in non-responders can be one of the pathogenetic mechanisms of development and maintenance of the inflammatory process in AxSp.

## Conclusion

Taken together, we conclude that E-MDSCs, as well as the Arg-1 expression in MDSCs can serve as biomarkers of the effectiveness of the response to bDMARD therapy, and also act as potential candidate markers in terms of an early predictor of response to bDMARD therapy.

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# СРАВНЕНИЕ ФЕНОТИПИЧЕСКИХ СВОЙСТВ ВРОЖДЕННЫХ ЛИМФОИДНЫХ КЛЕТОК НА РАЗНЫХ СТАДИЯХ РЕВМАТОИДНОГО АРТРИТА

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**Резюме.** Аутоиммунные заболевания на сегодняшний день занимают лидирующее место по частоте встречаемости в популяции, среди которых один процент занимает ревматоидный артрит (РА). Ремиссия при данном виде заболевания достигается крайне редко и требует постоянного использования фармакотерапии. В связи с этим необходимо подробно изучать патогенез РА для поиска новых мишеней лекарственных препаратов. Известно, что в развитии РА принимают участие Т-хелперы (Th)1 и Th17. Однако некоторые исследователи предполагают, что в развитии РА играют роль ILC. ILC являются «врожденными аналогами» Th, ввиду того, что данная субпопуляция синтезирует такие же цитокины. ILC1 является врожденными аналогами Th1, ILC2-Th2, ILC3-Th17. ILC представляют собой резидентные в тканях врожденные лимфоидные клетки, которые имеют функциональное разнообразие и регулируют направленность иммунного ответа с помощью продукции цитокинов.

В качестве материала мы использовали мононуклерные клетки периферической крови (МНК ПК) от пациентов (n = 19) и условно-здоровых доноров (n = 10). Группа пациентов была разделена в зависимости от терапии: ГИБП и МТХ, а также в зависимости от стадии РА (ранний и очень ранний артрит, развернутый и поздний). МНК ПК были окрашены моноклональными антителами и определялись как $Lin^{-}CD127^{+}$, в общей популяции оценивали количество $CD294^{+}$ILC (ILC2), $CD117^{-}CD294^{-}$ILC были идентифицированы как ILC1, а $CD117^{+}CD294^{-}$ILC были определены как ILC3.

Мы получили следующие результаты: количество ILC1 было достоверно снижено у пациентов, получавших МТ по сравнению с пациентами, находящимися на ГИБП и условно здоровыми донорами. Однако пациенты на МТХ с поздней стадией РА имели низкие уровни ILC2 и ILC3 по сравнению с

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пациентами на ГИБП. Доля ILC2 достоверно возрастала у пациентов на ранних стадиях РА по сравнению с пациентами с поздней стадией РА. Однако ILC1 были значительно снижены у пациентов, получавших МТХ, а ILC3 значительно увеличились у пациентов, получавших МТХ по сравнению с ГИБП.

Экспрессия PD1 на ILC1 была повышена по сравнению с пациентами, получавшими ГИБП. Однако ILC3 пациентов с поздними стадиями на МТХ имели повышенную экспрессию PD1 по сравнению с пациентами, принимавшими ГИБП. ILC3 доноров был значительно повышен по сравнению с пациентами на ГИБП.

*Ключевые слова: врожденные лимфоидные клетки, ревматоидный артрит, контрольные точки иммунного ответа, проточная цитометрия, цитокины*

# COMPARISON OF PHENOTYPIC PROPERTIES OF INNATE LYMPHOID CELLS AT VARIOUS STAGES OF RHEUMATOID ARTHRITIS

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**Abstract.** Autoimmune diseases currently take a leading place in terms of frequency of occurrence in the population, among which 1 percent is occupied by rheumatoid arthritis (RA). Remission in this type of disease is extremely rare and requires constant use of pharmacotherapy. Studying the pathogenesis of RA is necessary to study to search for new drug targets. It is known that T helpers 1 (Th) and Th17 are involved in the development of RA. However, some researchers suggest that ILCs play a role in the development of RA. ILCs are “innate analogues” of Th, due to the fact that this subpopulation synthesizes the same cytokines. ILC1 is innate analogs of Th1, ILC2-Th2, ILC3-Th17. ILCs are tissue-resident innate lymphoid cells that have functional diversity and regulate the direction of the immune response through the production of cytokines.

We used peripheral blood mononuclear cells (PBMCs) from patients (n = 19) and conditionally healthy donors (n = 10) as material. The group of patients was divided biologic disease-modifying anti-rheumatic drugs (bDMARDs) and Metotrexate (MTX) and of stage of RA (early and very early arthritis, advanced and late). PBMCs were stained with monoclonal antibodies. ILCs were identified as Lin-CD127$^+$, CD294$^+$ILCs (ILC2) were measured in the general population, CD117-CD294-ILCs were identified as ILC1, and CD117$^+$CD294$^-$ ILCs were identified as ILC3.

We obtained the following results: ILC1 was significantly reduced in patients treated with MTX comparison with patients on bDMARDs and healthy donors. However, patients on MTX with advanced RA had low levels of ILC2 and ILC3 compared to patients on bDMARDs. ILC2 significantly increased in patients with early stages of RA comparison with patients with advanced RA. However, ILC1 was significantly reduced in patients treated with MTX, and ILC3 increased significantly in patients treated with MTX comparison with bDMARDs. Expression of PD1 on ILC1 was increased compared to patients treated with bDMARDs. However, ILC3 patients with advanced stages on MTX had increased expression of PD1 comparison with patients taking bDMARDs. The ILC3 of donors was significantly increased comparison with patients on bDMARDs.

*Keywords: ILC, rheumatoid arthritis, immune checkpoint molecules, flow cytometry, cytokines*

The study was carried out within the framework of research project No. 122012000366-9 “Study of the immunopathogenesis of phenotypes of socially significant human diseases and polymorbidity as a basis for the development of new methods of personalized diagnosis and treatment”.

## Introduction

Today rheumatoid arthritis (RA) is one of the most common diseases among autoimmune diseases [8, 9]. RA is associated with progressive disability, systemic damage of organs and tissues, as well as with economic costs for society [6]. Until today, research

on rheumatoid arthritis is important and a significant problem due to low efficiency of medicines and the risk of developing unwanted effects. Therefore, it is necessary to study in detail the pathogenesis of RA and to search for new drug targets. Remission of RA is achieved extremely rarely and requires permanent use of pharmacotherapy [6]. It is interesting to research the role of innate lymphoid cells (ILC) in the development of autoimmune inflammation in RA, especially the role of plasticity of these cells. ILCs are tissue-resident innate lymphoid cells which have a functional diversity similar to T cells. In addition, ILCs regulate the direction of the immune response through the production of cytokines. Accordingly, understanding of these processes will allow the development of new therapeutic strategies aimed at reducing inflammation or enhancing antitumor immunity and based on the possible reprogramming of T cell populations towards one or another phenotype [1, 2].

ILCs are same as Th1 because they respond to intracellular pathogens, secrete IFNγ, and they depend on T-bet for their differentiation. ILC2s are like Th2 cells which produce high levels of interleukin (IL)-4, IL-5 and IL-13 in response to IL-33, IL-25 and thymic stromal lymphopoietin (TSLP). ILC-2s express high levels of the transcription factors GATA3 and RORα. ILC-3s are innate analogues of Th17 cells. It depends on RORγt [1, 2, 7, 11].

## Materials and methods

The study included 10 volunteers and 19 patients with RA divided into 3 groups according to the disease stage and treatment: (1) late and advanced stages 10 patients were treated with biologic disease-modifying anti-rheumatic drugs (bDMARDs); (2) late and advanced stages 5 patients were treated with methotrexate (MTX); (3) early and very early stages 4 patients were treated with MTX 9 ml of blood was collected from donors and patients. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood in a ficoll-urografin density gradient (1.077 g/ml). Isolated PBMCs were stained with fluorochrome-conjugated monoclonal antibodies: anti-Lineage (CD3/14/16/19/20/56) and anti-FceR1 alpha-FITC, anti-CD294-APC/Cy7, anti-CD127-PerCP/Cy5.5, anti-CD336-PE, anti-CD117-APC.C3. ILCs were defined as $Lin^-$ $CD127^+$, $CD294^+$ILCs were estimated as ILC2, $CD117^-CD294^-$ILCs were defined as ILC1, and $CD117^+CD294^-$ILCs were identified as ILC3. The cell phenotype was analyzed on a FACS Canto II flow cytometer (BD Biosciences, USA). Statistical processing of the obtained data was carried out using the GraphPad Prism 9.0.0 application package with one way ANOVA. Differences were considered significant at $p < 0.05$.

## Results and discussion

We found that ILC 1 is significantly reduced in patients treated with MTX in comparison with volunteers (D) and patients on bDMARDs in all stages of RA. However, we found that in patients with advanced and late stages of RA treated with MTX had significantly reduced ILC2 and ILC3 compared to volunteers and patients in bDMARDs (Figure 1). ILCs2 were significantly increased in patients in early and very early stages of RA in comparison with patients on late stage of RA on MTX and bDMARDs. However ILCs1 were significantly reduced in patients which were treated with MTX, but ILCs3 were significantly increased in patients on MTX comparison with patients in bDMARDs (Figure 2).

ILC1 of patients who were treated with MTX had significantly increased expression of PD1 in comparison with patients on bDMARDs. However we noted relative to ILC3 that expression of PD 1 is significantly increased in patients who were treated with MXT (late stages of RA) in comparison with patients with use bDMARDs. Also we found that ILC3 of donors are significantly increased in comparison with patients were treated with bDMARDs, however were compared to ILC1 and ILC2 donors and patients on bDMARDs we found trend. In the proportion of ILC2 $cKit^+$ we didn't obtain significant results (Figure 3).

It has been shown that various cells play a role in the development of RA, such as DCs, T and B cells and other cells. ILC have been seems have been involved in the pathogenesis of development RA too [2, 10, 11, 13].

This study demonstrates that numbers of ILCs depend on stage of RA as well as therapy. Some research reports that numbers of ILC1 and ILC3 is increased in the peripheral blood, synovial fluid, and lymph node of patients with RA, however numbers of ILC2 were low [5]. Apparently, the balance of ILC changes in patients with RA. In early stage of RA we observed ILC1 was low, but ILC2 is increased in the peripheral blood. These results are consistent with previous studies [12]. While the number of ILC2 in the synovial fluid (SF) of patients was decreased in active phase of RA, it was increased in remission [3, 12]. In later stage of RA we observed that numbers of ILC1 is significant increased. ILC 2 has been suggested to play a protective role synthesizing IL-4 and IL-5 activated Th2 to downregulate the inflammatory processes in RA [12]. ILCs2 secrete IL-10 and TBF b to reduce inflammation in gut by suppressing both ILC1 and ILC3 [2, 11, 13].

As known, patients with RA have an overactive immune response. These patients have a biased immune response, T helpers (Th)1 and Th17 which are significantly increased in comparison with Th2. ILCs are "innate analogues Th". ILCs locate on barrier

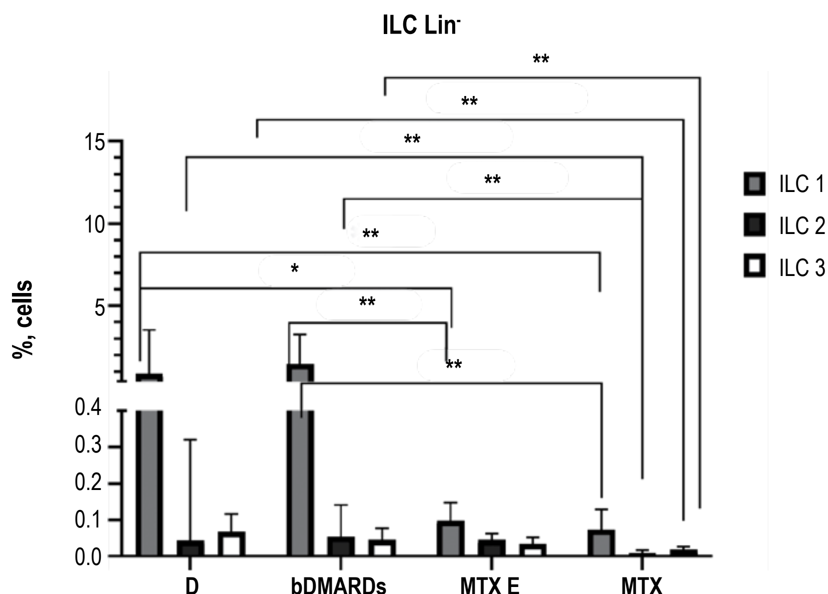


**Figure 1. Number of ILC Lin⁻. D, healthy donors, bDMARDs (biologic disease-modifying anti-rheumatic drugs), MTX E (patients who were treated MTX with early stage RA), MTX (patients who were treated MTX with advance stage RA)**

Note. Data are presented as median ± interquartile range with n = 10 (D), n = 12 (bDMARDs), n = 4 (MTX E.), n = 5 (MTX).*, significant differences are $p < 0.05$ by employing one-way ANOVA.

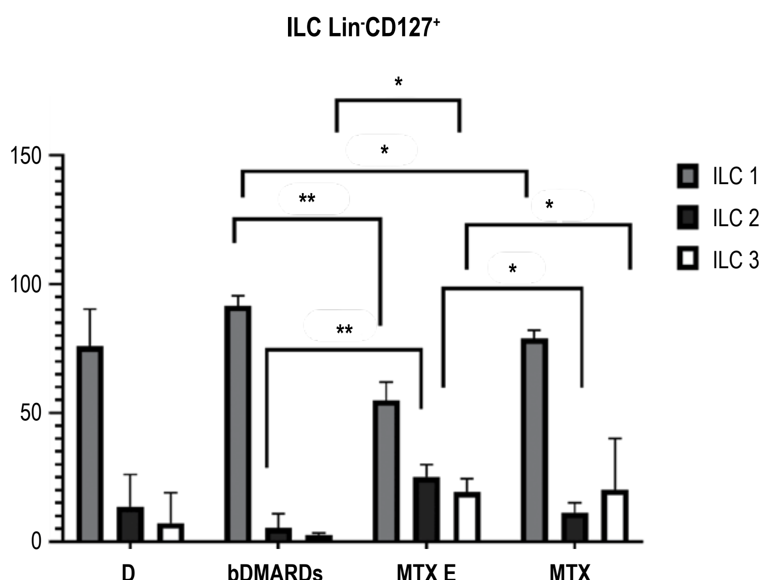


**Figure 2. Number of ILC Lin⁻CD127⁺. D, healthy donors, bDMARDs (biologic disease-modifying anti-rheumatic drugs), MTX E (patients who were treated MTX with early stage RA), MTX (patients who were treated MTX with advance stage RA)**

Note. Data are presented as median ± interquartile range with n = 8. *, significant differences are $p < 0.05$ by employing one-way ANOVA.

tissues and first respond on various stress signals by synthesizing cytokines to affect to TH. So we conclude that balance Th depends on ILCs and in that way anti-rheumatic therapy can balance the population of T helpers. [3]. Herman et al. [4] reported that MTX significantly reduces Th1 cells and modulates the immune status towards Th2 dominance. We obtained that balance of ILC depends on therapy as well. Patients which were treated with MTX have higher numbers of ILC2 and ILC3. However, Tamimoto et al. [10] reported that bDMARDs (rituximab) directs the immune response to Th 1. We obtained similar results that bDMARDs significantly increase ILC1. Thus numbers of ILC depends on stage and therapy of RA.

PD1 is an important negative regulator of differentiation that plays similar role on ILC as on other cells. The numbers of ILCs 2 depends on expression of PD 1 [11]. ILC synthesize high level of Th2 cytokines such as IL-5, IL-13, IL-9 in the absence PD1 or

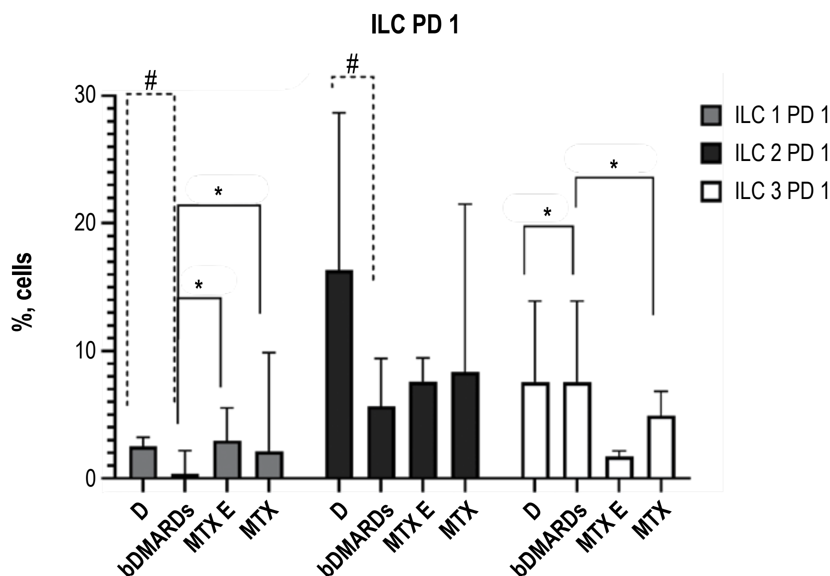


**Figure 3. Expressions of PD-1 and PDL-1 immune checkpoint molecules on ILC. D, healthy donors, bDMARDs (biologic disease-modifying anti-rheumatic drugs), MTX E (patients who were treated MTX with early stage RA), MTX (patients who were treated MTX with advance stage RA)**

PD-1-knockout (KO) mice, so that high lever PD1 can to inhibit of ILC [11]. We found that expression PD1 of ILC1 with patients on bDMARDs significantly reduced which can explain high level of numbers of ILC. Thus PD1 plays an important role to maintain the number of ILC.

## Conclusion

We found that the balance of ILC depends on therapy and stage of RA. However, further research is needed to confirm the relationship between the balance of ILC and antirheumatic drugs to improve the effectiveness of treatment.

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# СРАВНИТЕЛЬНЫЙ АНАЛИЗ ЭКСПРЕССИИ РАСТВОРИМОЙ ФОРМЫ РЕЦЕПТОРА IL-7 У ПАЦИЕНТОВ С АРТРОПАТИЕЙ

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**Резюме.** Артропатии являются одними из самых распространенных заболеваний, в основе которых лежит деструкция и ремоделирование хрящевой и костной ткани. Предшествующее деструкции воспаление может быть вызвано механической нагрузкой на суставы, аутоиммунными реакциями. В последнее время IL-7 рассматривают как один из ключевых цитокинов, способствующих продукции матриксных металлопротеиназ, катаболических ферментов, Т-клеточноопосредованной активации моноцитов, созреванию остеокластов. Растворимая форма рецептора к IL-7 может способствовать увеличению продолжительности жизни IL-7 и тем самым обеспечивать биодоступность цитокина и опосредовать эффект IL-7 на клетки. Целью данного исследования стало определение растворимой формы рецептора к IL-7 (sIL-7R) в плазме крови пациентов с ревматоидным артритом (RA), остеоартритом (OA), псориатическим артритом (PsA) и вульгарным псориазом (PS), а также здоровых индивидуумов. Пациенты с RA, вошедшие в исследование, имели умеренную и высокую активность заболевания согласно индексу DAS28. Пациенты с PsA преимущественно имели умеренную и низкую активность заболевания (DAS28) и характеризовались легкой и средней степенью тяжести заболевания (PASI). В соответствии с индексом PASI в исследование были включены пациенты с PS с легким и тяжелым течением заболевания. У всех пациентов с OA был метаболический фенотип, который сопровождается повышенным индексом массы тела.

sIL-7R определяли в плазме крови методом иммуноферментного анализа. Было обнаружено, что у пациентов с артропатией повышен уровень растворимой формы IL-7 относительно здоровых индивидуумов, исключение составила группа пациентов с PsA. Также высокая концентрация sIL-7R наблюдалась у пациентов с PS. Проведя анализ с учетом клинических характеристик пациентов, мы

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установили, что уровень sIL-7R повышался у пациентов с RA и PsA с высокой активностью заболевания по DAS28. Кроме того, были выявлены положительные корреляционные связи между концентрацией sIL-7R и DAS28 при RA и PsA. У пациентов с PsA со средне-тяжелой степенью тяжести (PASI) концентрация sIL-7R также была повышена относительно донорских значений. Напротив, у пациентов с PS высокий уровень sIL-7R фиксировался вне зависимости от степени тяжести заболевания. У пациентов с OA не было выявлено взаимосвязей между уровнем sIL-7R и клиническими параметрами.

Таким образом, повышенный уровень sIL-7R у пациентов с артропатией может говорить о вовлеченности IL-7 и его рецепторной системы в патогенез суставных заболеваний. Рецептор IL-7 может стать перспективной мишенью как в терапии заболеваний суставов, так и других аутоиммунных заболеваний, включая псориаз.

*Ключевые слова: растворимый рецептор IL-7, иммуноферментный анализ, псориатический артрит, псориаз, ревматоидный артрит, остеоартрит*

# COMPARATIVE ANALYSIS OF THE EXPRESSION OF THE SOLUBLE IL-7 RECEPTOR IN PATIENTS WITH ARTHROPATHY

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**Abstract.** Arthropathy is one of the most prevalent diseases, which are based on the destruction and remodeling of cartilage and bone tissue. The inflammation that precedes destruction can be caused by mechanical stress on the joints, or by autoimmune reactions. Recently, IL-7 is considered as one of the key cytokines that promote the production of matrix metalloproteinases, catabolic enzymes, T cell-mediated activation of monocytes, and maturation of osteoclasts. The soluble form of the IL-7 receptor can help prolong the lifespan of IL-7 and thereby it ensures the bioavailability of the cytokine and mediates effect of IL-7 on cells. The aim of this study was to determine the soluble form of the IL-7 receptor (sIL-7R) in the blood plasma of patients with rheumatoid arthritis (RA), osteoarthritis (OA), psoriatic arthritis (PsA) and psoriasis vulgaris (PS), as well as healthy individuals. The RA patients included in the study had moderate to high disease activity according to the DAS28 index. Patients with PsA predominantly had moderate and low disease activity (DAS28) and were characterized by mild to moderate disease severity (PASI). In accordance with the PASI index, patients with PS with mild and severe severity of the disease were included in the study. All patients with OA had a metabolic phenotype that is accompanied by an elevated body mass index.

sIL-7R was determined in blood plasma by enzyme-linked immunosorbent assay. It was found that in patients with arthropathy, the level of soluble form of IL-7 was increased relative to healthy individuals, with the exception of the group of patients with PsA. Also, a high concentration of sIL-7R was observed in patients with PS. Analyzing the clinical characteristics of the patients, we found that sIL-7R levels were elevated in RA and PsA patients with high disease activity by DAS28. In addition, positive correlations were found between the concentration of sIL-7R and DAS28 in RA and PsA. In patients with PsA with moderate severity of the disease (PASI), the concentration of sIL-7R was also increased relative to donor's values. On the contrary, in patients with PS, a high level of sIL-7R was noted regardless of the severity of the disease. In patients with OA, no relationship was found between sIL-7R levels and clinical parameters.

Thus, an elevated level of sIL-7R in patients with arthropathy may indicate the involvement of IL-7 and its receptor system in the pathogenesis of joint diseases. The IL-7 receptor may become a promising target both in the treatment of joint diseases and other autoimmune diseases, including psoriasis.

*Keywords: soluble IL-7 receptor, enzyme-linked immunosorbent assay, psoriatic arthritis, psoriasis, rheumatoid arthritis, osteoarthritis*

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## Introduction

Arthropathy is one of the most prevalent diseases, and it often irrevocably changes the quality of patient life [7]. It is believed that osteoarthritis (OA) is based on inflammation caused by inappropriate mechanical stress on the joints, rheumatoid arthritis (RA) is based on autoimmune inflammation, while in psoriatic arthritis (PsA) there is no relatively well-formed pathogenetic concept. In addition, the common feature of the three diseases is the destruction and remodeling of bone tissue and cartilage.

IL-7 is an important cytokine for the generation, development, maintenance and function of human T lymphocytes [11]. In addition to its role as a T cell homeostatic factor, it also promotes the production of IFN and IL-17, which adversely affects the course of Th1- and Th17-mediated diseases, including joint diseases.

It is known that IL-7 is produced not only by immune cells, but also by keratinocytes and chondrocytes. At the same time, cytokine production by chondrocytes is enhanced in response to stimulation by fibronectin filaments, IL-1 and IL-6. Through its receptor (IL-7R), IL-7 induces the production of matrix metalloproteinase-13 associated with the release of proteoglycans from cartilage. It can be assumed that IL-7 is indirectly involved in the destruction of joint tissue through autoimmune mechanisms [7]. IL-7 induces T cell-mediated activation of monocytes, and also increases the production of TNFα, which is an inhibitor of cartilage matrix synthesis and an inducer of cartilage degradation through an increase in the activity of fibroblasts that produce catabolic factors. IL-7 promotes the maturation of osteoclasts from monocytes through the induction of TNFα and an increase in RANKL expression [12], which may have an aggravating effect on the condition of the joints in OA, RA, and PsA. IL-7 expression is increased in OA [6]. In this disease, IL-7 has been found to mediate destruction of joint tissue through osteoclast activation as well as through its action on IL-7R$^+$ chondrocytes. Blockade of IL-7R led to a decrease in the intensity of cartilage tissue destruction *in vitro* and *in vivo* [8].

Soluble forms of receptors can be formed from the extracellular portion of a membrane-bound protein or as a result of alternative splicing. They are able to trigger the development of various immunological reactions through binding to the appropriate ligands, however, the effect may differ from the result of activation of the signaling pathway of a similar membrane-bound receptor [1]. The reason for this difference is the activation of different signaling pathways upon binding of cytokines to soluble and membrane forms of receptors [13].

The soluble form of the IL-7 receptor (sIL-7R) increases the bioavailability of IL-7 and enhances proliferative activity caused by activation of the membrane receptor signaling pathway [4]. An association of serum sIL-7R levels with the development of autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, and Schegen's syndrome, has been established [2, 5]. At the same time, in RA, a high level of sIL-7R is associated with a lack of response to non-steroidal anti-inflammatory drug therapy and systemic glucocorticosteroids, and with a poor prognosis for the patient. In addition, in RA, fibroblasts produced sIL-7R in response to TNFα, IL-1β, and IL-17. All of this indicates the need to study the role of sIL-7R in the development of joint diseases [9]. The aim of this study was to determine the soluble form of the IL-7 receptor (IL-7R) in the blood plasma of patients with rheumatoid arthritis (RA), osteoarthritis (OA), psoriatic arthritis (PsA) and psoriasis vulgaris (PS), as well as healthy individuals.

## Materials and methods

### Object of investigation

The study included patients with rheumatoid arthritis (RA, n = 14), osteoarthritis (OA, n = 16), psoriatic arthritis (PsA, n = 26), psoriasis vulgaris (PS, n = 12) and healthy individuals (n = 16). The recruitment of patients with rheumatoid arthritis and osteoarthritis was carried out on the Department of Rheumatology, Clinic of Immunopathology of RIFCI (Novosibirsk) with the participation of rheumatologists. The recruitment of patients with psoriatic arthritis was carried out on the State Budgetary Health Institution of the Novosibirsk Region "City Hospital No. 3" (Novosibirsk) with the participation of rheumatologists and dermatologists. The recruitment of patients with psoriasis was carried out on the Clinic "Allergo-city" (Novosibirsk) with the participation of dermatologist. Written informed consent was obtained from all participants included in the study prior to collection of peripheral blood samples. Peripheral blood samples were collected in the 6 mL vacuum tubes, containing $K_2$EDTA.

Characteristics of persons included in the study, are presented in Table 1. Thirteen patients with OA had an obesity: body mass index was more than 30; 3 patients had body mass index more than 25 and less than 30. Patents with RA characterized by moderate and high activity of the disease, Rg-stage II-IV. Patients with PsA had predominantly moderate and low activity of the disease (DAS28), mild and moderate severity (PASI). The group of psoriasis was presented by patients with mild and severe severity of the disease, according to PASI.

**TABLE 1. CHARACTERISTICS OF PERSONS INCLUDED IN THE STUDY**

| | Healthy control (n = 16) | Psoriasis (n = 12) | Psoriatic arthritis (n = 26) | Rheumatoid arthritis (n = 14) | Osteoarthritis (n = 16) |
|---|---|---|---|---|---|
| **Age, years** | 41.30±3.26 | 38.00±3.62 | 55.10±2.66 | 45.90±3.95 | 65.3±2.1 |
| **Gender: m/f (%)** | 6/10 (37.5/62.5) | 6/6 (50/50) | 11/15 (42.3/57.7) | 0/14 (0/100) | 0/16 (0/100) |
| **PASI**<br>**Severity:**<br>**– mild**<br>**– moderate**<br>**o severe**<br>**– severe** | – | 23.30±5.78<br><br>– 5 patients<br>– 0 patients<br><br>– 7 patients | 8.70±0.89<br><br>– 19 patients<br>– 7 patients<br><br>– 0 patients | – | – |
| **DAS28**<br>**Activity of the disease:**<br>**– low**<br>**– moderate**<br>**– high** | – | – | 3.75±0.21<br><br>– 7 patients<br>– 14 patients<br>– 3 patients | 5.00±0.35<br><br>– 0 patients<br>– 7 patients<br>– 7 patients | – |
| **VAS (mm)**<br>**BMI (kg/m²)** | – | – | – | – | 65.40±4.87<br>32.70±0.97 |
| **Rg-stage:**<br>**– II**<br>**– III**<br>**– IV** | | | | – 6 patients<br>– 6 patients<br>– 2 patients | – 6 patients<br>– 10 patients<br>– 0 patients |

Note. VAS, Visual Analogue Scale of pain; BMI, body mass index. The data are presented as Mean±Standard error.

**Evaluation of soluble IL-7 receptor**

Vacuum tubes with blood were performed centrifugation on 3000 rpm during 20 minutes to obtain plasma. Samples of plasma were collected and frozen. Prior to enzyme-linked immunosorbent assay, samples were rethawed and diluted in 5 times by 0,01M PBS (pH 7.0).

We used the ELISA kit for IL-7 receptor (Cloud-Clone Corp., USA), that is a sandwich enzyme immunoassay, to quantitatively measure human IL-7 receptor in the plasma samples, acting accordingly to the instruction. Measurement of absorbance of the enzyme-substrate reaction was performed at a wavelength of 450 nm with reference at a wavelength of 620 nm by a spectrophotometer Infinite F50 (Tecan, Austria). The concentration of IL-7 receptor in the samples was determined by counting the optical density of the samples to the standard curve (0.156-10 ng/mL) and multiplying the dilution factor.

**Statistical analysis**

Statistical data processing was carried out using the software "Statistica 6.0" (StatSoft, USA) and "GraphPadPrism 9.0" (GraphPad, USA), using nonparametric statistics methods (Mann–Whitney U test). For correlation analysis, the Spearman correlation coefficient was used. Differences were considered statistically significant at $p < 0.05$.

## Results and discussion

We found that the concentration of the soluble IL-7 receptor was increased in patients with arthropathy compared to healthy individuals, excluding patients with PsA (Table 2, p1). Nevertheless, content of the soluble IL-7 receptor in plasma of patients with PS was significantly higher than that in healthy individuals and patients with PsA. Patients with RA had a large concentration of the soluble IL-7 receptor; it significantly differed from values of patients with PsA. We suggested that observed differences may be linked to the severity and the activity of the diseases.

In the next step, we analyzed the soluble IL-7 receptor concentration in patients with PS, PsA and RA depending on the PASI and DAS28. It turned out that IL-7 receptor level was elevated in patients with PsA, having moderate to severe severity (Figure 1A) and high activity of the disease (Figure 1C) relatively donor's values.

Similar results were found for patients with RA: significant increasing of the soluble IL-7 receptor concentration was observed in patients with high activity of the disease (Figure 1D). On the contrary, the concentration of the soluble IL-7 receptor was increased in patients with PS regardless of the severity of the disease (Figure 1B). Moreover, IL-7 receptor level was strongly correlated with DAS28 values in

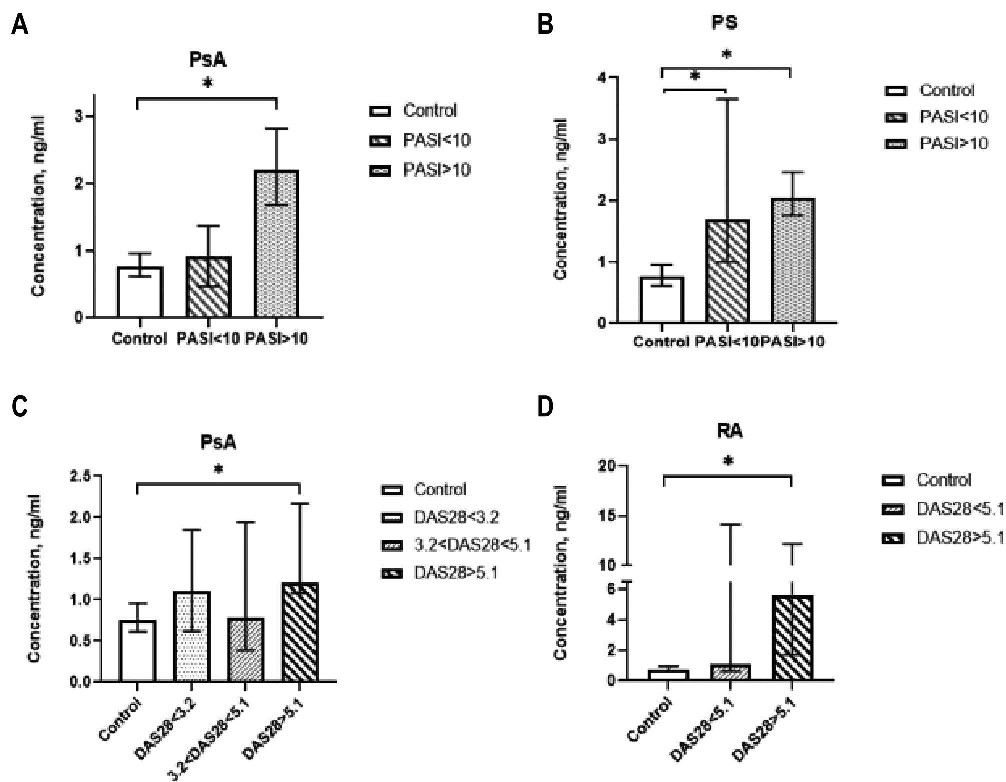


**Figure 1. Concentration of the soluble IL-7 receptor depending on the severity and activity of the disease**

Note. (A) Concentration of the soluble IL-7 receptor depending on PASI in psoriatic arthritis. (B) Concentration of the soluble IL-7 receptor depending on PASI in psoriasis. (C) Concentration of the soluble IL-7 receptor depending on DAS28 in psoriatic arthritis. (D) Concentration of the soluble IL-7 receptor depending on DAS28 in rheumatoid arthritis. *, significant differences, $p < 0.05$ (Mann–Whitney U test).

group of patients with RA ($R = 0.84$, $p < 0.05$). In patients with PsA, it was observed direct relationship between content of IL-7 receptor in plasma and PASI ($R = 0.48$, $p < 0.05$). No relationship has been found between clinical parameters (BMI, VAS pain) and concentration of IL-7 receptor in the plasma of patients with osteoarthritis.

It is known that sIL-7R enhances the activity of IL-7 and increases IL-7-induced proliferation of T lymphocytes and their survival [4, 7]. Our data also indirectly indicate this: in patients with severe and moderate PsA and RA, a significantly higher content of sIL-7R was observed compared with a mild form of the disease, and a correlation was found with the

**TABLE 2. CONCENTRATION OF THE SOLUBLE IL-7 RECEPTOR IN THE PLASMA OF HEALTHY INDIVIDUALS AND PATIENTS WITH ARTHROPATHY, Me ($Q_{0.25}$-$Q_{0.75}$)**

| Groups | sIL-7R, ng/mL | $p_1$ | $p_2$ | $p_3$ |
|---|---|---|---|---|
| **Healthy control** | 0.76 (0.61-0.96) | – | 0.13 | 0.0006 |
| **Psoriasis** | 1.89 (1.45-2.41) | 0.0001 | 0.022 | 0.52 |
| **Psoriatic arthritis** | 1.16 (0.58-1.94) | 0.13 | – | 0.015 |
| **Rheumatoid arthritis** | 2.68 (1.11-12.64) | 0.0006 | 0.015 | – |
| **Osteoarthritis** | 1.33 (1.09-2.61) | 0.001 | 0.103 | 0.236 |

Note. sIL-7R, soluble IL-7 receptor; $p_1$, p values of the difference from group of healthy control; $p_2$, p values of the difference from group of patients with psoriatic arthritis; $p_3$, p values of the difference from group of patients with rheumatoid arthritis.

level of sIL-7R and PASI in PsA and with DAS28 in rheumatoid arthritis. Similar data on the relationship between the severity of the course of the disease and the level of sIL-7R in the blood serum were found in systemic lupus erythematosus [3]. Moreover, in this disease, an inverse relationship was also found between the content of the soluble form of the receptor and the level of the complement component C1q.

These data allowed us to consider the content of sIL-7R as a prognostic factor in the course of the disease, which can be done based on our data on PsA and RA. However, for this, in our opinion, it is additionally necessary to evaluate the relationship between the sIL-7R content and laboratory parameters, such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and also to evaluate the response to conventional therapy in patients with high and low levels of the receptor in peripheral blood.

Patients with classic psoriasis vulgaris without joint involvement were characterized by significantly higher levels of sIL-7R compared to controls, while no association with disease severity was found. At the same time, the concentration of sIL-7R in psoriasis vulgaris is higher than in patients with the arthropathic form of the disease. It can be assumed that keratinocytes, even in limited forms of the disease, are characterized by a more pronounced ability to produce sIL-7R compared to chondrocytes in the arthropathic form of the disease.

In RA, the highest concentration of sIL-7R in the peripheral blood was observed compared with PsA and OA. It is known that sIL-7R α-chain polymorphism is associated with the development of RA, which indirectly indicates the involvement of the soluble form of the receptor of this cytokine in the development of this disease [9]. Patients with severe RA are generally characterized by a greater shift in CRP, ESR, as well as a more pronounced severity of the course of the disease compared to PsA, which may explain the higher level of sIL-7R in these patients [10].

## Conclusion

Thus, an elevated level of sIL-7R in patients with arthropathy may indicate the involvement of IL-7 and its receptor system in the pathogenesis of joint diseases. At the same time, in the case of autoimmune diseases, such as RA and PsA, the level of sIL-7R correlates with the activity of the disease. In the case of diseases without an autoimmune component and presence of metabolic disorders (osteoarthritis), the plasma concentration of sIL-7R is increased compared to donor values, however, it is not associated with either body mass index or VAS pain level. It is possible that sIL-7R reflects the inflammatory background in OA, that needs for further confirmation. A high concentration of sIL-7R in PS is detected even in mild psoriasis, which indicates its involvement in the pathogenesis of the disease at the early stage. Based on the obtained data, the IL-7 receptor can be considered a promising target for targeted therapy of various arthropathies and autoimmune diseases, including psoriasis.

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# ЭФФЕКТИВНОСТЬ И БЕЗОПАСНОСТЬ КУРКУМИНА У ПАЦИЕНТОВ С МЕТАБОЛИЧЕСКИМ ФЕНОТИПОМ ОСТЕОАРТРИТА: ПИЛОТНОЕ ИССЛЕДОВАНИЕ

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**Резюме.** Целью исследования была оценка эффективности и безопасности куркумина при остеоартрите, ассоциированном с метаболическим синдромом (МетС-ОА). Перед включением в исследование все пациенты подписали форму добровольного информированного согласия. Диагноз ОА ставился в соответствии с критериями Американской Коллегии Ревматологов, МетС – в соответствии с критериями Российского общества кардиологов.

Дизайн исследования: до, после. Основными критериями включения были наличие ОА и МетС, уровень оценки общего состояния здоровья и боли более 50 мм с использованием 100 мм визуальной аналоговой шкалы (ВАШ). Основными конечными точками были ВАШ общего здоровья. Другими исходами были ВАШ боли, различные подкатегории шкалы повреждения и ОА коленного сустава, Knee injury and Osteoarthritis Outcome Score (KOOS): подкатегория боли (KOOS боль), подкатегория другие симптомы (KOOS симптомы), подкатегория повседневных активностей (KOOS ПА), подкатегория активностей при спорте отдыхе sport and recreation (KOOS спорт и отдых) и подкатегория качества жизни (KOOS КЖ). Выраженность депрессии оценивалась с помощью опросника PHQ-9. Оценивалась доля пациентов, достигших минимального клинически значимого различия в изменении боли (МКЗР). Точкой разделения для МКЗР для боли было (а) 15 из 100 для абсолютного улучшения и 20% для относительного улучшения.

Пациенты принимали экстракт C. longa в дозе 1000 мг/день в течение 4 недель. Оценка параметров проводилась до лечения и через 4 недели терапии.

В исследование были включены 18 женщин с МетС-ОА. К концу терапии выявлялось достоверное уменьшение ВАШ общего здоровья в среднем на 33,9 мм (p = 0,001), ВАШ боли на 25 мм (p = 0,001). Наблюдалась тенденция к улучшению показателя PHQ-9 на 2,9 (p = 0,05). Среднее уменьшение KOOS боли было 11 (p = 0,001). KOOS симптомом – 9 (p = 0,025), KOOS ПА – 12,4 (p 0,001), KOOS спорт и отдых – 10,3 (p = 0,044), KOOS КЖ – 14,4 (p = 0,009). МКЗР боли и общего здоровья выявлено у 9 (56%) пациентов. Нежелательных событий не было.

Результаты этого исследования указывают на безопасность и эффективность экстракта C. Longa при МетС-ОА. Для подтверждения этих данных необходимы крупные контролируемые исследования.

*Ключевые слова: куркумин, ревматология, минимальное клинически значимое различие, метаболический синдром, депрессия, остеоартрит, коморбидность*

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# EFFICACY AND SAFETY OF CURCUMIN IN PATIENTS WITH METABOLIC PHENOTYPE OF OSTEOARTHRITIS: A PILOT STUDY

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**Abstract.** The aim of this study was to assess efficacy and safety of curcumin in metabolic syndrome-associated osteoarthritis (MetS-OA). All patients provided written informed consent. Knee OA was diagnosed according to American College of Rheumatology criteria; MetS was diagnosed according to Russian Scientific Society of Cardiology Guidelines. The study had before-and-after design. The main inclusion criteria were presence of knee OA and MetS, levels of global health assessment and pain assessment more than 50 mm using 0-100 visual analogue scale (VAS). The main outcome was VAS global. The other outcomes were VAS pain, Knee injury and Osteoarthritis Outcome Score (KOOS) consisting of five subscales: pain (KOOS pain), other symptoms (KOOS symptoms), activities in daily living (KOOS ADL), function in sport and recreation (KOOS Sport/Rec) and knee related Quality of life (KOOS QoL). The level of depression was measured using PHQ-9. For pain, proportion of patients achieving minimal clinically important improvement (MCII) was assessed using the cut-offs of (a) 15 of 100 for absolute improvement and 20% for relative improvement.

The treatment consisted of C. longa extract 1000 mg/day for 4 weeks. The assessments were performed on baseline and 4 weeks thereafter. Eighteen women with MetS-OA of the knee were included in the study.

At the end of treatment, there were significant improvements in the VAS global scale by an average 33.9 mm (p = 0.001), VAS pain by 25 mm (p = 0.001). There was a trend towards improvement in PHQ-9 by 2.9 (p = 0.05). The mean improvement in KOOS pain was 11 (p = 0.001). KOOS symptoms improved by 9 (p = 0.025), KOOS ADL – by 12.4 (p = 0.001), KOOS Sport/Rec by 10.3 (p = 0.044), and KOOS QOL by 14.4 (p = 0.009). The proportion of patients achieving clinically significant improvement (MCII) were nine (56%) for both global health and pain. There were no adverse events during the study. The findings of this study suggest clinical efficacy and safety of C. Longa in MetS-associated knee OA. There is a need for large controlled studies to confirm these results.

*Keywords: curcumin, rheumatology, minimal clinically important difference, metabolic syndrome, depression, osteoarthritis, comorbidity*

## Introduction

The World Health Organization (WHO) declares prevention and treatment of chronic non-communicable diseases (NCDs) as a priority of the third decade of the 21st century. Common NCDs are heterogeneous in terms of their pathogenesis and clinical manifestations. This heterogeneity is manifested as different endotypes (distinct patho-biological mechanisms of a disease) and phenotypes (observable characteristics or traits of a disease) of NCD [4, 8]. It now becomes evident that further improvements in the prevention, diagnostics, and treatment of NCDs are possible only in case of better understanding of the differences in various endotypes and phenotypes of asthma, rheumatoid arthritis, osteoarthritis and other NCDs.

Metabolic syndrome (MetS) – associated OA, also called metabolic OA, occurs as a result of metabolic disturbances caused by obesity, diabetes mellitus, insulin resistance, dyslipidemia, hyperuricemia, and arterial hypertension [8]. The prevalence of MetS ranges from 10 to 34% in people older than 18 years. In Russia, the estimated prevalence of MetS varies from 20% to 35% [5, 8].

A promising way to treat comorbidity is to use pleiotropic phytotherapeutic agents aimed at various pathogenetic pathways [3]. One of the popular phytomedicines, Curcuma longa (CL) and its active components (curcuminoids) has been shown to exert anti-inflammatory, immunomodulatory, and lipid lowering effects [1]. There have been many studies assessed the effects of CL in OA. In a meta-analysis treatment with CL was associated with clinically significant improvements of pain in knee O [6]. It should be noted that the studies on CL included patients with OA irrespective of their comorbidities or phenotype of OA.

There is a lack of studies evaluating effects of CL in a selected group of patients with particular phenotype of OA. Therefore, we undertook this exploratory study to assess efficacy and safety of CL in patients with MetS-associated OA.

## Materials and methods

The study protocol, patient information sheet, and case report form were approved by the Local Ethics Committee attached to the Scientific Research Institute of Fundamental and Clinical immunology. The main inclusion criteria were as follows:

– The diagnosis of knee OA made in accordance with ACR 1986 criteria [2].

– MetS diagnosed according to criteria of Russian Society of Cardiology:

Presence of abdominal obesity [waist circumference (WC) > 80 cm in women and > 94 cm in men] and two of the following criteria:

1) Arterial hypertension (arterial blood pressure ≥ 140/90 mm hg);

2) Triglycerides (TG) ≥ 1.7 mmol/L;

3) High density lipoprotein (HDL) cholesterol < 1.0 mmol/L in men and < 1.2 mmol/L in women;

4) Low density lipoprotein (LDL) cholesterol > 3.0 mmol/L;

5) Fasting hyperglycemia (blood glucose ≥ 6.1 mmol/L) or impaired glucose tolerance (plasma glucose ≥ 7.8 but ≤ 11.1 mmol/L 2 hours after meal).

– Pain level more than 50 mm on Visual Analogue Scale (VAS).

– Not taking non-steroidal anti-inflammatory drugs due to poor tolerability or lack of effect.

All patients underwent knee x-ray and assessment according to Kellgren-Lawrence classification. The study design was before-and-after study. Each participant was given oral curcumin 1000 mg daily in two doses for four weeks. The patients were not allowed to take non-steroidal anti-inflammatory drugs during the study. Paracetamol up to 3 g/day could be used as a rescue therapy.

The assessments and blood sampling were made on baseline visit (W0) and 4 weeks thereafter (W4). The primary end point was global assessment of health by the patient using 100 mm VAS. The secondary endpoints were:

– Knee injury and Osteoarthritis Outcome Score (KOOS) consisting of five subscales: pain (nine items), other symptoms (seven items), activities in daily living (ADL) (17 items), function in sport and recreation (Sport/Rec) (five items) and knee related Quality of life (QoL) (4 items). Likert scale was used so all items had five possible answer options scored from 0 (No Problems) to 4 (Extreme Problems). Each of the five scores was calculated as the sum of the items included. The scores were transformed to a 0-100 scale, with zero representing extreme knee problems and 100 representing no knee problems [7].

– Patient Health Questionnaire (PHQ-9) for the assessment of depression presence and severity. The PHQ-9 has nine Likert (0 to 3) questions. After the completion all items are summed thus giving range from 0 to 27, with 0 – indicating no depression, 27 – maximal depression. In accordance with PHQ-9, depressive symptomatology is classified as no depression (0-4), mild depression (5-9), moderate depression (10-14).

– Pain on 100 mm VAS.

We assessed the proportion of patients achieving minimal clinically important improvement (MCII) in global scale assessment and in VAS pain. The cut-offs for MCII changes were (a) 15 of 100 for absolute improvement and 20% for relative improvement [9]. The descriptive statistics is presented with mean and standard deviation/median and interquartile range for continuous variables, absolute number (percentage) for dichotomous variables. The differences between means before and after the treatment were assessed using paired t-test.

## Results and discussion

Eighteen women with knee OA and concomitant MetS were included in the study. The baseline demographic and clinical characteristics of the included patients are presented in Table 1. All patients had obesity and have had diagnoses of knee OA or hypertension for 8-10 years. All patients had radiographic knee OA, grades II-III according to Kellgren-Lawerence classification. The majority (88%) of patients had symptoms of mild to moderate depression.

Table 2 shows changes in VAS global, VAS pain, and PHQ-9 before and after the treatment. There

TABLE 1. BASELINE CHARACTERISTICS OF PATIENTS WITH KNEE OA AND MetS

| | Patients with knee OA and MetS, n = 18 |
|---|---|
| Age (years) | 65 (61-71) |
| Body mass (kg) | 90 (85-100) |
| Waist circumference (cm) | 107.5 (103.5-116.0) |
| Body mass index (kg/m²) | 34 (31.5-36.8) |
| Time since the diagnosis of OA (years) | 8 (5-11) |
| Time since the diagnosis of hypertension (years) | 10 (4-14) |

TABLE 2. VAS GLOBAL, VAS PAIN AND PHQ-9 BEFORE AND AFTER THE TREATMENT

| | Before treatment (W0) | After treatment (W4) | p |
|---|---|---|---|
| VAS global, mm | 57.6 (15.8) | 23.7 (20.5) | 0.001 |
| VAS pain, mm | 65.3 (19.4) | 40.3 (22.9) | 0.001 |
| PHQ-9 | 9.4 (4.9) | 6.5 (4.1) | 0.05 |

Note. The values represent mean (SD).

TABLE 3. KOOS SUBSCALES BEFORE AND AFTER THE TREATMENT WITH CURCUMIN

| | Before treatment (W0) | After treatment (W4) | p |
|---|---|---|---|
| **KOOS symptoms** | 49 (13.5) | 58 (14.9) | 0.025 |
| **KOOS pain** | 49.2 (12.1) | 60.9 (11.5) | 0.001 |
| **KOOS function in daily living** | 47.6 (10.2) | 60 (13.9) | 0.001 |
| **KOOS function in sport and recreation** | 24.3 (13.5) | 34.6 (13.6) | 0.044 |
| **KOOS knee related Quality of life** | 22.5 (13.6) | 36.9 (19.1) | 0.009 |

were statistically significant improvements in VAS global (-33.9 mm) and VAS pain (-25 mm) at the end of treatment. The proportion of patients achieving clinically significant improvement (MCII) were nine (56%) for both global health and pain. There was a trend towards improvement of depressive symptoms.

The mean values of KOOS subscales before and after treatment are shown in Table 3. There were significant improvements in all subcategories: KOOS symptoms, KOOS pain, KOOS function in daily living, KOOS function in sport and recreation, and KOOS knee related Quality of life.

No adverse event was registered during the study.

## Conclusion

In conclusion, we found significant improvement of global assessment of health, pain, function, and quality of life after treatment with curcumin of patients with MetS-associated knee OA. These results support efficacy and safety of curcumin in OA patients with comorbidity. However, as our findings were obtained in a non-controlled study, larger randomized double-blind controlled studies comparing curcuminoids with active control or placebo are needed.

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# ФЕНОТИПИЧЕСКАЯ ХАРАКТЕРИСТИКА ИНТРАЭПИТЕЛИАЛЬНЫХ ЛИМФОЦИТОВ ТОЛСТОЙ КИШКИ У ПАЦИЕНТОВ С БОЛЕЗНЬЮ КРОНА

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**Резюме.** Интраэпителиальные лимфоциты (IEL) играют важную роль в поддержании иммунного гомеостаза и обеспечивают первую линию защиты слизистых оболочек желудочно-кишечного тракта от антигенов, а также быстро реагируют на повреждение эпителия. В последнее время IEL рассматриваются в качестве ключевых медиаторов аберрантного иммунного ответа, характеризующегося стойкой иммунной активацией, воспалением и нарушением барьерной функции слизистых оболочек, что наблюдается при болезни Крона. В данном исследовании впервые приводится сравнительная характеристика субпопуляционного состава IEL толстой кишки у пациентов с болезнью Крона и здоровых доноров для последующего определения их потенциальной роли в патогенезе заболевания.

Материалом исследования явилась ткань толстой кишки и периферическая кровь 10 пациентов с болезнью Крона и 6 здоровых доноров. IEL выделяли из ткани методом клеточной диссоциации; циркулирующие лимфоциты получали из периферической венозной крови путем центрифугирования на градиенте плотности. Фенотип лимфоидных клеток оценивали с использованием моноклональных антител и метода проточной цитометрии.

Большинство IEL толстой кишки идентифицировалось как $CD3^+$T-лимфоциты, однако статистически значимые различия в их количестве отсутствовали в исследуемых группах. При этом установлены изменения субпопуляционного состава Т-клеток: у пациентов с болезнью Крона соотношение $CD3^+CD4^+$IEL и $CD3^+CD8^+$IEL составило 1:1 и коррелировало с процентом Т-лимфоцитов в периферической крови (R = 0,7; $p < 0,05$), в то время как в донорской ткани выявлялось ожидаемое преобладание $CD3^+CD8^+$Т-киллеров (соотношение достигало 1:2, $p < 0,05$). Наряду с этим повышение неклассических $\gamma\delta$IEL (преимущественно за счет $V\delta1^+$Т-клеток) и $CD161^+$Т-клеток в сочетании со снижением TNK-клеток наблюдалось как в толстой кишке ($p < 0,01$), так и в периферической крови ($p < 0,05$) пациентов с болезнью Крона относительно контрольной группы. При этом количество

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γδIEL коррелировало с локализацией (R = -0,6; p < 0,05) и течением заболевания (R = 0,7; p < 0,01) согласно Монреальской классификации.

У пациентов с болезнью Крона выявлены изменения состава IEL толстой кишки, которые характеризуют вовлечение субпопуляций Т-хелперов, γδТ-лимфоцитов и мукозально-ассоциированных $CD161^+$Т-клеток в патогенез аутоиммунного воспаления, что требует дальнейшего исследования для установления их патогенетической роли.

*Ключевые слова: интраэпителиальные лимфоциты, проточная цитометрия, болезнь Крона, толстая кишка, аутоиммунное воспаление, мукозальный иммунитет*

# PHENOTYPE CHARACTERISTIC OF COLONIC INTRAEPITHELIAL LYMPHOCYTES IN PATIENTS WITH CROHN'S DISEASE

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**Abstract.** Intraepithelial lymphocytes (IEL) play a critical role in maintaining the immune balance of the gut and provide the first line of mucosal defense against luminal antigens as well as rapidly respond to epithelial injury. Recently, IEL have received a lot of attention as key mediators of aberrant immune response resulted in persistent immune activation, inflammation and altered intestinal barrier function, seen in Crohn's disease (CD). This study describes for the first time subsets of colonic IEL in CD patients as compared to healthy controls aimed at characterization of altered IEL contribution to the pathogenesis of Crohn's disease.

The peripheral venous blood and colon tissues were obtained from 10 CD patients and 6 donors. IEL were isolated from the mucosa by incubation the tissue in a predigesting solution. Lymphoid cells phenotype was investigated using monoclonal antibodies and flow cytometry.

The majority of colonic IEL was identified as $CD3^+$T lymphocytes and no significant differences were found in their numbers in investigated groups. However, changes in T cell subsets composition have been shown: the ratio of $CD3^+CD4^+$IEL and $CD3^+CD8^+$IEL was 1:1 in colon of CD patients and correlated with T cells in peripheral blood (R = 0.7; p < 0.05) while donor tissues were characterized by expected $CD3^+CD8^+$T killers prevalence and the ratio reached 1:2 (p < 0.05). The increase of unconventional γδIEL (mainly due to $V\delta1^+$T cells) and $CD161^+$T cells in association with TNK cells decrease were revealed in colon (p < 0.01) as well as in peripheral blood (p < 0.05) of CD patients as compared to donors. Moreover, the number of colonic γδIEL was correlated with disease location (R = -0.6; p < 0.05), and disease behavior (R = 0.7; p < 0.01) according to Montreal classification.

The observed data indicates changes in colonic IEL composition in CD patients that may provide valuable insight into the contribution of T helpers, γδT cells and mucosa-associated $CD161^+$T cells in autoimmune intestinal inflammation but need further possible mechanisms discussion.

*Keywords: intraepithelial lymphocytes, flow cytometry, Crohn's disease, colon, autoimmune inflammation, mucosal immunity*

## Introduction

The mucosal immune system is the largest (about 400 m$^2$) autonomous immune structure, which has evolved as the first line of a body defense and represented by organized compartments and isolated lymphoid cells, classifying into intraepithelial lymphoid cells (IEL) and lamina propria lymphoid cells [8]. As 80% of the mucosal immunocompetent tissue is localized in the gut, intestinal IEL have received a lot of attention as key mediators of aberrant immune response seen in Crohn's disease (CD), a chronic autoimmune inflammatory bowel disease characterized by multifactorial aetiology and an uncontrolled adaptive immune reaction against intestinal microbiota [5]. Experimental data suggest that an imbalance between regulatory and cytolytic effector mechanisms results in a dysregulation of mucosal immunity, persistent immune activation, a

generation of a pro-inflammatory microenvironment, altered intestinal barrier function or even promotion of cancer development in CD patients [14].

On the one side, the location of IEL between epithelial cells, their effector memory and inflammatory phenotype as well as ability to destroy infected epithelial cells place them to protect the intestine from pathogens. On the other side, IEL-mediated epithelial cytolysis leads to ulceration, allowing bacterial invasion, and enhanced T cell activation in combination with regulatory cell reduction. Moreover, the interaction with non-immune epithelial cells and fibroblasts initiates tissue reorganization, including epithelial proliferation and fibrosis in gastrointestinal tract [3, 11].

As IEL immunobiology goes beyond the scope of classical immunology, limited data were reported about a role of IEL subsets in CD pathogenesis. It has not yet been clarified in what manner IEL subpopulations are changed during the initiation and development of adaptive antigen-specific autoimmune response and what are distinctive features of IEL distribution in CD patients. This article will focus on colonic IEL exhibiting properties of innate and adaptive immunity and describe for the first time IEL subsets in CD patients as compared to donors aimed at characterization of altered IEL contribution to immune dysregulation in intestinal mucosae.

## Materials and methods

The peripheral venous blood samples and colon tissues were obtained from 10 CD patients, aged 28.0 (22.7-36.8) years, of which 6 men and 4 women, during electively scheduled surgical resections in Minsk Regional Clinical Hospital (Republic of Belarus). The "Crohn's disease" diagnosis was confirmed by histopathological examination of the resected specimen. Patients had the following disease location: terminal ileum (n = 4), colon (n = 3), ileocolon (n = 3). Of these, 3 patients had an inflammatory disease behavior, 4 patients – stricturing and 3 patients – a penetrating/fistulous phenotype; 2 patients were also diagnosed perianal disease. None of the patients was taking steroids or other immunosuppressive drugs at the time of the operation. Control specimens were obtained from age- and sex-matched 6 donors undergoing surgery for large intestine. Written informed consent was provided by all individuals.

Tissues were processed within 2 h after resection. After removal of the muscular layer mucosal tissue was cut into pieces of 0.5 $cm^2$. IEL were isolated from the mucosa by shaking the tissue in a predigestion solution (Hank's balanced salt solution without $Ca^{2+}$ and $Mg^{2+}$ containing 10 mM HEPES, 5 mM EDTA, 5% fetal bovine serum, 1 mM dithioerythritol (Gibco, Germany)) for 20 min at 37 °C under continuous rotation followed sample application onto cell strainer (100 μm). Cell suspension containing IEL was centrifuged at 300 g for 10 min and resuspended to the required volume for immunophenotyping.

Peripheral blood lymphocytes (PBL) were isolated from heparinized venous blood samples by Histopaque-1077 (Sigma, Germany) density gradient centrifugation. Cells viability was determined by trypan blue exclusion.

The lymphoid cells subsets of IEL and PBL were determined using monoclonal antibody panels: CD45-FITC/CD4-RD1/CD8-ECD/CD3-PC5, CD45-FITC/CD56-RD1/CD19-ECD/CD3-PC5/CD5-PC7, γδTCR-FITC/αβTCR-PE/Vδ1TCR-PC7/CD3-AF750/Vδ2TCR-PB/CD45-KrO, CD127-FITC/Lin-PE/CD3-APC/CD161-PB/CD45-BrV (Beckman Coulter, USA). Monoclonal antibody reagents were added according to the manufacturer's instructions to 100 μL of cell suspension, and reaction mixtures were incubated at 20-25 °C for 15 min in the dark. Results were analyzed on 20000 lymphocytes using a 10-channel flow cytometer Cytoflex (Beckman Coulter, USA).

Statistical data processing was performed using Statistica 8.0. The median (Me), $25^{th}$ and $75^{th}$ percentiles were used as descriptive statistics of the studied groups. Significant differences between investigated groups were determined by nonparametric criteria Mann–Whitney U-test; p-values $< 0.05$ and $p < 0.01$ were considered as statistically significant. The correlation was estimated using Spearman's rank coefficient (R).

## Results and discussion

The comparative analyses of IEL and PBL numbers, cell viability and phenotype were done in investigated groups. After isolation IEL viability was not less than 86% in CD patients and not differed from one's in donor's tissue. But IEL yield per 1 g of tissue was significantly higher in colon of CD patients as compared to control group: 30.1 (27.9 ÷ 39.0) × $10^6$ *versus* 19.4 (15.6 ÷ 24.7) × $10^6$, respectively, indicating the activity of the autoimmune inflammation. An increase of colonic IEL may disrupt the balance between immune tolerance and immune response to self-antigens, including microbiota, which can result in damage of the intestinal epithelium. At the same time there were no significant differences in PBL total numbers in investigated groups.

As lymphoid cells are a highly heterogeneous population the following subsets have been investigated in colon and peripheral blood: NK cells, TNK cells, γδT cells ($V\delta1^+T$, $V\delta2^+T$ and $V\delta3^+T$ subpopulations), mucosa-associated innate lymphoid $Lin^-CD161^+$ cells and $B_1$ cells were identified as innate immunity cells while T helpers, T killers and $B_2$ cells – as the main

**TABLE 1. CHARACTERISTIC OF LYMPHOID CELLS IN COLON AND PERIPHERAL BLOOD OF CD PATIENTS AND CONTROL GROUP, Me ($Q_{0.25}$÷$Q_{0.75}$) ‰**

| Lymphoid cells | IEL | | PBL | | p-value |
|---|---|---|---|---|---|
| | CD patients | Control group | CD patients | Control group | |
| | 1 | 2 | 3 | 4 | |
| $CD3^+$T cells, % | 74.7 (65.1 ÷ 80.1) | 70.4 (50.2 ÷ 78.1) | 75.2 (66.3 ÷ 78.3) | 72.1 (67.9 ÷ 70.3) | n. s. |
| $CD3^+CD4^+$ T helpers, % | 47.9 (17.9 ÷ 54.7) | 30.8 (25.2 ÷ 36.6) | 63.2 (59.8 ÷ 65.6) | 60.2 (57.6 ÷ 62.7) | n. s. |
| $CD3^+CD8^+$ T killers, % | 47.1 (36.3 ÷ 51.1) | 66.9 (63.9 ÷ 70.5) | 33.4 (25.5 ÷ 33.7) | 33.3 (27.3 ÷ 39.3) | $p_{1-2} < 0.01$ |
| $\gamma\delta TCR^+$T cells, % | 24.0 (17.3 ÷ 35.6) | 13.5 (9.9 ÷ 16.4) | 8.5 (2.5 ÷ 10.6) | 3.1 (2.1 ÷ 5.5) | $p_{1-2} < 0.01$ $p_{3-4} < 0.05$ |
| $CD19^+$B cells, % | 21.1 (14.9 ÷ 26.3) | 12.6 (7.9 ÷ 17.3) | 12.8 (11.8 ÷ 14.7) | 13.1 (10.8 ÷ 15.3) | n. s. |
| $CD19^+CD5^+$ $B_1$ cells, % | 5.6 (3.7 ÷10.0) | 11.1 (10.0 ÷12.2) | 16.8 (14.9 ÷ 19.7) | 20.8 (17.1 ÷ 24.5) | n. s. |
| $CD56^+$ NK cells, % | 14.1 (11.8 ÷ 19.8) | 27.3 (18.4 ÷ 35.7) | 14.3 (12.8 ÷ 17.9) | 10.1 (7.2 ÷ 13.1) | n. s. |
| $CD3^+CD56^+$ TNK cells, % | 13.7 (3.8 ÷ 19.3) | 26.6 (24.9 ÷ 33.3) | 0.2 (0.1 ÷ 0.3) | 5.8 (7.2 ÷ 13.1) | $p_{1-2} < 0.01$ |
| $CD161^+$ cells, % | 24.1 (20.7 ÷ 34.7) | 5.4 (2.6 ÷ 15.0) | 19.7 (16.3 ÷ 23.1) | 9.6 (5.8 ÷ 13.5) | $p_{1-2} < 0.01$ $p_{3-4} < 0.05$ |

Note. Me ($Q_{0.25}$÷$Q_{0.75}$), median, 25th and 75th percentiles; IEL, intraepithelial lymphocytes; PBL, peripheral blood lymphocytes; n, patients' number in a group; p-value, statistically significant test result; n. s., not significant; CD, cluster of differentiation; TCR, T cell receptor; NK cells, natural killer cells; TNK cells, natural killer T cells.

population of acquired immunity cells. The results of lymphoid cells phenotype are presented in Table 1.

The majority of colonic IEL was determined as $CD3^+$T lymphocytes and no significant differences were found in their numbers in investigated groups. However, changes in T cell subsets composition have been shown: the ratio of $CD3^+CD4^+$IEL and $CD3^+CD8^+$IEL was 1:1 in colon of CD patients and correlated with T cells in peripheral blood ($R = 0.7$; $p < 0.05$) while donor tissues were characterized by expected $CD3^+CD8^+$T killers prevalence and the ratio reached 1:2 ($p < 0.01$).

Currently the generally accepted approach to the immune status assessment is the characterization of lymphoid cells in the peripheral blood. However, the investigation of local mucosal gut immunity under immunopathological conditions remain highly relevant as well as its following comparison with circulating lymphoid cells for the identification the relationship between local reactions and systemic effects.

The obtained results are consistent Hu et al. (2017), indicating the absolute majority of $CD3^+$T lymphocytes among IEL and a decrease in their percentage in lamina propria, but at the same time demonstrating the active involvement of T lymphocytes of both compartments in inflammatory bowel diseases pathogenesis [5]. IEL localization contributes to their rapid response to antigenic structures, including microbiota components that come into contact with the gut epithelium. Mucosal T lymphocytes have a predominantly effector phenotype, exhibit various cytotoxic activities, including alloreactive and virus-specific, provide assistance to B cells, play a role in maintaining tolerance, and regulate the function of epithelial cells, thereby fulfilling the role of immune surveillance or the first line of defense. However, the presence of autoreactive T cells clones in CD patients leads to the fact that instead of the first line of defense, epithelial damage occurs as a result of an imbalance between immunological tolerance and effector immune response [12].

In healthy gut tissues $\alpha\beta$T lymphocytes are mainly represented by cytotoxic $CD3^+CD8^+$T cells, which play an important protective role in the detection and elimination of damaged epithelial cells and anti-infective protection. While in CD patients there is an infiltration of the epithelial layer by $CD3^+CD4^+$T helpers what was also confirmed in this study as T helpers were mainly determined in the colon of CD patients (Table 1). This presumably is associated with chronic antigenic stimulation by opportunistic bacteria in the gut and indicates an autoimmune inflammatory response. Autoimmune inflammation in CD patients is mainly associated with increased activation and aberrant proliferation of

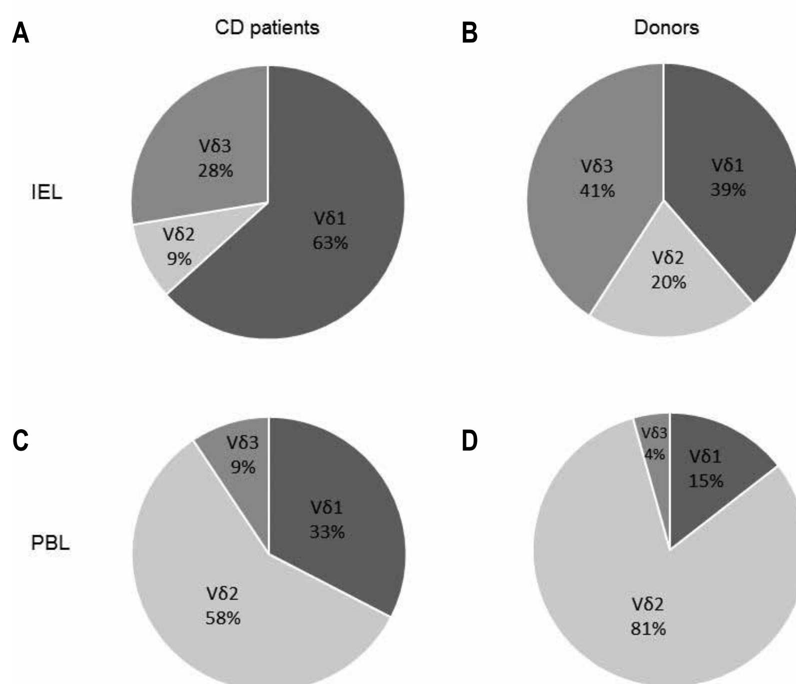


**Figure 1. γδT cells subsets (%) in colon and peripheral blood of CD patients and control group**

Note. (A) γδIEL subsets in colon of CD patients. (B) γδIEL subsets in colon of control group. (C) γδT cells subsets in peripheral blood of CD patients. (D) γδT cells subsets in peripheral blood of control group.

T helper types 1 and 17, the effector reactions of which are mediated by the production of pro-inflammatory cytokines (IL-12, IL-23, IFNγ and IL-17). T helpers are the main mediators of cellular immunity and play a key role in the activation of other immune cells such as B cells and cytotoxic T cells by modulating an antigen-specific immune response [6].

However, a growing number of evidence points to the role of cytotoxicity mechanisms in intestinal tissue injury: T killers realize their effector reactions through the appearance of such highly active biomolecules as perforin, granzymes, granulisin, Fas-ligand and tumor necrosis factor α. Moreover, pro-inflammatory cytokines released by T killers may be involved in disrupting the epithelial barrier by inducing epithelial cell apoptosis and increasing intestinal permeability. High levels of cytotoxicity support the existing concept that CD affects the entire gastrointestinal tract [1].

The investigation of T cells subsets based on the type of T cell receptors (αβTCR or γδTCR) expression revealed a statistically significant increase in the number of γδT cells in colon ($p < 0.01$) as well as in peripheral blood ($p < 0.05$) of CD patients as compared to donors (Table 1). Moreover, the number of colonic γδIEL was correlated with disease location ($R = -0.6$; $p < 0.05$), and disease behavior ($R = 0.7$; $p < 0.01$) according to Montreal classification what confirmed the involvement of unconventional γδT lymphocytes in CD immunopathogenesis.

Due to the high heterogeneity of γδT cells, a detailed subsets analysis of circulating and resident unconventional T cells was carried out. As seen in Figure 1, $V\delta1^+$T cells subset was prevailed (> 60%) among colonic γδIEL (Figure 1A) and significantly elevated in peripheral blood among CD patients (Figure 1C) in combination with decreased $V\delta2^+$T cells numbers as compared to control group (Figure 1B and D, $p < 0.05$).

Unlike αβT lymphocytes, γδT cells combine cells' properties of innate and acquired immunity: their receptor apparatus is partially similar to that of antigen-presenting cells and NK cells in expression of pattern recognition and killer/inhibitor receptors, however, this population is able to recognize antigenic structures via a specific T cell receptor, similarly to classical T lymphocytes [2]. An established persistent increase in γδT lymphocytes rate in colon and peripheral blood of CD patients is consistent with Regner et al. [12] characterizing the non-thymic origin of some lymphoid cells of the gut mucosa, at times reaching up to 50% of intestinal lymphoid cells. Experimental studies are rather contradictory in confirming the involvement of γδT lymphocytes in CD pathogenesis which is partly due to the high heterogeneity of this population, and therefore their clinical significance has not been fully elucidated [10].

Taking into consideration the predominantly cytotoxic $V\delta1^+$T cells profile, γδT cells can be assumed as one of the potential mechanisms of mucosal barrier

damage and gut inflammation contributing to the chronicity and systemic reactions to antigens of the gastrointestinal microbiota. Most authors support the hypothesis of aberrant activation of γδT lymphocytes due to inflammatory microenvironment formed by changes in αβT cells subsets in the intestinal mucosa, as well as microbial stimulation what resulted in their active homing through the intestinal epithelium via an occludin-dependent mechanism and involvement in disease progression by realizing their cytotoxic effector reactions. In addition, excessive epithelial regeneration mediated by γδT cells may contributes to the formation of pseudopodia, atypical serrated surface, and the development of serious disease complications, such as colorectal cancer [9].

Besides γδT cells, CD patients also display a significantly higher percentage of mucosa-associated innate lymphoid $CD161^+$ cells in colon and peripheral blood than healthy individuals (Table 1). CD161 is a C-type lectin-like type-II transmembrane protein that is expressed on the majority of natural killer (NK) cells, innate T cells (mucosal-associated invariant T cells, invariant natural killer T cells, γδT cells), some adult peripheral blood αβT cells as well as on innate lymphoid cells, restricting to those cells with a secretion of interleukin-17, and therefore to type 17 phenotype [7]. The reasons behind the preferential accumulation of $CD161^+$ cells in the intestine are not completely understood. Apart from the presence of $CD161^+$ cells in the gut, a number of studies demonstrated that CD161 was preferentially expressed on circulating T cells that exhibited gut-homing properties like expression of chemokine receptor 9 or integrin α4β7. In fact, CD161 was proposed to be involved in lymphocyte transendothelial migration and thereby could facilitate trafficking of CD161-expressing T cells to the intestine [4].

The interesting revealed fact was the decrease of circulating and colonic TNK cells reflecting a possible reduction of immunoregulatory mechanisms as this population has a critical role in peripheral tolerance. TNK cells represent a minor subset of T lymphocytes that share cell-surface proteins with conventional T cells and NK cells. A lack of TNK cells may result in a defective regulation of luminal bacteria and as a consequence overt bacterial invasion into the intestine and chronic inflammatory responses. Accordingly, in the presence of TNK cells, dendritic cells were found to produce more IL-10 and lose the ability to produce IL-12. It is unclear what percentages of IEL are in fact TNK cells in human colon, presumably, it may vary from 17 to 45%. The biological function of TNK cells is paradoxical, because these cells can rapidly produce large amounts of T helper type 1 (Th1), Th2, and regulatory cytokines. TNK cells may thus promote or suppress cell-mediated immunity in different conditions. But the exact function of TNK cells in the intestine and whether they may also have regulatory functions in intestinal inflammation remain uncertain [13].

## Conclusion

In colonic IEL composition the changes in major (T helpers) and minor (γδT cells, TNK cells and mucosa-associated $CD161^+$ cells) populations of intraepithelial T lymphocytes have been revealed in CD patients, while B lymphocytes and NK cells may be not so pathogenetically significant and further research is required to establish their role in disease immunopathogenesis. Taking into account that the intestinal environment is tolerant to most foreign harmless antigens, the identified changes in the quantitative composition of lymphoid subpopulations may be trigger mechanisms for disruption of mucosal barrier integrity, translocation of intestinal bacteria, and an increase in the number of local and systemic inflammatory reactions. The established correlation suggests the migration of mucosal lymphoid cells and their circulation in the peripheral blood what can be used as diagnostically significant markers of disease progression and opens up new possibilities for the use of mucosal lymphoid cells in targeted therapy and preventive medicine.

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# ГИПЕРСЕГМЕНТАЦИЯ ЯДЕР НЕЙТРОФИЛОВ КРОВИ У БОЛЬНЫХ С ЛОКАЛИЗОВАННЫМ И РАСПРОСТРАНЕННЫМ РАКОМ ГОРТАНИ И ГОРТАНОГЛОТКИ

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**Резюме.** Нейтрофильные гранулоциты обладают широким спектром функциональной активности. В последние годы обсуждается функциональная значимость нейтрофилов в развитии и течении злокачественных новообразований. Показано, что при опухолевом росте нейтрофильные гранулоциты могут играть двоякую роль, проявляя про- или противоопухолевую активность. Результаты клинических и экспериментальных исследований свидетельствуют о возможности перепрограммирования нейтрофилов под влиянием факторов микроокружения. Цель исследования – оценка структурных и функциональных особенностей нейтрофильных гранулоцитов у больных с разной степенью распространенности рака гортани и гортаноглотки. Проведено обследование 41 пациента (мужчины в возрасте от 35 до 67 лет) с впервые выявленным раком гортани и гортаноглотки. После окончательной верификации диагноза пациенты были разделены на подгруппы в соответствии с классификацией TNM: первую подгруппу с локализованным опухолевым процессом ($T_{1-3}N_0M_0$) составили 14 пациентов, вторая подгруппа состояла из 27 пациентов с распространенным опухолевым процессом ($T_3N_{1-2}M_0$). В периферической крови оценивали относительное и абсолютное количество нейтрофилов, определяли нейтрофильно-лимфоцитарное соотношение (NLR). Подсчитывали относительное содержание в крови нейтрофилов с разной степенью сегментированности ядер, цитохимически определяли активность кислородозависмых и кислородонезависмых механизмов микробицидности (миелопероксидазы, катионных белков, щелочной фосфатазы и степень активации нейтрофилов в НСТ-тесте). Концентрацию интерлейкина-8 определяли с помощью иммуноферментного анализа. Выявлено, что у больных раком гортани и гортаноглотки на фоне повышенной в сравнении со здоровыми концентрации интерлейкина-8 возрастает численность циркулирующих в крови нейтрофиль-

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ных гранулоцитов (p = 0,045) и NLR (p = 0,033). В популяции нейтрофилов по мере распространения опухолевого процесса увеличивается доля клеток, обладающих гиперсегментированными ядрами (p < 0,001) и повышенным цитотоксическим потенциалом. Выявлена прямая корреляционная связь средней силы (r = 0,42, p = 0,03) между индексом Т, отражающим объем опухоли, и относительным содержанием гиперсегментированных нейтрофилов. На основании результатов настоящего исследования можно утверждать, что такой простой и доступный для анализа лабораторный параметр, как степень сегментации ядер нейтрофильных гранулоцитов, может быть использован в качестве одного из критериев, позволяющих оценивать и прогнозировать особенности течения опухолевого процесса.

*Ключевые слова: рак гортани, рак гортаноглотки, объем опухоли, нейтрофилы, гиперсегментация ядер, цитотоксичность нейтрофилов, IL-8*

# HYPERSEGMENTATION OF NEUTROPHIL NUCLEI IN PERIPHERAL BLOOD OF PATIENTS WITH LOCALIZED AND ADVANCED CANCER OF THE LARYNX AND LARYNGOPHARYNX

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**Abstract.** Neutrophilic granulocytes have a wide spectrum of functional activity. In recent years, the functional significance of neutrophils in the development and course of malignant neoplasms has been discussed. It has been shown that neutrophilic granulocytes can play pro- or antitumor activity. The aim of the study was to assess the structural and functional features of neutrophils in patients with varying degrees of prevalence of cancer of the larynx and laryngopharynx. Forty-one patients (aged 35-67) with newly diagnosed cancer of the larynx and laryngopharynx were examined and divided into subgroups according to the TNM classification: the first subgroup (14 patients) with a localized tumor process consisted; and the second subgroup (27 patients) with a widespread tumor process. The relative and absolute number of neutrophils was assessed, and the neutrophil-lymphocyte ratio (NLR) was determined. The content of neutrophils with varying degrees of nuclear segmentation in the blood was calculated, the activity of myeloperoxidase, cationic proteins, alkaline phosphatase, and the degree of neutrophil activation in the NBT test was determined cytochemically. Concentration of interleukin-8 was determined using ELISA. In patients with cancer of the larynx and laryngopharynx the number of neutrophils (p = 0.045) and NLR (p = 0.033), as well as serum concentration of interleukin 8 (p = 0.011), increased compared to healthy individuals. The proportion of cells with hypersegmented nuclei in the neutrophil population (p < 0.001) and cytotoxic potential increased with the spread of tumor process. A direct correlation (r = 0.42, p = 0.03) was found between the T index, which reflects the volume of the tumor, and the content of hypersegmented neutrophils. It can be argued that such a simple and accessible laboratory parameter as the degree of segmentation of the nuclei of neutrophilic granulocytes can be used as one of the criteria to assess and predict the course of the tumor process.

*Keywords: cancer of the larynx, cancer of the laryngopharynx, tumor volume, neutrophil, hypersegmentation of nuclei, cytotoxicity of neutrophils, IL-8*

## Introduction

Neutrophilic granulocytes (NGs), also called polymorphonuclear or segmented leukocytes, are the dominant cell population among circulating leukocytes. Their most important function is considered to be antimicrobial protection implemented in the innate immune system through phagocytosis, secretory degranulation, and the formation of neutrophil extracellular "traps" (NETs) [1].

In recent years, information has appeared that NGs, along with the phagocytic ability, have a wide variety of functional activities. It has been shown that neutrophils are able to produce cytokines, modulate the activity

of cells in the microenvironment, and thus actively participate in the pathogenesis of various diseases, including cancer [1, 8]. Relatively recently, it has become clear that neutrophilic granulocytes can play a dual role during tumor growth, exhibiting either pro- or antitumor activity [1]. The subpopulation of neutrophils with antitumor properties is commonly referred to as N1, with protumor properties as N2 [1, 4, 7]. The results of clinical and experimental studies demonstrate the ability of neutrophils to transform under the influence of tumor microenvironment factors [4, 7]. Currently, close attention of researchers is directed to the search for morphological, phenotypic, and molecular genetic markers of pro- and antitumor NGs phenotypes and to assess the possibility of using these cells as diagnostic and therapeutic targets [2, 4, 6].

***The aim of this study*** was to evaluate the structural and functional features of neutrophilic granulocytes circulating in the blood in patients with varying degrees of prevalence of cancer of the larynx and laryngopharynx.

## Materials and methods

On the basis of the endoscopic department of the Research Institute of Oncology of the Tomsk Research Medical Center of the Russian Academy of Sciences (Tomsk), 41 patients with newly diagnosed cancer of the larynx and hypopharynx (men aged 35 to 67 years) were examined before starting antitumor therapy. After the final verification of the diagnosis, all patients with cancer of the larynx and laryngopharynx were divided into subgroups in accordance with the TNM classification, in which the T (tumor) criterion characterizes the size of the primary tumor, the N (nodus) criterion describes the state of regional lymph nodes, and the M (metastasis) criterion indicates the presence or absence of distant metastases. The first subgroup with a localized tumor process (T1-3N0M0) included 14 patients, the second subgroup consisted of 27 patients with a widespread tumor process (T3N1-2M0). As a control group, 55 apparently healthy individuals (men aged 39 to 69 years) were examined, for whom the inclusion criteria were: the absence of malignant and benign neoplasms in history, the absence of chronic diseases of the oropharynx and nasopharynx, the absence of acute respiratory viral infections within 3 weeks before the examination, sanitized oral cavity (absence of carious teeth and bleeding gums). All examined persons underwent the procedure of signing the informed consent to participate in the scientific and practical research.

The total number of leukocytes was determined in the blood and the leukocyte formula was calculated using a Carl Zeiss Axioskop 40 FL microscope in the standard mode and the ratio of the relative number of neutrophils and lymphocytes (Neutrophil-Lymphocyte Ratio – NLR) was determined. The percentage of active neutrophils and the intensity of activation of oxygen-dependent mechanisms of cytotoxicity were assessed using the NBT-test in the form of an average cytochemical coefficient (ACC) according to the L.S. Kaplow [3]. Cytochemically, the intracellular expression of neutrophil marker enzymes (myeloperoxidase, alkaline phosphatase) and cationic proteins involved in the implementation of the cytotoxic function was determined [3]. In addition, when analyzing 100 neutrophilic granulocytes, groups of cells with varying degrees of nuclear segmentation were identified: 1) cells with nuclei containing 2-3 segments; 2) neutrophils containing 4 segments; 3) neutrophils containing 5 or more segments (hypersegmented nuclei). To analyze the structural features, the average segmentation coefficient (ASC) was introduced, which reflects the distribution of neutrophils with different nuclear morphology. The average segmentation coefficient was determined by the formula:

$$ASC = (1 \cdot a + 2 \cdot b + 3 \cdot c)/N,$$

where a is the relative number of neutrophils with a nucleus consisting of 2-3 segments; b is the relative number of neutrophils with a nucleus consisting of 4 segments; c is the relative number of hypersegmented neutrophils with a nucleus of 5 or more segments; N is the number of counted cells.

In blood plasma, ELISA was used to determine the concentration of interleukin (IL)-8, one of the main chemokines for NG. To determine the concentration of IL-8, a set of reagents Interleukin-8-ELISA-BEST (CJSC Vector-Best, Russia) was used.

For statistical analysis of the obtained data, the software package "SPSS Statistics 17.0" was used. The results were processed using the Mann-Whitney U-test and presented as a median and quartiles – Me ($Q_{0.25}$-$Q_{0.75}$). Differences between groups were considered significant at a significance level not exceeding 0.05. Spearman's paired correlation analysis was used to identify the relationships between the parameters.

## Results and discussion

Statistically significant differences in the group of patients with cancer of the larynx and laryngopharynx from the group of healthy ones were manifested in the shift of the leukocyte formula towards the predominance of neutrophilic cells: 59.00 (54.00-66.00) % – in patients and 55.00 (51.00-61.00) % in healthy people (p = 0.045). Changes in the hemogram were also reflected in the increase in NLR: in the group of patients, this indicator was 2.12 (1.66-3.00), while in healthy people it was 1.75 (1.31-2.13), p = 0.033. In the last decade, many publications have appeared on the change in NLR in cancers of various localizations, and our data are consistent with the results of other

authors, who recorded an increase in this indicator in tumors of various localizations [13].

It should be noted that an increase in the relative and absolute number of neutrophils in the blood was noted against the background of an increase in the concentration of IL-8, the content of which was 17.36 (15.14-41.45) pg/mL in patients with cancer of the larynx and laryngopharynx compared with 10.75 (6.86-32.95) pg/mL in healthy people (p = 0.011). Information about the increased concentration of IL8 in the blood serum of cancer patients has been presented in recent years in a number of publications, while the position on the possibility of using an elevated level of this cytokine as a negative prognostic criterion in various malignant neoplasms is very well substantiated [6].

Analysis of the structural features of neutrophils, conducted in this study, revealed in patients with cancer of the larynx and laryngopharynx an increased number of NGs circulating in the blood with four, five or more segments of the nuclei (Table 1).

The oxygen-dependent cytotoxicity of NGs was assessed by the results of a spontaneous NBT-test, reflecting the activity of NADPH oxidase, and by the activity of myeloperoxidase (Table 2). The relative number of NBT-positive neutrophils and the average cytochemical coefficient (ACC) in this test in patients with cancer of the larynx and laryngopharynx were increased in comparison with the control group, mainly due to cells with an average and high degree of activation (Table 2). Myeloperoxidase activity was also increased in NGs in cancer patients (Table 2). Similar features were revealed when assessing the state of oxygen-independent mechanisms of neutrophil cytotoxicity: cytochemical coefficients characterizing the content of cationic proteins and alkaline phosphatase in patients with laryngeal cancer exceeded the corresponding values in the healthy group (Table 2). The ability to implement cytotoxic reactions is regarded ambiguously when considering the pro- and antitumor effects of NGs. On the one hand, this cytotoxicity can be realized in relation to tumor target cells, on the other hand, it can contribute to disruption of the structural organization of the extracellular matrix, abnormal angiogenesis, and metastasis [2].

Analyzing the relationship between the parameters characterizing the morphofunctional status of NGs with the clinical characteristics of the tumor process, determined using the TNM classification, we found that in the subgroup of patients with advanced tumor process (T3N1-2M0), the number of hypersegmented cells (5 or more segments) exceeded the corresponding indicator patients with localized cancer of the larynx and laryngopharynx (T1-3N0M0): (10.0 (7.5-14.5)) % and (4.0 (2.0-8.5)) %, respectively (p = 0,04). When conducting a paired correlation analysis, a direct relationship of medium strength (r = 0.42, p = 0.03) was revealed between the T index, which reflects the volume of the tumor, and the relative content of hypersegmented neutrophils.

Currently, the prevailing opinion is that a segmented nucleus is necessary for neutrophils for accelerated migration into tissues, while the mechanisms and functional significance of the hypersegmentation phenomenon are not fully understood [5]. It is generally accepted that hypersegmented forms of NGs (more than 5 segments) appear in the blood in pathological conditions [5]. Nuclear hypersegmentation is one of the earliest, most sensitive and specific signs of megaloblastic anemia, and is also observed in myelodysplastic syndrome, chronic infections, and conditions associated with a decrease in the concentration of granulocyte colony-stimulating factor (G-CSF) [5]. It is well known that NGs are the first immunocompetent cells migrating from blood vessels to the area of tissue damage or infectious inflammation. It has been proven that, due to their pronounced ability to extravasation and interstitial migration, NGs, along with the implementation of

**TABLE 1. DISTRIBUTION OF NEUTROPHILS ACCORDING TO THE DEGREE OF NUCLEAR SEGMENTATION IN PATIENTS WITH CANCER OF THE LARYNX AND LARYNGOPHARYNX, Me ($Q_{0.25}$-$Q_{0.75}$)**

| Indicator | Study group | | p |
|---|---|---|---|
| | Cancer of the larynx and laryngopharynx (n = 41) | Healthy volunteers (n = 55) | |
| **Neutrophils with a moderate degree of segmentation (2-3 segments), %** | 48 (36-60) | 76 (70-82) | p < 0.001 |
| **Neutrophils with a high degree of segmentation (4 segments), %** | 39 (31-50) | 20 (18-29) | p < 0.001 |
| **Hypersegmented neutrophils (5 and > segments), %** | 11 (7-17) | 1 (0-2) | p < 0.001 |
| **Average segmentation coefficient (ASC)** | 1.66 (1.47-1.79) | 1.24 (1.18-1.33) | p < 0.001 |

Note. n, the number of examined individuals, p, the level of significance of differences.

**TABLE 2. INDICATORS CHARACTERIZING THE CYTOTOXIC POTENTIAL OF NEUTROPHILS IN PATIENTS WITH CANCER OF THE LARYNX AND LARYNGOPHARYNX, Me ($Q_{0.25}$-$Q_{0.75}$)**

| Indicator | Study group | | p |
|---|---|---|---|
| | Cancer of the larynx and laryngopharynx (n = 41) | Healthy volunteers (n = 55) | |
| **NBT-positive neutrophils, %** | 20 (10-30) | 10 (10-12) | 0.042 |
| **ACC in the NBT-test** | 0.28 (0.13-0.57) | 0.12 (0.10-0.16) | 0.003 |
| **Myeloperoxidase activity, ACC** | 1.92 (1.74-2.12) | 1.58 (1.39-1.80) | 0.010 |
| **Cationic proteins, ACC** | 1.61 (1.44-1.68) | 1.34 (1.22-1.51) | 0.039 |
| **Alkaline phosphatase, ACC** | 0.66 (0.61-0.79) | 0.33 (0.25-0.40) | < 0.001 |

Note. n, the number of examined patients; p, the level of significance of differences; ACC, the average cytochemical coefficient.

phagocytosis, are actively involved in tissue remodeling during wound healing, angiogenesis, tumor formation, and metastasis [9]. Moreover, neutrophils very quickly have to switch between different modes of migration. The segmented nucleus allows cells to change their shape and easily overcome intercellular contacts as part of tissue cell layers. However, other cells with non-segmented nuclei, such as lymphocytes and monocytes (mononuclear cells), are also able to migrate into tissues [11]. It is believed that the severity of segmentation of the NGs nuclei characterizes the degree of their maturity, and the hypersegmented nucleus, which has increased flexibility, indicates the readiness of the cell to complete the life cycle [5]. Interestingly, "old" neutrophils migrate faster to the sites of inflammation [9]. To date, there is no unequivocal answer about the functional significance of hypersegmented neutrophils present in the tumor growth zone. Experimental studies have shown that nuclear hypersegmentation, which is a morphological sign of more mature neutrophils, is characteristic of cells with antitumor properties, i.e. for N1-neutrophils [4]. At the same time, there is evidence that the presence of a large number of hypersegmented neutrophils in tumor tissue is associated with an unfavorable outcome of oncological diseases [6].

There is still no unequivocal answer to the question: are the morphofunctional features of cells formed during differentiation in the bone marrow, or are mature neutrophils, exposed to any factors in the systemic circulation, capable of plasticity? Experimental studies have demonstrated the possibility of *in vitro* induction of a hypersegmented neutrophil phenotype, corresponding in its functional characteristics to the antitumor subpopulation N1 [10, 12].

The results of this study indicate that the number of cells with hypersegmented nuclei circulating in the blood increases as the tumor process spreads, and simultaneously with an increase in the degree of nuclear segmentation, the cytotoxic potential of NGs increases. Questions about the functional significance and biological feasibility of morphological modifications of the nuclear apparatus of neutrophils circulating in the blood during malignant growth remain open. Based on the results of this study, it can be argued that such a simple and accessible for analysis laboratory parameter as the degree of segmentation of NCs nuclei can be used as one of the criteria to assess and predict the course of the tumor process.

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# ФУНКЦИОНАЛЬНАЯ АКТИВНОСТЬ МОНОЦИТАРНОГО ЗВЕНА ИММУНИТЕТА ПРИ АДЕНОКАРЦИНОМЕ ЖЕЛУДКА

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**Резюме.** Рак желудка входит в десятку по распространенности и занимает 4-е место по причинам смертности во всем мире. Наиболее распространенным и при этом самым агрессивным вариантом рака желудка является аденокарцинома (АКЖ). Моноцитарное звено иммунитета обеспечивают основную линию борьбы организма со злокачественными клетками, при этом у пациентов с АКЖ является недостаточно изученным.

Цель исследования — оценить показатели функциональной активности моноцитов у пациентов с аденокарциномой желудка на разных стадиях заболевания.

Обследовано 164 человека, среди которых 85 был поставлен диагноз аденокарцинома желудка I-IV стадии. Также в исследовании приняли участие 79 практически здоровых доноров. Функциональную активность и кислород-зависимый фагоцитоз моноцитов оценивали хемилюминесцентным методом. В качестве индуктора хемилюминесценции использовали люминол. Активация респираторного взрыва осуществлялась опсонизированным зимозаном.

У больных аденокарциномой желудка выявлено в состоянии покоя (спонтанная хемилюминесценция) увеличение показателей времени выхода кривой на максимум интенсивности хемилюминесценции (Tmax = 7957 с), площади под кривой хемилюминесценции (Squr = $0,2 \times 10^6$), индекса активации (1,89 у. е.) и снижение максимального значения интенсивности хемилюминесценции (Imax = 424 у. е.) относительно контрольной группой (Tmax = 5533 с, Squr = $0,011 \times 10^6$, индекс активации = 0,88 у. е., Imax = 424 у. е., $p < 0,05$). При индуцировании хемилюминесценции у больных АКЖ фиксируется статистически значимое преобладание Squr ($0,46 \times 10^6$, в контрольной группе Squr = $0,031 \times 10^6$). Также в группе пациентов с аденокарциномой желудка моноцитарный фагоцитоз снижен более чем в 2 раза (29% против 84% в контрольной группе, $p < 0,05$). При анализе исследуемых параметров в зависимости от стадии заболевания было установлено, что нарушение хемилюминесцентной реакции у больных аденокарциномой желудка фиксируется уже на ранней стадии. При этом у больных с IV стадией аденокарциномы желудка показатели спонтанной и индуцированной хемилюминесценции более чем в 2 раза отличаются от показателей контрольной группы и пациентов на I стадии заболевания. Выявленные особенности свидетельствует о снижении эффективности иммунных реакций моноцитар-

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ного звена при аденокарциноме желудка уже на ранних стадиях заболевания и могут использоваться для выявления ранних признаков иммунных нарушений и оптимизации терапевтических подходов при данном заболевании.

*Ключевые слова: аденокарцинома желудка, моноциты, хемилюминесцентная активность, иммунитет, иммунодефицит, макрофаги*

# FUNCTIONAL ACTIVITY OF THE MONOCYTE IMMUNE LINK IN GASTRIC ADENOCARCINOMA

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**Abstract.** Stomach cancer is in the top ten in terms of prevalence and ranks 4th in terms of causes of death worldwide. The most common and most aggressive variant of gastric cancer is adenocarcinoma. The monocytic link of immunity provides the main line of the body's fight against malignant cells, while in patients with adenocarcinoma it is insufficiently studied. The purpose of the study was to evaluate the functional activity of monocytes in patients with gastric adenocarcinoma at different stages of the disease.

Individuals (n = 164) were examined, among whom 85 were diagnosed with stage I-IV stomach adenocarcinoma. The study also involved 79 apparently healthy donors. The functional activity and oxygen-dependent phagocytosis of monocytes were assessed by the chemiluminescent method. Luminol was used as a chemiluminescence inducer. The respiratory burst was activated with opsonized zymosan.

In patients with stomach adenocarcinoma, at rest (spontaneous chemiluminescence), an increase in the time the curve reached the maximum intensity of chemiluminescence (Tmax = 7957 s), the area under the chemiluminescence curve (Squr = $0.2 \times 10^6$), the activation index (1.89 c. u.) and a decrease in the maximum value of chemiluminescence intensity (Imax = 424 c. u.) relative to the control group (Tmax = 5533 s, Squr = $0.011 \times 10^6$, activation index = 0.88 c. u., Imax = 424 c. u., $p < 0.05$) were seen. When chemiluminescence is induced in patients with stomach adenocarcinoma, a statistically significant predominance of Squr is fixed ($0.46 \times 10^6$, in the control group Squr = $0.031 \times 10^6$). Also, in the group of patients with stomach adenocarcinoma, monocytic phagocytosis was reduced by more than 2 times (29% *vs* 84% in the control group, $p < 0.05$). When analyzing the studied parameters, depending on the stage of the disease, it was found that the violation of the chemiluminescent reaction in patients with stomach adenocarcinoma is fixed already at an early stage. At the same time, in patients with stage IV stomach adenocarcinoma, the indicators of spontaneous and induced chemiluminescence are more than 2 times different from those in the control group and patients at stage I of the disease. The identified features indicate a decrease in the effectiveness of immune reactions of the monocytic link in stomach adenocarcinoma already in the early stages of the disease and can be used to detect early signs of immune disorders and optimize therapeutic approaches in this disease.

*Keywords: gastric adenocarcinoma, monocytes, chemiluminescent activity, immunity, immunodeficiency, macrophages*

## Introduction

Oncological diseases remain one of the most pressing socioeconomic problems worldwide [11]. According to the WHO for 2020, stomach cancer is among the ten most common and ranks 4th in terms of causes of death worldwide [12]. And although in the developed countries of the world there has been a trend towards a decrease in the incidence of this pathology, in developing countries, stomach cancer continues to occupy a leading position in terms of mortality among oncological diseases. Stomach cancer has a low survival rate due to late diagnosis, with survival rates directly correlated with the stage of the disease. Russia is among the top three countries in terms of morbidity and mortality for this disease. Also, for stomach cancer, pronounced regional variability is characteristic. According to MSROI named after P.A. Herzen for 2020, the highest percentage of the prevalence of this disease is recorded in the Central, Volga and Siberian federal districts, while this disease is least common in the North Caucasian and Far

Eastern regions. The Krasnoyarsk Territory among the territories of the Siberian federal district is the leader in terms of incidence of stomach cancer [7]. Currently, the mechanisms of the pathogenesis of gastric cancer remain poorly understood. The etiology of this disease is characterized by multifactoriality, with special attention being paid to the infection of the body with *Helicobacter pylori*, recognized by WHO as a carcinogen [6]. Adenocarcinoma is the most common and aggressive type of stomach cancer [1, 4].

The immune system, and primarily phagocytic leukocytes, provide the body's main line of defense against malignant cells. The monocyte is the most active phagocyte in peripheral blood, with a phagocytic index approximately 3 times higher than that of neutrophils. A distinctive feature of monocytes is their high activity in an acidic environment, as well as the preservation of viability after the phagocytization process. Monocytes perform various functions – cytokine production, pathogen clearance, antigen presentation, are involved in wound healing and providing pro- and antitumor response [2] chemiluminescent activity. Chemiluminescent analysis is based on the registration of the emission of light quanta caused by the production of free radicals and reactive oxygen species by phagocytic cells at rest (spontaneous chemiluminescence), as well as in response to a stimulus or stimulus (induced chemiluminescence). There are works in the literature devoted to the chemiluminescent activity of neutrophils in various malignant diseases, however, such studies on monocytes in stomach adenocarcinoma are rare. Given the asymptomatic nature of the course of stomach cancer in the early stages, the identification of markers of impaired immune response, including the phagocytic activity of monocytes, can expand laboratory capabilities for detecting this malignant disease during population screening.

**The aim of the study** was to evaluate the features of the functional activity of monocytes in stomach cancer, depending on the stage of the disease.

## Materials and methods

As part of the goal, 164 people were examined, of which 85 were patients with stomach adenocarcinoma (SAC) aged 22-69 years. The control group consisted of 79 practically healthy donors without gastroenterological complaints (aged 20-68 years). Diagnosis of stomach adenocarcinoma was carried out by oncologists in the Krasnoyarsk regional oncological dispensary on the basis of a comprehensive instrumental and morphological examination. The study included patients with SAC stages I, II, III, IV.

The study was conducted with the permission of the Ethics Committee of the FRC KSC SB RAS (protocol no. 4 dated 02.08.2019). In working with the examined patients, the ethical principles required by article 24 of the Constitution of the Russian Federation and the Declaration of Helsinki by the World Medical Association. Each participant signed an informed consent form for the study confirming their voluntary participation in the study.

Venous blood for research was taken in the morning from 8 to 9 o'clock, on an empty stomach, from the cubital vein, into vacutainer tubes with sodium heparin solution (5 U/mL). Peripheral blood monocytes were obtained by the standard method of adhesion to flat surfaces from mononuclear cells isolated from heparinized venous blood by ficoll-urografin density gradient centrifugation ($\rho = 1.077$).

The assessment of spontaneous and induced chemiluminescence of monocytes was carried out for 90 minutes on a 36-channel CL 3606 chemiluminescent analyzer (Russia). The study of oxygen-dependent phagocytosis of blood monocytes was also carried out by the chemiluminescent method. The following characteristics were determined: the time of appearance of the chemiluminescence intensity maximum (Tmax), the maximum value of the chemiluminescence intensity (Imax), and the area of the chemiluminescence curve (S). Luminol was used as a chemiluminescence enhancer. Opsonized zymosan served as the respiratory burst inducer. The enhancement of chemiluminescence induced by opsonized zymosan was estimated by the ratio of the area of induced (S induced) to the area of spontaneous (S spont.) chemiluminescence and was designated by the activation index. Statistical data processing was carried out using the Statistica for Windows 8.0 (StatSoft Inc., USA, 2008) and Microsoft Excel 2007 (Microsoft, USA) application packages. Processing of the received data included calculation of non-parametric data: median (Me) and quartiles ($Q_{0.25}$-$Q_{0.75}$). Statistical differences between the data were assessed using the nonparametric Mann–Whitney U test. The critical level of significance when testing statistical hypotheses was taken equal to $p < 0.05$.

## Results and discussion

The study of the chemiluminescent activity (CA) of monocytes will allow us to evaluate the characteristics of a respiratory burst in a spontaneous and induced state in stomach adenocarcinoma. It is believed that the functional activity of phagocytes depends on CA, the higher the chemiluminescent activity, the greater the functional ability of the cells.

At the first stage, the chemiluminescent activity of monocytes was studied in patients with gastric adenocarcinoma relative to the control group (Table 1). It was found that the index of maximum intensity (Imax) during spontaneous chemiluminescence is lower compared to the control

**TABLE 1. PARAMETERS OF THE CHEMILUMINESCENT REACTION OF MONOCYTES IN PATIENTS WITH STOMACH ADENOCARCINOMA (SAC) COMPARED WITH THE CONTROL GROUP, Me ($Q_{0.25}$-$Q_{0.75}$)**

| Indicator | Patients with SAC (n = 85) | Control group (n = 79) |
|---|---|---|
| **Imax spontaneous, c. u.** | 424* (295.6-614.7) | 486 (77.7-690.4) |
| **Tmax spontaneous, s** | 7957* (7313-10465) | 5533 (3505-9992) |
| **Squr spontaneous (× $10^6$)** | 0.2* (0.140-0.305) | 0.011 (0.006-0.022) |
| **Imax induced, c. u.** | 1183 (657-1284) | 1395.6 (412.9-1637.3) |
| **Tmax induced, s** | 5496 (5085-5869) | 5485 (3104-6185) |
| **Squr induced (× $10^6$)** | 0.46* (0.39-0.96) | 0.031 (0.01-0.07) |
| **Activation index** | 1.89* (0.91-2.07) | 0.88 (0.6-1.3) |
| **Monocytic phagocytosis** | 29%±5% * | 84% ±7% |

Note. *, statistically significant differences between the indices of patients with stomach cancer and the control group (p < 0.001).

**TABLE 2. INDICATORS OF SPONTANEOUS AND INDUCED CHEMILUMINESCENT RESPONSE OF MONOCYTES IN PATIENTS WITH STOMACH ADENOCARCINOMA (SAC) COMPARED WITH THE CONTROL GROUP, Me ($Q_{0.25}$-$Q_{0.75}$)**

| Indicator | Control group (n = 79) | SAC I stage (n = 19) | SAC II stage (n = 22) | SAC III stage (n = 24) | SAC IV stage (n = 20) |
|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** |
| **Imax spontan., c. u.** | 486.8 (77.7-690.4) | 454 (342-597) | 425 (296-615) | 390 (305-506) | 362 (280-477) |
| | | | | | $p_{1-5} < 0.05$ |
| **Tmax spontan., s** | 5523 (3505-9992) | 6401 (6355-10060) | 7951 (7313-10465) | 9057 (7678-11263) | 9285 (7569-11406) |
| | | $p_{1-2} < 0.001$ | $p_{1-3} < 0.001$ | $p_{1-4} < 0.001$ | $p_{1-5} < 0.001$ $p_{2-5} < 0.05$ |
| **Squr spontan. (× $10^6$)** | 0.01 (0.006-0.022) | 0.12 (0.099-0.240) | 0.20 (0.14-0.30) | 0.29 (0.20-0.34) | 0.33 (0.21-0.37) |
| | | $p_{1-2} < 0.001$ | $p_{1-3} < 0.001$ | $p_{1-4} < 0.001$ | $p_{1-5} < 0.05$ $p_{2-5} < 0.001$ |
| **Imax induced, c. u.** | 1375.6 (412.0-1637.3) | 1077 (819-1202) | 1173 (657-1284) | 1186 (1101-1324) | 1181 (956-1425) |
| **Tmax induced, s** | 5385 (3104-6185) | 5584 (5499-6977) | 5436 (5085-5869) | 5768 (5288-6497) | 5850 (5070-6719) |
| **Squr induced (× $10^6$)** | 0.03 (0.01-0.07) | 0.34 (0.29-0.38) | 0.44 (0.39-0.95) | 0.69 (0.6-0.7) | 0.72 (0.66-0.77) |
| | | $p_{1-2} < 0.001$ | $p_{1-3} < 0.001$ | $p_{1-4} < 0.001$ | $p_{1-5} < 0.001$ $p_{2-5} < 0.05$ |
| **Activation index, c. u.** | 0.86 (0.6-1.3) | 1.7 (1.4-2.2) | 1.79 (0.90-2.06) | 2.0 (1.90-2.27) | 2.4 (2.0-2.6) |
| | | $p_{1-2} < 0.001$ | $p_{1-3} < 0.001$ | $p_{1-4} < 0.001$ | $p_{1-5} < 0.001$ |

group, which characterizes the low functionality of monofilaments in SAC. The time for the appearance of the spontaneous chemiluminescence maximum (Tmax) in patients with stomach adenocarcinoma is higher than in the control group, which indicates a prolongation of the time required for monocyte activation. The area under the curve of spontaneous and induced chemiluminescence (CL), as well as the activation index, were also increased in patients with SAC relative to the control. When studying the phagocytic activity of monocytes in patients with stomach adenocarcinoma, a decrease in the number of functionally mature phagocytic cells relative to the control group was recorded (Table 1).

In the second stage, the indicators of the chemiluminescent activity of monocytes in patients with stomach adenocarcinoma depending on the stage of the disease were studied (Table 2). The index of maximum intensity (Imax) in spontaneous CL of monocytes decreased in proportion to the stage of the disease, reaching statistical significance in stage IV of SAC relative to the control group. The Tmax of spontaneous CL, Squr of spontaneous and induced CL, as well as the monocyte activation index, on the contrary, progressively increased from stage I to stage IV of stomach adenocarcinoma, exceeding the control group by more than two times at the last stage ($p < 0.05$). Statistical differences between the studied parameters between I and IV stages of SAC were also revealed ($p < 0.05$) (Table 2).

The development of stomach adenocarcinoma is promoted by many etiological factors, accompanied by the development of inflammatory and atrophic changes in the gastric mucosa [3, 6, 8]. The literature presents studies of the state of the immune system in patients with atrophic gastritis, during which activation of cellular and humoral immunity was revealed [5, 10]. According to the authors, the activation of immunity in gastritis is aimed at eliminating concomitant infectious agents and destructive changes in mucosal cells. If we assume that atrophic gastritis can transform into a malignant state, then the decrease in immunological reactions in stomach adenocarcinoma may, on the one hand, be a consequence of the depletion of the internal reserves of the immune system, on the other hand, be an additional factor in the transformation of healthy cells of the gastric mucosa into malignant ones with prolonged exposure to risk factors. Changes in the immune system can also contribute to oxidative stress in the blood plasma, which is detected in both atrophic and malignant pathological conditions of the stomach [3, 9].

## Conclusion

The study made it possible to identify pronounced dysfunctions of the monocytic link of the immune systems in stomach adenocarcinoma already in the early stages of the disease. The syndrome of immune deficiency in tumor diseases is a nonspecific and universal link in the pathological process, which is once again confirmed by our results. Therefore, signs of immune deficiency in the presence of symptoms of gastric pathology increase the likelihood of detecting stomach cancer at an early stage. Given the high prevalence and significance of this disease, a study to identify the pathogenetic mechanisms of the formation and progression of stomach adenocarcinoma can contribute to the expansion of diagnostic approaches and optimization of therapeutic treatment.

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# АНГИОГЕННЫЙ ПОТЕНЦИАЛ ЦИРКУЛИРУЮЩИХ НЕЙТРОФИЛОВ ПЕРИФЕРИЧЕСКОЙ КРОВИ ПРИ РАКЕ ПОЧКИ

**Мягдиева И.Р.[1], Абакумова Т.В.[1], Долгова Д.Р.[1], Горшков О.Ю.[2], Генинг Т.П.[1]**

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**Резюме.** В настоящее время актуальным является изучение роли нейтрофилов при раке почки. Их роль в канцерогенезе неоднозначна. Являясь одним из наиболее распространенных лейкоцитов крови, нейтрофилы играют важную роль в прогрессировании рака посредством множества механизмов, включая стимулирование ангиогенеза, иммуносупрессии и метастазирования рака. Нейтрофилы синтезируют и высвобождают проангиогенные факторы, которые способны прямо или косвенно стимулировать рост и миграцию эндотелиальных клеток, что, в свою очередь, вызывает образование новых кровеносных сосудов из ранее существовавших. Продукция нейтрофилами различных факторов, в том числе и проангиогенных, опосредована экспрессией генов данных молекул. Функциональная гетерогенность характеризуется различиями в паттернах экспрессии генов нейтрофилов. Целью данного исследования была оценка ангиогенного потенциала циркулирующих нейтрофилов при раке почки. Объектом исследования явились нейтрофилы крови пациентов с верифицированным раком почки светлоклеточного типа на I стадии (T1N0M0G1, n = 28, медиана возраста 60), II стадии (T2N0M0G2, n = 15, медиана возраста 61) и III стадии (T3N0M0G2, n = 15, медиана возраста 63) до хирургического лечения. Группу контроля составляли условно здоровые доноры (n = 15, медиана возраста 54). Методом иммуноферментного анализа оценивались уровни IL-8 и VEGF-A в сыворотке крови. Экспрессия генов CXCL8 и VEGF-A в циркулирующих нейтрофилах была определена методом количественной ПЦР с обратной транскрипцией. В результате проведенного нами исследования выявлено повышение уровня IL-8 и VEGF-A в сыворотке крови пациентов с раком почки во всех исследуемых группах по сравнению с группой контроля. Мы наблюдали прямую корреляционную связь между уровнем IL-8 и VEGF-A в сыворотке у пациентов с раком почки (r = 0,429; p = 0,016), которая подтверждает взаимосвязь данных ангиогенных факторов. Было установлено значимое повышение экспрессии гена CXCL8 циркулирующими нейтрофилами у пациентов на II (2,91, $Q_{0,25}$-$Q_{0,75}$: (1,296-4,99), p = 0,02) и III (1,93, $Q_{0,25}$-$Q_{0,75}$: (0,755-11,36, p = 0,014) стадии рака почки по сравнению с контрольной группой (1,50, $Q_{0,25}$-$Q_{0,75}$: (0,80-4,05)), однако экспрессия гена VEGF-A циркулирующими нейтрофилами не отличалась от аналогичных показателей в группе контроля. На основании полу-

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ченных результатов можно предположить, что циркулирующие в крови нейтрофилы при раке почки осуществляют свой ангиогенный потенциал через продукцию IL-8.

*Ключевые слова: нейтрофилы, ангиогенез, VEGF-A, IL-8, фенотип нейтрофилов, рак почки*

# ANGIOGENIC POTENTIAL OF CIRCULATING PERIPHERAL BLOOD NEUTROPHILS IN KIDNEY CANCER

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**Abstract.** The role of neutrophils in kidney cancer is currently being studied. Their role in carcinogenesis is ambiguous. As one of the most abundant blood leukocytes, neutrophils play an important role in cancer progression through multiple mechanisms, including promotion of angiogenesis, immunosuppression, and cancer metastasis. Neutrophils synthesize and release pro-angiogenic factors that are able to directly or indirectly stimulate the growth and migration of endothelial cells, which in turn causes the formation of new blood vessels from pre-existing ones. The production of various factors by neutrophils, including proangiogenic ones, is mediated by the expression of the genes of these molecules. Functional heterogeneity is characterized by differences in neutrophil gene expression patterns. The aim of this study was to evaluate the angiogenic potential of circulating neutrophils in kidney cancer. The object of the study were blood neutrophils of patients with verified clear cell kidney cancer at stage I (T1N0M0G1, n = 28, median age 60), stage II (T2N0M0G2, n = 15, median age 61) and stage III (T3N0M0G2, n = 15, median age 63) before surgery. The control group consisted of apparently healthy donors (n = 15, median age 54). Serum levels of IL-8 and VEGF-A were assessed by enzyme immunoassay. Expression of the CXCL8 and VEGF-A genes in circulating neutrophils was determined by reverse transcription quantitative PCR. As a result of our study, an increase in the level of IL-8 and VEGF-A in the blood serum of patients with kidney cancer in all studied groups compared with the control group was revealed. We observed a direct correlation between serum levels of IL-8 and VEGF-A in patients with kidney cancer (r = 0.429; p = 0.016), which confirms the relationship of these angiogenic factors. A significant increase in CXCL8 gene expression by circulating neutrophils was found in patients on II (2.91, $Q_{0.25}$-$Q_{0.75}$: (1.296-4.99), p = 0.02) and III (1.93, $Q_{0.25}$-$Q_{0.75}$: (0.755-11.36, p = 0.014) stages of kidney cancer compared with the control group (1.50, $Q_{0.25}$-$Q_{0.75}$: (0.80-4.05)). However, VEGF-A gene expression by circulating neutrophils did not differ from those in the control group. Blood neutrophils in kidney cancer exercise their angiogenic potential through the production of IL-8.

*Keywords: neutrophils, angiogenesis, VEGF-A, IL-8, neutrophil phenotype, kidney cancer*

## Introduction

The most common type of kidney cancer (KC) is clear cell (about 70% of cases). KC is considered to be an immunogenic tumor but is known to mediate immune dysfunction to a large extent by causing the entry of immunoinhibitory cells, such as regulatory T cells and myeloid suppressor cells, into the tumor microenvironment [4]. The microenvironment of a tumor can contribute to its development from initiation to metastasis [7]. Neutrophils (Nph) are the most common population of granulocytes in human blood and, as a rule, make up a significant proportion of tumor-infiltrating immune cells in KC [3, 13]. In response to chemokine signals, neutrophils quickly migrate from the bloodstream to the focus of inflammation [11]. These tumor-associated Nph (TANs) are the main effector cells of the tumor microenvironment [9]. Nph can support tumor growth through various mechanisms, including suppression of T cell activation, stimulation of genetic instability, tumor cell proliferation, angiogenesis, and metastasis [8].

Nph are known to play an important role in stimulating tumor angiogenesis through the production of pro-angiogenic factors, including MMP-9, Bv8, vascular endothelial growth factor A (VEGF-A), and chemokines [3]. The proangiogenic activity of Nph is crucial in the early stages of tumor progression, since Nph-produced MMP-9 triggers angiogenesis, facilitating the mobilization of VEGF-A and subsequent binding to VEGFR2 [3].

In addition to its pro-inflammatory function, IL-8 enhances the proliferation, survival, and migration of endothelial cells, thereby activating and maintaining the development of angiogenesis [2]. IL-8 and its receptors are widely expressed by both tumor cells and a variety of non-malignant cells present in the tumor microenvironment, including tumor-associated macrophages, Nph, and endothelial cells [2, 6]. It was found that elevated serum levels of IL-8 in KC were associated with the prevalence of the process and worse overall survival [12]. The production of various factors by neutrophils, including proangiogenic ones, is mediated by the expression of the genes of these molecules. The functional heterogeneity of neutrophils is characterized by differences in the patterns of neutrophil gene expression [10].

**The aim of the study** was to assess the angiogenic potential of circulating Nph in KC.

## Materials and methods

The object of the study was blood Nph of patients with verified KC, clear cell type I stage (T1N0M0G1, n = 28, median age 60), II stage (T2N0M0G2, n = 15, median age 61) and III stage (T3N0M0G2, n = 15, median age 63) before surgical treatment. The control group consisted of apparently healthy donors (n = 15, median age 54). Informed consent to participate in the study was obtained from all patients. The study was approved by the Ethics Committee of the Institute of Medicine, Ecology and Physical Culture of Ulyanovsk State University (protocol No. 1 dated January 15, 2020).

Nph was isolated from leukocyte suspension on a double density gradient of sterile ficoll-verografin solutions. The isolated Nph were washed from the gradient three times with sterile saline sodium chloride solution and adjusted to a concentration of $5 \times 10^6$ cells/ml. The purity of the Nph fraction was 92–94%. The viability of Nph in the test with 0.5% trypan blue was 95%. From the general blood test, the following were calculated: the absolute number of leukocytes in patients with KC, median 6.36 ($Q_{0.25}$-$Q_{0.75}$: 5.00-7.18), neutrophils, median 3.072 ($Q_{0.25}$-$Q_{0.75}$: 0-4.14). RNA was isolated from the peripheral blood Nph fraction using SileksMagNA magnetic particles (LLC Sileks, Moscow, Russia) at a KingFisher automated nucleic acid isolation station. After isolation, the reverse transcription reaction was set up. Expression of CXCL8 and VEGF-A genes was determined by quantitative PCR with reverse transcription using primers (CJSC Evrogen, Moscow, Russia). The level of IL-8 and VEGF-A in serum was determined using ELISA (CJSC Vector-Best-Volga, Russia). Sets of quantitative indicators, the distribution of which differed from normal, were described using the values of the median (Me) and the lower and upper quartiles ($Q_{0.25}$-$Q_{0.50}$). The statistical significance of differences was assessed using the Mann–Whitney U test. In order to study the relationship between quantitative variables, the calculation of the Spearman correlation coefficient and the linear regression model were used. Statistical processing was performed using Statistical 13.

## Results and discussion

As a result of our study, an increase in the level of IL-8 in the blood serum of patients with KC in all the studied groups compared with the control group was revealed (Table 1). High concentrations of IL-8 have also been found by Wu L. et al. (2021) in serum and tissue samples from patients with various types of cancer and have been shown to correlate with tumor progression and worse overall survival. High levels of IL-8 in the blood promote the migration of Nph into the tumor, and blocking the signaling of this chemokine, according to Schimek V. et al. (2022), suppresses this process. Accordingly, an increase in the level of this chemokine in patients with KC can lead to an increase in Nph chemotaxis into the tumor.

We also found an increase in the serum level of VEGF-A at all stages of KC relative to the control group (Table 1), which is consistent with previous studies [5]. In addition, the studies of Apte R.S. et al. (2019) found that a high serum level of VEGF-A correlates with invasiveness, vascular density, me-

**TABLE 1. SERUM LEVELS OF IL-8 AND VEGF-A AT VARIOUS STAGES OF KIDNEY CANCER, Me ($Q_{0.25}$-$Q_{0.75}$)**

| Indicator \ Group | Control n = 22 | Stage I n = 28 | Stage II n = 15 | Stage III n = 15 |
|---|---|---|---|---|
| IL-8, pg/mL | 11.04 (10.30-14.99) | 94.26 (36.07-387.50) p = 0.00001 | 409.08 (29.23-422.15) p = 0. 00023 | 96.37 (61.83-275.22) p = 0.0001 |
| VEGF-A, pg/mL | 136.34 (91.01-168.95) | 372.99 (282.99-545.82) p = 0.0001 | 337.52 (329.33-692.18) p = 0.00047 | 227.34 (227.34-399.49) p = 0.0017 |

Note. Statistical significance p was calculated relative to the control group; the values were considered statistically significant at $p \leq 0.05$.

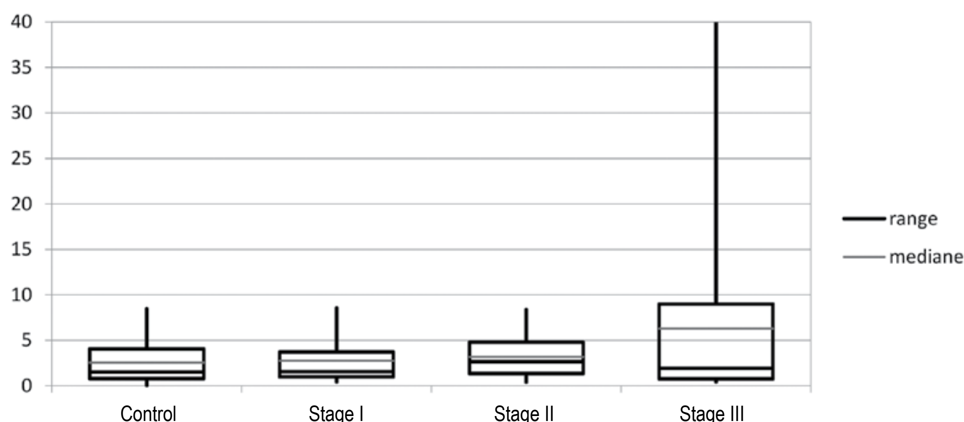


**Figure 1. CXCL8 gene expression in circulating neutrophils**

tastasis, and relapses in KC and can serve as a biomarker for the development of angiogenesis.

We observed a direct correlation between the level of IL-8 and VEGF-A in serum in patients with KC ($r = 0.429$; $p = 0.016$), which confirms the relationship of these angiogenic factors. It is known that IL-8 promotes a proinflammatory state, stimulates angiogenesis and is a powerful chemoattractant for Nph [7, 14], and also activates pro-angiogenic pathways, which is confirmed by previous studies by Zhou N.A.N. et al. (2016).

The phenotype of neutrophils is characterized by gene expression in them [10]. We found a significant increase in CXCL8 gene expression by circulating neutrophils in patients on II (2.91, $Q_{0.25}$-$Q_{0.75}$: (1.296-4.99), $p = 0.02$) and III (1.93, $Q_{0.25}$-$Q_{0.75}$: (0.755- 11.36, $p = 0.014$) of the KC stage compared with the control group (1.50, $Q_{0.25}$-$Q_{0.75}$: (0.80-4.05)) (Figure 1), which may indicate an increase in the ability to produce IL-8 Nph in the studied groups.

From previous studies by Apte R.S. et al. (2019) it is known that Nph synthesize VEGF-A, which is stored in them and released during inflammatory angiogenesis. VEGF-A is one of the factors that determine protumor (N2) neutrophil polarization [7]. In studies by Amorim C. et al. (2022) also demonstrated that a high level of VEGF-A released by neutrophils is characteristic of the N2 phenotype. As a result of our analysis, the expression of the VEGF-A gene by circulating Nph in KC did not differ from similar indicators in the control group (Figure 2). Expression of the VEGF-A gene (Me = 1.47, $Q_{0.25}$-$Q_{0.75}$: (0.645-3.96)) by circulating Nph in patients with KC was significantly lower than the expression of the CXCL8 gene (Me = 2.34, $Q_{0.25}$-$Q_{0.75}$: (0.904-8.32), $p = 0.0023$). Analysis of the linear regression model of the VEGF-A gene expression Nph and the serum

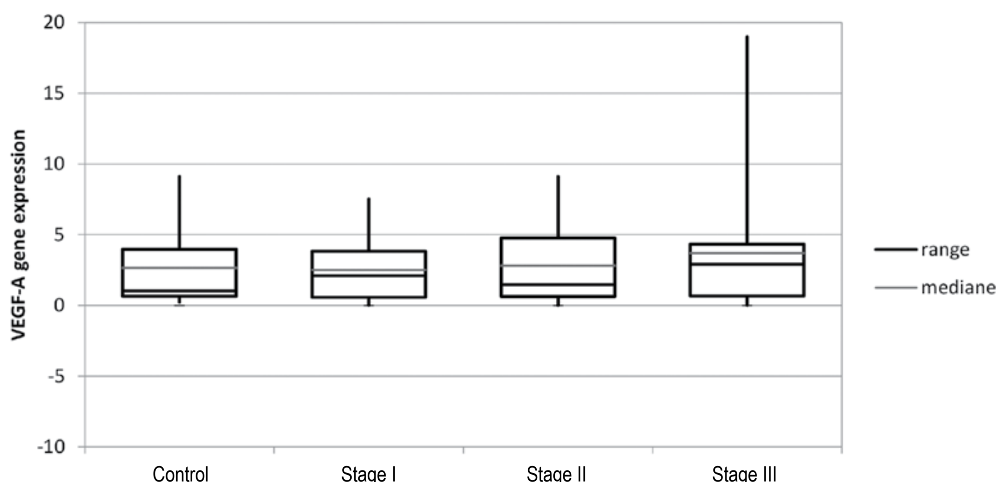


**Figure 2. VEGF-A gene expression in circulating neutrophils**

level of IL-8 in the group of patients at stage II showed an inverse relationship between these indicators ($R^2 = 0.8$, $p = 0.0054$).

## Conclusion

Thus, our results suggest that Nph circulating in the blood during KC exercise their angiogenic potential through the production of IL-8, and are not the main producers of serum VEGF-A. An elevated serum level of IL-8 probably contributes to the formation of a protumor phenotype of circulating Nph. It can be assumed that an increase in the level of this chemokine in the future will lead to a change in the expression of proangiogenic factor genes in neutrophils and their protumor polarization in the process of recruitment to the tumor.

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# IL-4 И ЕГО ПОЛИМОРФИЗМ (*IL4*-589C/T) ПРИ ЦЕРВИКАЛЬНОЙ НЕОПЛАЗИИ

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**Резюме.** Переход неоплазии шейки матки (CIN) в рак шейки матки происходит при активном участии IL-4, для которого показано как про-, так и противоопухолевое действие при опухолях различной локализации. Экспрессия цитокинов регулируется на уровне транскрипции в промоторной области гена. Показано, что генотип *IL4* (589C/T) (rs2243250) ассоциирован с развитием рака желудка и молочной железы. Вклад вариаций генотипа *IL4* в развитие CIN еще не изучен. Цель исследования – оценить риск развития неоплазии шейки матки по наличию полиморфизма *IL4* (589C/T) и уровню IL-4.

Объектом исследования служили циркулирующие нейтрофилы, сыворотка и геномная ДНК 36 больных CIN и 20 женщин без дисплазии (группа сравнения). С помощью ИФА определяли уровень IL-4 в лизате нейтрофилов и сыворотке крови. Оценивали фагоцитарную активность и способность нейтрофилов к адгезии (CD11b). Аллель-специфическую ПЦР в реальном времени с использованием зондов Taq-Man использовали для анализа *IL4* 589C/T (rs2243250). Статистическую обработку проводили с помощью программ Statistica 13 и Jamovi 1.6.5.0.

В результате исследования установлено, что уровень IL-4 в сыворотке крови и циркулирующих нейтрофилах у больных с CIN достоверно выше, чем в группе сравнения. Аллель -589C* гена *IL4* и генотип ТТ чаще встречаются в группе с CIN (55,5%), чем в контроле (25%). При этом установлена прямая связь между наличием полиморфизма и повышенной адгезивной способностью и с показателями фагоцитарного числа циркулирующих нейтрофилов. Анализ частоты встречаемости *IL4* C589T методом «случай-контроль» показал, что шансы формирования CIN у носителей аллеля -589C и генотипа ТТ составили 3,75 (95% ДИ: 1,013-13,880, Хи-квадрат = 4,161, p = 0,042). Аллель -589C* и ТТ генотип *IL4*, уровни нейтрофилов и сывороточного IL-4 связаны с инфекцией ВПЧ. С помощью модели бинарной логистической регрессии показана возможность использования уровней IL-4 в циркулирующих нейтрофилах и полиморфизма *IL4* (589C/T) для дифференциальной диагностики пациентов с CIN ($\chi^2$ = 15,6, p = 0,001). Значимость их сочетания оценивали с помощью анализа ROC-кривых (IL-4 в нейтрофилах; *IL4* (-589C*), вероятность 75%).

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Таким образом, *IL-4* (589C/T) связан с адгезивной и фагоцитарной активностью циркулирующих нейтрофилов. У ВПЧ-инфицированных пациентов полиморфизм гена *IL-4* (589C/T) может служить маркером раннего выявления и прогноза CIN.

*Ключевые слова: IL-4, полиморфизм IL4 (589C/T), цервикальная интраэпителиальная неоплазия, нейтрофилы, ВПЧ, дифференциальная диагностика*

# IL-4 AND ITS POLYMORPHISM (*IL4*-589C/T) IN CERVICAL NEOPLASIA

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**Abstract.** The transition of cervical neoplasia (CIN) to cervical cancer occurs with the active participation of IL-4, for which both pro- and antitumor effects have been shown with tumors of various localizations. The expression of cytokines is regulated at the transcriptional level in the promoter region of the gene. It has been shown that the genotype *IL4* (589C/T) (rs2243250) is associated with the development of gastric and breast cancer. The contribution of IL-4 genotypic variations to the development of CIN has not yet been studied. The aim of the study was to assess the risk of developing cervical neoplasia by the presence polymorphism of *IL4* (589C/T) and the level of IL-4. The object of the study was circulating neutrophils, serum and genomic DNA of 36 patients with CIN and 20 women without dysplasia (comparison group). Using ELISA, the level of IL-4 was determined in neutrophil lysate and serum. Phagocytic activity and adhesive ability (CD11b) of neutrophils were assessed. Allele-specific real-time PCR using Taq-Man probes was used to analyze of the *IL4* 589C/T (rs2243250). Statistical processing was carried out using Statistica 13 and Jamovi 1.6.5.0. As a result of the study, it was found that the level of IL-4 in serum and circulating neutrophils in patients with CIN is significantly higher than in the comparison group. The -589C* allele of the *IL4* gene and the TT genotype are more common in the group with CIN (55.5%) than in the control (25%). At the same time, a direct relationship was established between the presence of polymorphism and increased adhesive ability and with indicators of the phagocytic number of circulating neutrophils. Analysis of the incidence of *IL4* C589T by the «case-control» method showed that the chances of CIN formation in carriers of the -589C allele and the TT genotype were 3.75 (95% CI: 1.013 – 13.880, Chi-square = 4.161, p = 0.042). The -589C* allele and TT *IL4* genotype, neutrophil and serum IL-4 levels are associated with HPV infection. Using a binary logistic regression model, we demonstrated the possibility of using IL-4 levels in circulating neutrophils and IL-4 gene polymorphism (589C/T) for the differential diagnosis of patients with CIN ($\chi^2 = 15.6$, p = 0.001). Significant significance for their combination was assessed by ROC-curve analysis (IL-4 in neutrophils; *IL4* (-589C*), 75% probability. Thus, the *IL4* (589C/T) is associated with the adhesive and phagocytic activity of circulating neutrophils. In HPV-infected patients, *IL4* gene polymorphism (589C/T) can serve as a marker for early detection and prognosis of CIN.

*Keywords: IL-4, IL4 polymorphism (589C/T), neutrophils, cervical intraepithelial neoplasia, HPV, differential diagnosis*

## Introduction

Infection with certain strains of human papillomavirus (HPV) is associated with a high risk of malignant transformation, and HPV-associated cervical intraepithelial neoplasia (CIN) can become an invasive cancer. Up to 32% of cases there is a transition from CINIII to cervical cancer [2]. In this case, factors that regulate tumor growth and modulate immunological control can play a special role. Interleukin 4 (IL-4) has an antitumor effect [13], however elevated levels of IL-4 have been found in tumor tissues in patients with breast, renal cell, prostate, colon and lung cancers [8, 11]. IL-4 is mainly produced by macrophages and T lymphocytes [1], as well as by neutrophils (Nph) [3]. At the same time, it causes hyperproduction of antibodies, which leads to

increased tumor growth due to "antibody screening" of tumor cell antigens [9]. At the same time, the tumor growth-inhibiting effect of IL-4 associated with blockade of the cell cycle, increased expression of MHC on tumor cells, and a decrease in the expression of oncogenes was shown [12].

The expression of cytokines is regulated at the transcriptional level in the promoter region of the gene. The functional polymorphism of the promoter region of the *IL4* gene at position -590C → T and the association of increased protein production with the *T allelic variant of the gene were shown [10]. In a detailed subgroup analysis by cancer type, the *IL4* genotype (589C/T) (rs2243250) was associated with the risk of developing gastric and breast cancer [4, 14]. At the same time, Wang et al. (2012) showed that carriers of the Q576R G *IL4R* allele were associated with a significantly reduced risk of cervical cancer. To date, the contribution of *IL4* genotypic variations to the development of CIN has not been studied.

**The aim of the study** was to assess the risk of developing cervical neoplasia by the presence of *IL4* polymorphism (589C/T) and the level of IL-4 in circulating neutrophils.

## Materials and methods

The study included 36 patients with verified CIN who were examined and then treated at the Regional Clinical Oncological Center in Ulyanovsk, and 20 women without dysplasia (comparison group), corresponding in age to patients in the study group and not having a history of oncological diseases (Table 1). The study was conducted in compliance with the principles of voluntariness and confidentiality, in accordance with the requirements of the ethics commission of the Institute of Medicine, Ecology and Physical Culture of Ulyanovsk State University (protocol No. 3, dated March 15, 2015).

The level of IL-4 was determined by ELISA in the Nph lysate and serum before the start of treatment (CJSC Vector-Best-Volga, Russia). Genomic DNA was isolated from peripheral blood leukocytes using the DNA Express Blood kit (Litekh, Russia). The study of the phagocytic activity of Nph was carried out by quantitative determination of the absorption and digestion capacity of Nph during 30 min incubation with *Saccharomyces cerevisiae*. The ability of Nph to adhere was assessed by the expression of the surface marker CD11b. Allele-specific real-time PCR using Taq-Man probes was used to analyze the polymorphic variant of the promoter region of the *IL4* gene 589C/T (rs2243250) (Litekh, Russia).

The frequencies of alleles and genotypes of polymorphic loci, as well as the correspondence of the distribution of the observed frequencies of genotypes to those theoretically expected according to the Hardy–Weinberg equilibrium, were checked using the $\chi^2$ test. To assess the relative risk of developing a disease/event, the OR value (odds ratio) was calculated in case-control studies. Sets of quantitative indicators, the distribution of which differed from normal, were described using the values of the median (Me) and the lower and upper quartiles ($Q_{0.25}$-$Q_{0.75}$). The Mann–Whitney U test was used to compare independent populations in cases where there were no signs of normal data distribution. The construction of a predictive risk model for the outcome of a malignant neoplasm was performed using the binary logistic regression method. The statistical significance of the resulting model was determined using the $\chi^2$ test. The quality of the predictive model obtained using ROC

**TABLE 1. CHARACTERISTICS OF PATIENTS INCLUDED IN THE STUDY**

| Characteristic | Number of patients (%) |
|---|---|
| Mean age, median 51.5 years (IQR, $Q_{0.25}$-$Q_{0.75}$; 39-59) | n = 56 (100%) |
| Diagnosis : | |
| Without dysplasia | 20 (35,7%) |
| CIN (dysplasia II-III, cancer in situ) | 36 (64.3%) |
| HPV-positive | 52 (92,8%) |
| Complete blood count of patients with CIN before treatment:<br>leukocytes, $*10^9$/L, median 6.2 (IQR, $Q_{0.25}$-$Q_{0.75}$; 5.2-7.7)<br>neutrophils, $*10^9$/L, median 10.6 (IQR, $Q_{0.25}$-$Q_{0.75}$; 8.6-11.6) | |

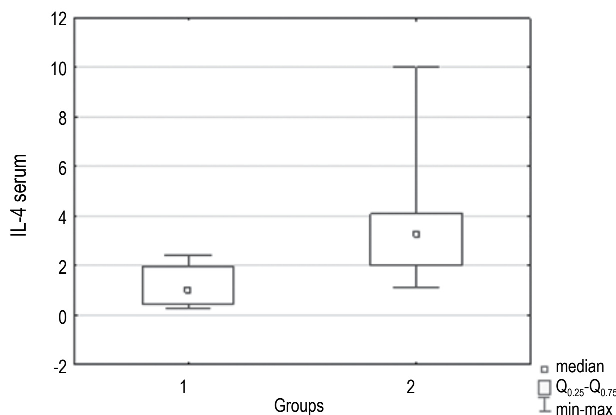


**Figure 1. Level of IL-4 in the blood serum of the comparison group (1) and patients with CIN (2)**

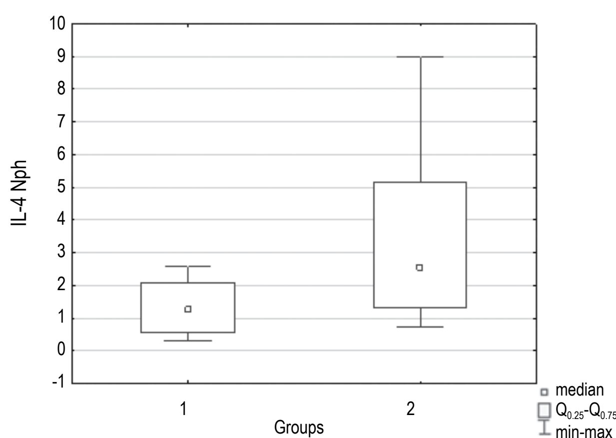


**Figure 2. IL-4 level in circulating neutrophils of the comparison group (1) and patients with CIN (2)**

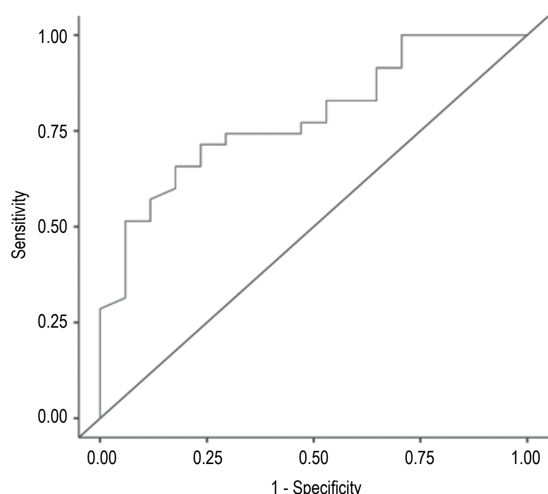


**Figure 3. ROC-curve for the regression model of the differential diagnosis of CIN, taking into account the indicators: the level of IL-4 in neutrophils and *IL4* (-589C*)**

analysis was assessed based on the area under the ROC curve with a standard error and 95% confidence interval (CI) and the level of statistical significance (Jamovi 1.6.5.0). Statistical processing was carried out using Statistica 13.

## Results and discussion

Today, there is an opinion that IL-4 is a "tumor-promoting molecule". Serum levels of IL-4 are usually elevated in cancer patients [7, 15]. According to Harris et al. (2019), through IL-4 receptors have a direct stimulating effect on epithelial tumor cells. As a result of the study, we also found a significantly increased serum level of IL-4 ($p = 0.003$) in patients with CIN compared with the values in the comparison group (Figure 1).

The relationship between IL-4 and circulating Nph seems to be quite complex and poorly understood. An analysis of literature data suggests that IL-4 actively influences the morphofunctional state of Nph: it limits the migration of Nph into tissues, inhibits its survival in tissues, delays their apoptosis, activates *de novo* actin synthesis and rearrangement of the Nph cytoskeleton [5]. According to Guo et al. (2013), IL-4 also recruits Nph through the activation of cytokine-induced chemoattractant and adhesion molecules. We found a significant increase in the level of IL-4 in circulating Nph ($p = 0.0008$) compared with the control group (Figure 2).

A study of the polymorphism of the promoter gene, which determines the level of IL-4 production in patients with neoplastic processes, showed an association of IL-4 SNP with the risk of developing bladder cancer, gastric and pancreatic cancer, and metastatic kidney cancer [6]. At the same time, both an increase and a decrease in the frequency of the TT genotype in patients with breast cancer were noted [10]. Analysis of the polymorphism of the *IL4* promoter region (589C/T) in patients with CIN has not been performed to date. In our study, the -589C* allele of the *IL4* gene and the TT genotype were more common in the group with CIN (55.5%) than in the control (25%).

At the same time, a direct relationship was established between the presence of polymorphism and an increase in adhesive ability ($r = 0.409$, $p = 0.004$) and with indicators of the phagocytic number of circulating Nph ($r = 0.428$, $p = 0.002$). Analysis of the incidence of functional polymorphism C-589T of the *IL4* gene by the "case-control" method showed that the chances of CIN formation in carriers of the -589C allele and the TT genotype were

3.75 (95% CI: 1.013 – 13.880, Chi-square = 4.161, p = 0.042). Allele -589C* and TT *IL4* genotype (r = -0.397, p = 0.004), IL-4 level in Nph (r = 0.386, p = 0.009) and in serum (r = 0.458, p = 0.0002) are associated with HPV infection. The level of IL-4 in Nph and serum is not associated with the C-589T polymorphism of the *IL4* gene.

Using a binary logistic regression model, we demonstrated the possibility of using indicators of the level of IL-4 in circulating Nph and *IL4* gene polymorphism (589C/T) for the differential diagnosis of patients with CIN ($\chi^2 = 15.6$, p = 0.001). Significant significance in their combination was assessed by ROC-curve analysis (IL-4 in Nph, OR 1.693 95% CI 1.048-2.74, p = 0.032; *IL4* (-589C*), OR 4.317 95% CI 1.059-17.60, p = 0.041). The area under the curve (AUC) of this model was 0.785, and CIN could be diagnosed with a 75% probability (Figure 3). The values of the selected indicators allow us to classify patients according to the degree of risk of CIN in combination with sensitivity (0.800) and specificity (0.471).

## Conclusion

The *IL4* gene polymorphism (589C/T) is associated with the adhesive and phagocytic activity of circulating Nph. In HPV-infected patients, *IL4* gene polymorphism (589C/T) can serve as a marker for early detection and prognosis of CIN.

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# УРОВНИ МЕСТНЫХ ЦИТОКИНОВ КАК ПРОГНОСТИЧЕСКИЙ ФАКТОР РАННЕГО РЕЦИДИВА НЕМЫШЕЧНО-ИНВАЗИВНОЙ КАРЦИНОМЫ МОЧЕВОГО ПУЗЫРЯ

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**Резюме.** Цель работы – дать оценку локального уровня цитокинов в качестве возможных прогностических факторов раннего рецидивирования немышечно-инвазивного мочевого пузыря (НМИРМП). В исследование включено 75 больных: 51 с первичным и 24 – с рецидивным НМИРМП; в обеих группах были опухоли высокой и низкой степени злокачественности (HG и LG). Больных с первичным НМИРМП наблюдали в течение 9 мес. после лечения: ТУР и адъювантная химиотерапия (№6). Из образцов опухолевой ткани готовили супернатанты, в которых определяли уровни цитокинов (IL-1β, IL-6, IL-10, IL-18, TNFα, IFNγ, IL-8) методом ИФА. Результаты исследования показали, что у больных с первичным НМИРМП рецидивы развились в 15 случаях (46,8%) LG и в 11 (45%) – HG-опухолей; не выявлено различий в зависимости от степени злокачественности. В исходно рецидивных опухолях как HG, так и LG, уровни цитокинов были максимальными: в LG они превышали первичные от 7,1 (IFNγ) до 300 (IL-6) раз, в HG – от 2,0 (IL-10) до 9,7 (IL-6) раз. Уровни IL-1β, IL-6, IL-10, IFNγ, IL-8 были выше в тех первичных LG-опухолях, которые рецидивировали через 6-9 мес. наблюдения, чем в нерецидивировавших, хотя их содержание было значительно ниже, чем в исходно рецидивных опухолях (от 2,6 раз для IFNγ до 150 раз для IL-6). Сходная тенденция, хотя и не по тем же цитокинам, наблюдалась в HG-опухолях: тканевые уровни IL-6, IL-10, IL-18 и TNFα были выше в опухолях, рецидивировавших через 6-9 мес. после лечения. Повышение уровней двух цитокинов было общим для LG- и HG-опухолей (IL-6 и IL-10), что можно рассматривать в качестве нового фактора

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негативного прогноза. Таким образом, рецидивирование LG и HG НМИРМП связано с некоторыми иммунологическими механизмами, а именно с локальной гиперпродукцией цитокинов, особенно IL-6 и IL-10, хотя IL-1β, IL-8, IFNγ могут играть роль при LG, а IL-18, TNFα – при HG-опухолях. Учитывая общие сигнальные пути IL-6 и IL-10 (JAK/STAT), эти транскрипционные факторы могут быть потенциальными мишенями для новых эффективных подходов к лечению.

*Ключевые слова: цитокины, микроокружение опухоли, прогноз, немышечно-инвазивный рак мочевого пузыря, раннее рецидивирование*

# LOCAL CYTOKINE LEVELS AS PROGNOSTIC FACTORS FOR EARLY RELAPSE OF NON-MUSCLE-INVASIVE BLADDER CARCINOMA

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**Abstract.** The aim of our study is to assess the local cytokine levels as prognostic factors for early relapse in NMIBC patients. 75 patients with NMIBC were enrolled in the study: 51 with primary NMIBC and 24 with initially recurrent NMIBC, LG and HG tumors were diagnosed in each group. Patients with primary NMIBC were monitored during 9 months after treatment: TURB and chemotherapy (No. 6). During TURB samples of tumors were taken, supernatants were obtained and tissue cytokine levels were measured (IL-1β, IL-6, IL-10, IL-18, TNFα, IFNγ, IL-8) by ELISA test. The results showed that in patients with primary NMIBC early relapses were diagnosed in 15 (46.8%) of LG tumors and in 11 (45%) of HG tumors matching that there was no difference depending upon tumor grade. In initially recurrent tumors of both LG and HG NMIBC the amounts of cytokines were maximal: in LG tumors they exceeded the primary ones from 7.1 (IFNγ) to 300 (IL-6) while in HG – from 2.0 (IL-10) to 9.7 (IL-6). The amounts of IL-1β, IL-6, IL-10, IFNγ, IL-8 were higher in those LG primary tumors which relapsed in 6-9 months compared to the ones which didn`t, though their levels were much lower than in initially manifested relapse (from 2.6 times for IFNγ to 150 times for IL-6). A similar trend, though not for all the same cytokines, was observed in HG tumors: tissue levels of IL-6, IL-10, IL-18 and TNFα were higher in tumors which relapsed in 6-9 months after treatment. The increase of 2 cytokines` levels were common for both LG and HG tumors (IL-6 and IL-10). This finding might be considered as a new prognostic factor of the early relapse. We conclude that relapse of LG and HG NMIBC is related to some immune mechanisms, namely to local hyperproduction of cytokines, especially IL-6 and IL-10, though IL-1β, IL-8, IFNγ could have an impact on LG and IL-18, TNFα – on HG tumors. Taking into account common signaling pathways of IL-6 and IL-10 like JAK/STAT, these transcription factors might be potential targets for new effective approaches to treatment.

*Keywords: cytokines, tumor microenvironment, prognosis, non-muscle-invasive bladder carcinoma, early relapse*

## Introduction

Chronic inflammation is considered to be one of the main triggers of carcinogenesis in many types of malignant tumors. Local hyperproduction of cytokines by tumor cells or activated macrophages and lymphocytes contributes to tumor microenvironment (TME) formation which plays a crucial role in immunoediting of tumor growth [3, 6, 12]. It is well known that cytokines are able to enhance neoangiogenesis and epithelial-mesenchymal transition (EMT), and disrupt

the extracellular matrix, as well as to cause the migration to TME of immunosuppressive cells like M2, Tregs or MDSC [13]. Inflammatory cytokines can cause epigenetic modifications that upregulate the expression of oncogenes or downregulate the expression of tumor-suppressor genes [5]. IL-10, IL-6, TGF-β are described by many researchers as prooncogenic, nevertheless cytokines, proclaimed as immunostimulating (IL-1β, TNFα, IFNα and γ and some others) seem to possess dual function in tumor-bearing organism and demonstrate promotion not only of immune response but also of tumor growth [8].

Relapse of malignant tumors following months or even years after treatment including surgery and radio- and/or chemotherapy is usually considered to be a problem of insufficiently radical approach. But nowadays more and more data proves the biologic causes of tumor recurrence, emphasizing the role of cancer stem cells [9] and cytokines of TME [7, 10]. At the time of the first referring for medical care 75-80% of patients with urothelial bladder carcinoma have non-muscle-invasive tumor (NMIBC), i.e. located in mucosa (Ta, CIS), or submucosa (st. T1). Though it is the initial stage of bladder cancer, the tumor has a marked tendency to recur even when the treatment is started timely.

According to European Organization for the Research and Treatment of Cancer (EORTC) the most often type of tumor progression in NMIBC is relapse occurring in 37% of patients with low risk, in 65% of patients with intermediate risk and in 84% of patients with high risk after transurethral bladder resection (TURB) and adjuvant chemotherapy. In the last case early relapses are typically diagnosed during 6-12 months after surgery and chemotherapy, especially in high grade (HG) carcinomas; such patients need more aggressive treatment. That is why the search for new prognostic factors which might also serve as targets for treatment of NMIBC is an topical medical and biological problem.

**The aim of our study** is to assess the local cytokine levels as prognostic factors for early relapse in NMIBC patients.

## Materials and methods

Seventy-five patients with NMIBC were treated in the Department of Urology of National Medical Research Centre for Oncology, Rostov-on-Don, Russia. The group of 51 patients (46 men (90.2%) and 5 women (9.8%) aged 40-83 years) had primary NMIBC which was histologically verified as papillary urothelial carcinoma: low grade (LG, n = 31 or 60.8%) and high grade (HG, n = 20 or 39.2%). All the patients were in high and intermediate risk groups according to EORTC; they received surgical treatment (TURB) and 6 courses of adjuvant intravesical chemotherapy. Patients were monitored during 9 months after treatment. Patients of the group with initially recurrent NMIBC (n = 24), had LG tumors (n = 15 or 62.5%) and HG tumors (n = 9 or 37.5%). They were also subjected to surgical treatment and chemotherapy according to the accepted standards. Prior to enrollment in the study, all participants gave written informed consent.

The samples of tumors were taken from all the patients during TURB, disintegrated by BD Medimachine (USA), supernatants were obtained by centrifugation and stored at -80 °C. After thawing sandwich Elisa test was performed with cytokine kits to estimate the levels of IL-1β, IL-6, IL-10, IL-18, TNFα, IFNγ, IL-8 (Vector-Best, Russia) by Uniplan Reader (Russia). Total protein amount was measured by biuret test on analyzer Sinnowa Medical Science and Technology Co (China). Cytokine levels were expressed in pg/mL per 1 g protein. Statistical analysis was performed by program STATISTICA 13 (StatSoftInc., USA). Since our data had no Gaussian (normal) distribution they were represented as median with interquartile range, 25 and 75 percentiles – Me ($Q_{0.25}$-$Q_{0.75}$). Intergroup differences were estimated by Mann–Whitney U test. Statistically significant differences were accounted when $p < 0.05$.

## Results and discussion

During the period of clinical monitoring of the patients with primary NMIBC early relapses were diagnosed in 15 (46.8%) of them with LG tumors and in 11 (45%) with HG tumors matching that there was no difference depending upon tumor grade. Patients without these early relapses were regarded as groups 1 in LG and 4 in HG (better prognosis) and with newly diagnosed early relapses as groups 2 in LG and 5 in HG (worse prognosis) while patients with initially recurrent tumors formed groups 3 in LG and 6 in HG.

Comparative analysis of tissue cytokine contents between these groups with different course of the disease is shown in Table 1. In relapse tumors (groups 3 and 6) of both LG and HG NMIBC the amounts of most of cytokines were maximal: in LG tumors they exceeded the primary ones from 7.1 (IFNγ) to 300 (IL-6) while in HG – from 2.0 (IL-10) to 9.7 (IL-6). It is worth noting that in HG tumors some cytokines demonstrated no difference between the recurrent and the primary ones (IFNγ) and IL-18 level was higher in relapse HG tumors than in primary ones of the group 4, but not 5.

We found out that primary tumors which manifested relapse after 6-9 months after treatment also contained higher cytokine levels than primary tumors without relapse during this period and it was typical for both groups. The amounts of IL-1β, IL-6, IL-10, IFNγ, IL-8 were statistically significantly higher in those LG primary tumors which relapsed in 6-9 months compared to the ones which didn`t,

**TABLE 1. TISSUE CYTOKINE LEVELS IN TUMORS OF NMIBC PATIENTS WITH DIFFERENT COURSE OF THE DISEASE, Me ($Q_{0.25}$-$Q_{0.75}$)**

| Cytokine level (pg/mL per 1 g ofprotein) | Groups of patients | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | NMIBC LG | | | | NMIBC HG | | | |
| | 1 Primary without relapse, n = 16 | 2 Primary with relapse in 6-9 months, n = 15 | 3 Relapse, n = 15 | p | 4 Primary without relapse, n = 11 | 5 Primary with relapse in 6-9 months, n = 9 | 6 Relapse, n = 9 | p |
| IL-1β | 13.3 (10.2-17.9) | 19.8[1] (19.1-48.9) | 149.2[1, 2] (83.0-215.4) | $p_{2-1}$ = 0.038 $p_{3-1}$ = 0.001 $p_{3-2}$ = 0.001 | 31.3[1] (17.3-41.0) | 38.5[2] (26.7-49.6) | 83.8[3, 4, 5] (60.8-91.1) | $p_{6-4}$ = 0.035 $p_{6-5}$ = 0.04 |
| IL-6 | 1.1 (0.6-1.5) | 2.6[1] (2.2-7.3) | 330.2[1, 2] (167.8-492.6) | $p_{2-1}$ = 0.025 $p_{3-1}$ = 0.001 $p_{3-2}$ = 0.001 | 3.0[1] (1.3-3.9) | 6.2[2, 4] (4.5-9.2) | 29.2[3, 4, 5] (29.1-31.7) | $p_{5-4}$ = 0.027 $p_{6-4}$ = 0.011 $p_{6-5}$ = 0.008 |
| IL-10 | 3.2 (2.1-4.2) | 5.9[1] (4.9-9.9) | 55.4[1, 2] (29.4-81.4) | $p_{2-1}$ = 0.039 $p_{3-1}$ = 0.012 $p_{3-2}$ = 0.019 | 3.6 (1.6-3.9) | 5.1[4] (4.2-5.6) | 7.2[3, 4, 5] (6.1-10.5) | $p_{5-4}$ = 0.04 $p_{6-4}$ = 0.039 $p_{6-5}$ = 0.037 |
| IL-18 | 59.7 (41.8-90.0) | 57.3 (31.7-81.5) | 641.0[1, 2] (478.2-903.9) | $p_{3-1}$ = 0.014 $p_{3-2}$ = 0.016 | 22.8[1] (20.3-34.4) | 87.6[2, 4] (83.9-124.1) | 69.0[3, 4, 5] (46.9-80.4) | $p_{5-4}$ = 0.012 $p_{6-4}$ = 0.001 $p_{6-5}$ = 0.035 |
| TNFα | 10.1 (5.9-13.8) | 10.0 (7.1-17.5) | 212.0[1, 2] (113.3-310.7) | $p_{3-1}$ = 0.001 $p_{3-2}$ = 0.001 | 5.5[1] (4.5-7.2) | 10.9[4] (8.3-12.7) | 22.7[3, 4, 5] (21.9-42.7) | $p_{5-4}$ = 0.032 $p_{6-4}$ = 0.018 $p_{6-5}$ = 0.012 |
| IFNγ | 6.9 (4.9-8.4) | 19.0[1] (11.1-38.9) | 49.0[1, 2] (28.9-69.2) | $p_{2-1}$ = 0.021 $p_{3-1}$ = 0.017 $p_{3-2}$ = 0.039 | 15.2[1] (11.0-20.9) | 11.2 (5.3-19.4) | 10.8[3] (9.8-16.1) | |
| IL-8 | 13.0 (8.4-15.3) | 26.4[1] (22.4-37.8) | 663.4[1, 2] (341.6-985.2) | $p_{2-1}$ = 0.028 $p_{3-1}$ = 0.001 $p_{3-2}$ = 0.001 | 69.4[1] (47.5-113.2) | 42.6[2, 4] (38.4-45.7) | 190.9[3, 4, 5] (125.7-329.2) | $p_{5-4}$ = 0.043 $p_{6-4}$ = 0.014 $p_{6-5}$ = 0.022 |

though their levels were much lower than in initially manifested relapse (2,6 times for IFNγ and 150 times for IL-6). A similar trend, though not for all the same cytokines, was observed in HG tumors: tissue levels of IL-6, IL-10, IL-18 and TNFα were statistically significantly higher in tumors which relapsed in 6-9 months after treatment. This finding might be considered a new prognostic factor of the early relapse. Based on the values of the ranged data represented as LQ and UQ we tried to specify the limits of cytokines` indicators possibly prognostic for early relapse during the period of monitoring. For LG tumors the value appeared to be IL-1β > 18.0, IL-6 > 1.5, IL-10 > 4.2, IFNγ > 8.4, IL-8 > 15.3 pg/mL per 1 g of protein and for HG tumors IL-6 > 3.9, IL-10 > 3.9, IL-18 > 34.4, TNFα > 7.2 pg/mL per 1 g of protein. We observed that the increase of 2 cytokines` levels were common for both LG and HG tumors (IL-6 and IL-10). So they might be used as a possible prognostic factors for early relapse in patients with NMIBC though verification on more broad groups and more prolonged monitoring is needed.

Accounting the biologic properties of both cytokines, it is rather expected. IL-6 induces invasion and metastasis through the Janus kinase (JAK) signal transducer and activator of transcription (STAT) pathway and thus is able to activate a wide array of signaling pathways and transcription factors promoting EMT, fostering the acquisition of mesenchymal features in cancer cells [1]. The other type of its`

prooncogenic activity is support of cancer stem cells which are considered to be a pool for relapse forming from treatment-resistant tumor cells [11].

IL-10 is known to be an immunosuppressive factor of TME due to suppression of T cell proliferation, modulation of APCs, preservation of the activity/stability of Treg cells, though it remains unclear if it can stimulate them [4]. Notably, both IL-6 and IL-10 are involved in STAT3 activation matching that pro-inflammatory and anti-inflammatory cytokines can work through the same signaling pathway, although they perform very distinct functions and their downstream mechanisms are different. In the review cited above the authors point out that in macrophages, both IL-10 and IL-6 induce the activation of SOCS3, and this could be the target for future therapeutic approaches.

From our previous experience, high levels of IL-6 and IL-10 in TME were factors of poor prognosis in patients with melanoma and esophageal carcinoma [2, 14, 15].

## Conclusions

We consider that relapse of LG and HG NMIBC is related to some immune mechanisms, namely to local hyperproduction of cytokines, especially IL-6 and IL-10, though IL-1β, IL-8, IFNγ could have an impact on LG and IL-18, TNFα – on HG tumors. Taking into account common signaling pathways of IL-6 and IL-10 like JAK/STAT, these transcription factors might be potential targets for new effective approaches to treatment.

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# ОСОБЕННОСТИ ИММУНОЛОГИЧЕСКИ АКТИВНОГО И «ХОЛОДНОГО» ФЕНОТИПОВ ИНВАЗИВНОЙ КАРЦИНОМЫ ШЕЙКИ МАТКИ РАННИХ СТАДИЙ ПО ДАННЫМ СЕКВЕНИРОВАНИЯ ТРАНСКРИПТОМА

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**Резюме.** Вопросы молекулярной классификации, иммунной гетерогенности и существования различных иммунофенотипов вирус-ассоциированного рака шейки матки (РШМ), в особенности его наиболее ранних клинических и доклинических форм – цервикальных интраэпителиальных неоплазий (ЦИН), – остаются недостаточно исследованными. Цель данной работы заключалась в анализе транскриптомных профилей инвазивного РШМ на начальных этапах его прогрессии, различающихся иммунологическими характеристиками, спектром сигнальных путей и составом микроокружения. Транскриптомный анализ проводился с использованием РНК-секвенирования на платформе Illumina. Панель образцов нативной ткани, полученных в ходе хирургической операции, включала: ВПЧ-положительные ЦИН 1-3-й степени, инвазивный РШМ IA1-IIB стадий и морфологически нормальный эпителий. Транскриптомные профили далее были проанализированы с помощью биоинформатических инструментов, включая поиск дифференциально экспрессированных генов (DESeq2), анализ сигнальных путей (Gene Set Enrichment, GAGE), извлечение клеточного состава (xCell), позиционный анализ дифференциально экспрессированных геномных регионов (PREDA). На первоначальном этапе иерархический кластерный анализ выявил гетерогенность транскриптома образцов РШМ ранних стадий, а именно их распределение по двум кластерам; метод K-means подтвердил наличие трех функционально различных паттернов генов с координированно изменяющейся экспрессией. Сравнительный анализ обогащения сигнальных путей в двух опухолевых кластерах инвазивного РШМ (‘A’ и ‘B’) относительно группы ‘C’, представленной преимущественно ЦИН, показал, что опухолевая прогрессия в кластерах ‘A’ и ‘B’ может основываться на различных иммунных

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механизмах. xCell анализ подтвердил различия в изменении численности популяций иммунных и стромальных клеток, а также суммарных показателей влияния микроокружения, при сравнении кластеров РШМ и ЦИН. По результатам PREDA установлено, что транскриптомные различия ассоциированы с различными хромосомными регионами и ко-локализованы с определенными семействами генов, вовлеченных в регуляцию иммунного ответа. Таким образом, на транскриптомном уровне выявлено существование различных иммунофенотипов РШМ ранних стадий, что может иметь значение для развития методов таргетной и иммунной противоопухолевой терапии.

*Ключевые слова: вирус-ассоциированный рак шейки матки, профилирование транскриптома, опухолевая инвазия, опухолевое микроокружение, интраэпителиальные неоплазии, сигнальные пути, иммунный инфильтрат, иммуносупрессия*

# CHARACTERISTICS OF IMMUNE-ACTIVE AND IMMUNE-SILENT PHENOTYPES OF EARLY-STAGE CERVICAL CARCINOMA AS REVEALED BY TRANSCRIPTOME SEQUENCING

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**Abstract.** Molecular classification, immune heterogeneity, and the existence of distinct immunophenotypes of virus-associated cervical cancer (CeCa) remain as-yet weakly explored issues, and this is particularly true of its earliest clinical stages and pre-invasive forms: cervical intraepithelial neoplastic (CIN) lesions. The goal of the study was to identify transcriptomic landscapes of invasive CeCa at its initial progression that differ substantially in their immune-related characteristics, patterns of signaling pathways and composition of the microenvironment. Transcriptome profiling was carried out using RNA-sequencing on Illumina platform. A panel of surgical-derived tissue samples comprised human papillomavirus-positive CIN grade 1-3, cancer of FIGO IA1-IIB stages, and morphologically normal epithelium. Transcriptomic profiles were analyzed with the use of bioinformatics tools, such as gene set enrichment (GAGE) for signaling pathways, xCell enrichment for cell composition identification, and PREDA positional analysis of genomic data. Hierarchical clustering revealed heterogeneity of transcriptomic profiles within the early-stage CeCa, namely, the existence of two clusters of tumor samples and three functional patterns of genes showing coordinately altered expression. Pathway enrichment analysis on genes differently expressed between the two clusters/groups of CeCa samples (‘A’ and ‘B’) and CIN (group ‘C’) suggested that invasive tumor progression in groups ‘A’ and ‘B’ might rely on immunologically dissimilar mechanisms. xCell analysis confirmed heterogeneity of changes in the abundancies of cell populations when comparing CeCa sample groups and CIN, along with differences in immune and stromal scores. PREDA demonstrated that these transcriptomic differences could be linked to different chromosomal regions and co-localized with particular gene families and potentially the reported virus integration hotspots. Overall, the existence and detectability of different transcriptomic immune-based phenotypes of invasive CeCa at its initial stages of progression is shown, which may provide new options to broaden the knowledge and applicability of target and immune anti-cancer therapy.

*Keywords: virus-associated cervical cancer, transcriptome profiling, tumor invasion, tumor microenvironment, pre-invasive lesions, signaling pathways, immune infiltration, immune suppression*

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## Introduction

According to WHO data on global incidence and mortality rates of oncological diseases and the results of the Global Cancer Observatory database analysis, cervical cancer (CeCa) is the fourth most common cancer in women and therefore continues to be a major health problem [2]. One of the reasons for this may arise from insufficient understanding of pathogenesis mechanisms hampering elaboration of new diagnostic and therapy approaches. Rapid advance in cancer immunotherapy boosts an ever-increasing interest toward CeCa due to its virus-associated nature. Current high-throughput techniques provide researchers with an opportunity to construct immune-based, or tumor microenvironment (TME)-based, classification systems, which serve an essential basis for designing immunotherapeutic approaches [11]. On the whole, classification models proposed by researchers on the basis of expression profiles of immune-related genes and/or immune infiltration patterns are in accord with the concept of “hot”, “cold”, and “altered” tumors.

It should be noted, however, that the problem of immune heterogeneity and the possibility of detecting diverse immune-related phenotypes have only recently come under discussion with respect to CeCa. By analyzing The Cancer Genome Atlas datasets, some authors define human papillomavirus (HPV) associated CeCa as the “hot” subtype evolving on the basis of chronically inflamed TME and showing high rates of immune infiltration, Th1-dominant cytokine profile, an activated interferon status, high level of genomic instability and hence high neoantigen load [8, 15, 16]. Importantly, high immune activity in a tumor site is believed to invoke intrinsic inhibitory checkpoint mechanisms, which tumor cells take advantage of to combat immune response. However, such categorization of CeCa immunophenotypes presently seems insufficient to account for all the processes of active immunosuppression within this “hot” tumor type, which may be the cause of low-to-moderate efficiency of immunotherapy in CeCa patients [3].

Along with the problem of elaborating CeCa molecular classification and refining its immunophenotypes, there are still many debatable questions regarding the earliest stages of CeCa progression (precancer lesions, microinvasive cancer). Since most CeCa molecular profiling studies addressed more advanced, metastatic and recurrent disease stages, it remains largely unknown how the diversity of CeCa immune “portraits” is formed and what are the putative determinants. Despite the widely studied mechanisms behind the action of HPV-oncogenes, there is still no clear understanding of how an immunologically latent infection transforms into a “hot”, heavily infiltrated and inflamed neoplasia in certain cases and why the latter acquires further an immunosuppressive and exhausted phenotype [5].

Addressing the above issues, we performed whole-transcriptome sequencing (RNA-Seq) and bioinformatics analysis of a cervical tissue sample panel consisted of mostly preclinical cancerous and precancerous lesions. We were aimed at showing detectability of distinct transcriptomic patterns that may reflect the formation of different immunophenotypes at early stages of CeCa invasion and progression, as well as gaining deeper understanding of the role of innate immune and proinflammatory pathways in promotion of invasive tumor growth.

## Materials and methods

Tissue samples were obtained from patients with high-risk HPV(+) cervical intraepithelial neoplasia (CIN) of grade 1-3 (CIN3 comprised carcinoma *in situ*, n = 5) and early invasive squamous cell carcinoma of the cervix at FIGO stages IA1-II (including microinvasive cancer with stromal invasion < 3 mm in depth, n = 9) during a colposcopy-directed biopsy or surgery; morphologically normal cervical epithelium (n = 2) was also included. The diagnosis was based on comprehensive physical examination, extended colposcopy findings, cytology and histopathology tests, in full compliance with the approved standards for the diagnosis and treatment of patients with gynecological malignancies. All women engaged in this this study were informed and gave voluntary written consent. Cervical tissue samples were placed in a stabilization reagent immediately after excision. Total RNA was isolated using TriZOL (Invitrogen). The quality and quantity of isolated RNA were assessed based on 28S:18S rRNA ratio using Fragment Analyzer system (Advanced Analytical) and NanoDrop-2000.

cDNA libraries were constructed using TruSeq stranded Ribo-Zero kit (Illumina). The adaptors-ligated purified fragments were loaded onto the flow cell using MiSeq v3 sequencing kit; 75 bp end-reads were generated on the MiSeq platform (Illumina). Raw paired-end reads were filtered (sequence quality control was done with the FastQC tool). Then, the

filtered reads were mapped to the reference human genome (GRCh38/p13, NCBI) using STAR aligner to generate BAM-files and, further, calculate read counts. HTSeq package was used to assess the abundance of transcripts in Counts Per Million reads mapped (CPM). Genes with minimum counts of 0.5 in at least one sample were considered for analysis. The generated RNA-Seq datasets has been deposited in the NCBI Gene Expression Omnibus (GEO) with accession ID GSE223804.

The top 1000 most variable genes were selected for hierarchical clustering and heatmap construction. K-means clustering was performed using 2000 most variable genes and 3 clusters considered the most optimal choice. Total transcriptional profiles were compared among the samples via principal component analysis (PCA) along the first two principal components. DESeq2 software was applied to study the differential gene expression. The genes with the base 2 log fold change value |logFC| larger than 1.0 and an adjusted p-value $< 0.1$ were identified as Differentially Expressed Genes (DEGs). Gene ontology (GO) functional enrichment analysis were carried out on DEGs using Gene Ontology biological processes with an adjusted p-value of $< 0.05$ and gene count of $> 2$ considered as the thresholds.

Pathway analysis for patient group comparisons was performed using the Generally Applicable Gene set Enrichment (GAGE) method, and the genes were annotated according to GO biological processes. The minimum and maximum gene set sizes were set to 15 and 2000 respectively, and the pathway significance cutoff was set to 0.2. The top 30 pathways were retrieved for each pairwise group comparison. Identification of co-expression networks and sub-modules was performed using weighted gene co-expression network analysis (WGCNA). Cell-type enrichment analysis of bulk transcriptomes was performed using xCell deconvolution method, and enrichment scores of 64 immune and stroma cell types across samples were obtained. PREDA Position RElated Data Analysis (PREDA) package was conducted to identify genomic regions significantly enriched with upregulated or downregulated genes. The study was performed on the equipment of the Unique Scientific Installation (No. 075-15-2021-665).

## Results and discussion

### Identification of DEGs and pathway enrichment

To search for the biologically relevant transcriptomic alterations that may reflect the formation of distinct immune-related molecular phenotypes upon transition from a pre-invasive lesion to an invasive tumor, we initially examined distribution of samples between pathologic groups according to their transcriptomic profiles. Unsupervised hierarchical clustering yielded a heatmap with two main clusters that overall matched the expected separation among CIN and cancer groups (Figure 1A, see 2nd page of cover), and PCA confirmed the results. However, it turned out that the tumor group was clearly heterogeneous and could be further subdivided into two sub-groups, which cannot be accounted for by pathological staging.

Therefore, a transcriptomic comparison was next performed between the three groups of specimens identified on the basis of similarity of their gene expression profiles. Groups A and B were designated as “tumorous” (A1-A5 consisted of microinvasive and invasive CeCa only, group B also included one CIN3), while group C (C1-C7) was considered as “control”, since it comprised both Norm cases and most part of pre-invasive CIN cases. K-means clustering approach confirmed successful distribution of groups A, B, and C between the three patterns of coordinately expressed genes; GO enrichment analysis defined the functional differences between these patterns (Figure 1B, see 2nd page of cover).

Pairwise differential expression analysis resulted in 809 down- and 552 upregulated DEG in group A *vs* C comparison, 679 down- and 217 upregulated DEG in group B *vs* C comparison, and 434 down- and 488 upregulated DEG in group A *vs* B comparison, showing a trend of a higher ratio of down-regulated over up-regulated genes in tumor groups. This is, overall, in concordance with the current concept that HPV-dependent carcinogenesis rests on epigenetic reprogramming and silencing of vast regions of the host genome. DEG were found to be enriched in different GO functions for each pair of groups. Group C expectedly exhibited a higher expression level of genes mediating epithelial terminal differentiation. Group A was distinguished by up-regulation of the immune response-related genes involved primarily in the innate immunity reactions and members of cytokine-dependent signaling pathways. Group B showed elevated expression of many genes employed in various forms of (intra-)cellular motility and cardiovascular system-associated processes, whereas group A samples showed up-regulation of genes functionally related to the chromatin structure (DNA packaging and conformational changes accompanying

the cell cycle progression, and response to stress). This provides opportunity to presume that the early-stage A and B tumors may utilize different mechanisms to sustain progression and may thus constitute different phenotypes. In light of this assumption, we analyzed the range of signaling pathways on a more rigorous basis with the use of GAGE approach.

According to GAGE, repression of the epithelial differentiation program was a common feature distinguishing both groups A and B. Besides, it emerged that group 'A' datasets displayed higher prevalence of DNA/chromatin- and immunity-related gene sets (Table 1). Regarding chromatin-associated processes, GAGE reveals these include not only spatial chromatin organization per se (at the level of DNA-histone interactions), but activation of chromatin remodeling and epigenetic regulation (such as post-transcriptional silencing) as well; with detection of DNA damage repair (DDR) signaling no less important. One can suppose the tumor evolution in this case is accompanied with the large-scale epigenetic repression of oncosuppressor genes, with DDR reflecting a higher degree of genomic instability and a mutator phenotype. The observed enrichment in immune-related gene sets most likely implies a primary role of interferon-dependent and other innate immunity mechanisms, which could be elicited by genomic instability and, in turn, could foster the adaptive immunity arm with tumor progression. Similarly, a marked up-regulation of innate immunity pathways (such as DNA sensor-mediated or interferon-induced), along with DDR "heating-up" the immune TME, has been evidenced by other researchers who explored transcriptomic profiles of more advanced CeCa stages using publicly available databases [7, 12, 13].

We found it curious that the early consecutive stages of cervical cancer progression, differing actually in their invasion status, demonstrated activation of a specific set of genes with immune annotations. Activity of a number of these genes is known to be abrogated by HPV-oncoproteins in a chronic infection setting [8, 9]. On the other hand, it is well established that chronic antigen stimulation can turn on inhibitory immune checkpoint mechanisms and lead to immune exhaustion/suppression thereby restraining anti-tumor response. We therefore looked at the expression profile of individual genes recognized as immune checkpoint modulators: indeed, an immune-active group A displayed significantly enhanced expression of several markers (e.g., BTN3A1/2, SLAMF7, TIGIT); an increasing trend was also observable for PD-L2 expression. Unlike A, group B displayed significant elevation of an immunosuppressive HMGB1 gene and CD73, but reduced GZMB expression.

To get closer to the systemic-level understanding of relations between the observed gene expression changes and CeCa phenotypes at initial progression stages, a search for gene co-expression networks (modules) was done with the use of WGCNA. Ten different modules of the highly correlated genes were obtained, and subsequent GO enrichment analysis of these modules pointed out that the mechanisms guiding developmental programs, including angiogenesis, cell adhesion and migration, might be functionally important to the formation of CeCa phenotypic traits at early disease stages; the mechanisms of chromatin conformation maintenance and remodeling turned out to be tightly coupled with the morphogenetic processes. A separate module was enriched for immune effector pathways.

**Identification of differentially expressed chromosome regions**

The above results suggest that the chromatin structure-associated processes play a role in diversifying the molecular portrait of CeCa. Furthermore, in HPV-dependent cancers, the proximity effect of HPV-integration sites may influence the functional control mechanisms of genome expression [14]. To discern chromosomal patterns of highly or weakly expressed genomic regions specific for groups A and B tumors, we applied PREDA [4] and found that down-regulated DEG tended to group into relatively extended blocks, while up-regulated DEG were more scattered throughout the genome. From a functional standpoint, many of these regions overlapped with tandemly arranged gene families that shared common functions in the innate immune response, inflammation, cell death, invasion, and cell identity. For example, interleukin (2q11-q12), chemokine (4q13), and siglec gene clusters were found to distinguish group A from both B and C.

Several other group A-distinguishing gene families were functionally linked by their role in antiviral response and cytosolic DNA sensing: these are interferon-inducible IFIT family genes (10q23), TRIM gene cluster (11p15), and IFN cluster (19q13). Furthermore, group A-specific genome regions contain collections of genes implicated in inflammasome activity and various forms of inflammatory cell death such as pyroptosis (GSDM cluster, pro-inflammatory

**TABLE 1. SIGNALING PATHWAY ANALYSIS BY GAGE APPROACH FOR SAMPLE CLUSTERS 'A', 'B', AND 'C' PAIRWISE COMPARISONS**

| Direction | Pathways | Genes | adj p-value |
|---|---|---|---|
| **'A' *versus* 'C' comparison** | | | |
| Up | DNA conformation change | 243 | 0.016 |
| | Chromatin assembly | 127 | 0.016 |
| | DNA packaging | 158 | 0.016 |
| | Nucleosome assembly | 86 | 0.023 |
| | Protein-DNA complex assembly | 189 | 0.027 |
| | Response to virus | 230 | 0.051 |
| | DNA repair | 419 | 0.051 |
| | Defense response to symbiont | 177 | 0.062 |
| | Chromosome segregation | 248 | 0.062 |
| | Adaptive immune response | 273 | 0.1 |
| | DNA recombination | 225 | 0.1 |
| | Type I interferon signaling pathway | 58 | 0.14 |
| | Cellular response to type I interferon | 58 | 0.14 |
| | Posttranscriptional gene silencing | 116 | 0.16 |
| Down | Epidermis development | 232 | 0.002 |
| | Epidermal cell differentiation | 161 | 0.002 |
| | Keratinocyte differentiation | 125 | 0.003 |
| **'A' *versus* 'B' comparison** | | | |
| Up | Nucleosome organization | 115 | 9.9e-05 |
| | Chromatin assembly | 124 | 0.00014 |
| | DNA replication-dependent nucleosome organization | 19 | 0.0025 |
| | Protein-DNA complex assembly | 184 | 0.00034 |
| | DNA conformation change | 240 | 5e-04 |
| | Negative regulation of gene expression, epigenetic | 78 | 0.002 |
| | Chromosome segregation | 244 | 0.0015 |
| | Gene silencing | 185 | 0.0031 |
| | RDNA heterochromatin assembly | 28 | 0.011 |
| | Mitotic sister chromatid segregation | 132 | 0.0057 |
| | Chromatin remodeling | 155 | 0.0059 |
| | DNA-dependent DNA replication | 161 | 0.0081 |
| | Nuclear division | 329 | 0.013 |
| | DNA repair | 411 | 0.02 |
| **'B' *versus* 'C' comparison** | | | |
| Down | Keratinocyte differentiation | 109 | 2.5e-05 |
| | Epidermal cell differentiation | 144 | 2.5e-05 |

**TABLE 2. xCELL ENRICHMENT SCORES FOR SUBCLUSTERS (‘A’, ‘B’, OR ‘C’) OF CERVICAL TISSUE SAMPLES**

| Cell population | A | B | C |
|---|---|---|---|
| **CD4⁺ memory T cells** | 0.016 (0.001-0.048)* | 0.0021 (0.000-0.008) | 0.002 (0.000-0.015) |
| **CD4⁺ naive T cells** | 0.050 (0.014-0.104)* | 0.0343 (0.014-0.089) | 0.009 (0.000-0.037) |
| **CD4⁺T cells** | 0.021 (0.000-0.044)* | 0.005 (0.000-0.019) | 0.001 (0.000-0.010) |
| **CD8⁺T cells** | 0.024 (0.000-0.043) | 0.003 (0.000-0.013) | 0.003 (0.000-0.012) |
| **CD8/CD4** | 1.126 | 0.667 | 2.270 |
| **Tregs** | 0.016 (0.000-0.038) | 0.018 (0.000-0.054) | 0.009 (0.000-0.059) |
| **CD8/Treg** | 1.481 | 0.177 | 0.380 |
| **Th1 cells** | 0.050 (0.000-0.090) | 0.004 (0.000-0.018) | 0.007 (0.000-0.015) |
| **Th2 cells** | 0.078 (0.003-0.129)* | 0.002 (0.000-0.004) | 0.019 (0.000-0.078) |
| **B cells** | 0.059 (0.000-0.190)* | 0.002 (0.000-0.008) | 0.000 |
| **Class-switched mem B cells** | 0.025 (0.000-0.084)* | 0.001 (0.000-0.003) | 0.001 (0.000-0.006) |
| **Macrophages M1** | 0.018 (0.000-0.044)* | 0.003 (0.000-0.011) | 0.000 |
| **Macrophages M2** | 0.002 (0-0.006) | 0.005 (0-0.015)* | 0.001 (0-0.002) |
| **M1/M2** | 8.530 | 0.650 | 0.064 |
| **conventional DC** | 0.002 (0.000-0.001) | 0.016 (0.000-0.051) | 0.024 (0.000-0.083) |
| **inhibitory DC** | 0.019 (0.000-0.053) | 0.148 (0.120-0.165)* | 0.055 (0.000-0.135) |
| **activated DC** | 0.181 (0.110-0.266)* | 0.044 (0.000-0.147) | 0.025 (0.000-0.042) |
| **Pericytes** | 0.049 (0.000-0.134) | 0.129 (0.100-0.212) | 0.045 (0.000-0.127) |
| **Endothelial cells** | 0.029 (0.004-0.083) | 0.067 (0.025-0.146) | 0.029 (0.000-0.072) |
| **Epithelial cells** | 0.113 (0.030-0.184) | 0.052 (0.000-0.100) | 0.165 (0.108-0.278) |
| **Keratinocytes** | 0.071 (0.046-0.093) | 0.030 (0.000-0.057) | 0.100 (0.066-0.139) |
| **ImmuneScore** | 0.081 | 0.018 | 0.008 |
| **StromaScore** | 0.062 | 0.158 | 0.057 |
| **MicroenvironmentScore** | 0.143 | 0.176 | 0.066 |

Note. *, $p < 0.05$ (Wilcoxon–Mann–Whitney test).

caspases-1, caspases-4, caspases-5, and caspases-12, cIAP1/2).

Several chromosome regions specifically expressed in either A or B groups showed functional linkage to invasion, as they contained clustered protease gene families or their inhibitors (e.g., SPINK, KLK, MMP). Group B was distinguished by the engagement of CEACAM gene cluster in 19q13 region, as well as SCCA locus (18q21) containing two apoptotic inhibitors and immune modulators, SERPINB3 and SERPINB4. A number of identified group-specific regions spanned the reported integration hotspots [14] suggesting that the differences in HPV integration breakpoints may guide molecular phenotype determination of early CeCa.

**Transcriptome-based analysis of cell population composition**

We compared the cell population content between the sample groups defined by similarity of their gene expression profiles using xCell algorithm [1]. Considering infiltrating T cells, substantial differences were derived for the $CD4^+T$ subset, with its score showing a significant increase in group A (Table 2). Although the counts of $CD4^+$ naïve T cells were comparably elevated in both A and B groups relative to C, the $CD4^+$ memory T cell score increased only

in group A suggesting that not only $CD4^+$ cells are increasingly recruited at the tumor site, but in contrast to B, group-A tumors exhibited a more efficient immune response.

Regarding Th1/Th2-differentiation, A and B groups showed opposite changes: group A was distinguished by a sharp increase in enrichment scores of both Th1 and Th2 gene sets, this increase being higher for the Th1. In group B, Th1 score didn't alter and Th2 score was dramatically decreased. Tregs showed a clear trend to an increase in both A and B groups pointing at a developing immunosuppression in both cases. A different landscape of alterations was observed for $CD8^+$T cell population: unlike group-A that displayed an abruptly increased signal from the total $CD8^+$ cell population and its na ve $CD8^+$T cell subset, group B had reduced frequencies of those cell types indicating $CD8^+$T cell exclusion.

Interestingly, however, while in group C samples, $CD8^+$T cells were about twofold more prevalent than $CD4^+$T cells, A and B tumors showed a notable decline of the CD8/CD4 ratio. The observed trend also agrees with divergent changes of the CD8/Treg ratio: group A demonstrated an increase of CD8/Treg ratio to the values averagely higher than 1, while this ratio fell to negligibly low levels in group B tumors. Group A also showed a clear enrichment across the B cell-differentiation lineage, and a significant increase in the level of class-switched B cells may indicate that the recruited B cells become actively engaged with a specific antibody-mediated response.

Specific differences between groups A and B compared with C were seen in the abundances of dendritic cells (DC) and macrophages (M), particularly their different functional or polarization states (Table 2). Immature DC (iDC) constituted a substantial proportion of conventional DC (cDC) in the pre-invasive group C, which could be explained from a concept that, by the time of CIN3 establishment, the processes of DC activation become suppressed as the result of prolonged action of HPV-oncogenes. However, in group A the percentage of activated DC (aDC) greatly increased, while that of iDC cells decreased. Conversely, in group B the frequencies of iDC cells were significantly elevated suggesting that these tumors likely exhibit exacerbated impairment of DC maturation, which could be one of the underlying causes of deviating T and B cell responses described above. A relatively high prevalence of aDC-specific gene set seen in group A might be related to the higher rates of DDR-signaling, interferon- and pro-inflammatory patterns, which create a more conducing milieu for DC maturation and favor T/B cell infiltration. As to M1/M2-differentiation, group A and B tumors also represented different phenotypes: group A displayed an apparent M1-polarity, whereas group B showed a significantly increased percentage of M2-macrophages.

Quantities of non-immune cells (epithelial, stromal lineages) indicated that group A tumors corresponded to a more differentiated epithelial phenotype, while group B specimens had a more extensive microvascular network. The combined xCell-scores (ImmunoScore, StromalScore, and MicroenvironmentScore) revealed a detectably higher TME impact in both A and B cancerous groups than in C, but group A demonstrated a significantly increased influence from immune infiltration, while group B conversely exhibited an expanded role of tumor stroma. Given that A and B tumors comprised mainly the earliest invasive stages, these groups can be viewed as not only different immunophenotypes, but likewise as different scenarios of transition from intraepithelial growth toward active invasion with different sources of immune suppression. Overall, these findings highlight the dual, complicated role of immune-activating and inflammatory processes in viral-driven biology of CeCa invasive progression. Overstimulation of protective mechanisms in early periods may be one of the triggers and contributors of CeCa invasion. At the same time, at later, advanced stages this could become manifest in the form of a widely documented immune-active but chronically inflamed and exhausted molecular subtype [6, 10].

## Conclusion

In conclusion, we have shown the feasibility of detecting consistent transcriptomic immune-related patterns manifested at different levels (i.e., gene enrichment, signaling mechanisms, co-expression modules, cell type enrichment, genome positioning) for the earliest stages of invasive CeCa progression. These transcriptomic landscapes can be taken as a starting point for further investigation of cervical cancer molecular subtypes and the diversity of underlying mechanisms, this being of potential value in point of developing treatment approaches to preclinical or early forms of invasive cervical cancer, as well as stratifying advanced cancer patients for targeted therapy.

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# ОЦЕНКА ПРЯМЫХ ВЗАИМОДЕЙСТВИЙ ОПУХОЛЕВЫХ КЛЕТОК, МИЕЛОИДНЫХ СУПРЕССОРНЫХ КЛЕТОК И PD-1- И TIM-3-ЭКСПРЕССИРУЮЩИХ Т-КЛЕТОК У БОЛЬНЫХ МНОЖЕСТВЕННОЙ МИЕЛОМОЙ

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Резюме. Уход атипичных плазматических клеток (ПК) из-под иммунного надзора при множественной миеломе (ММ) опосредован разнообразными механизмами, среди которых существенные роли играют индукция Т-клеточного истощения и экспансия миелоидных супрессорных клеток (МСК). При этом в настоящее время все еще нет данных о возможном влиянии МСК на индукцию Т-клеточного истощения. Целью настоящей работы была оценка возможной взаимосвязи относительного содержания атипичных ПК, МСК и фенотипически истощенных $PD-1^+$ и $TIM-3^+$T-клеток в костном мозге (КМ) и периферической крови (ПК) больных ММ при различных стадиях заболевания. Образцы ПК (n = 88) и КМ (n = 56) были получены у больных ММ (впервые выявленные (n = 6), пациенты в ремиссии (n = 71) и с прогрессирующим течением (n = 11)). Методом проточной цитометрии была проведена оценка относительного содержания Т-клеток, экспрессирующих ингибиторные рецепторы PD-1 и TIM-3, полиморфноядерных МСК (ПМЯ-МСК, $Lin^-CD14^-HLA\text{-}DR^-CD33^+CD66b^+$), моноцитарных МСК (М-МСК, $CD14^+HLA\text{-}DR^{low/-}$), ранних МСК (Р-МСК, $Lin^-HLA\text{-}DR^-CD33^+CD66b^-$) и атипичных ПК ($CD45^{dim}CD38^+CD138^+CD56^+CD19^-CD117^+CD27^-CD81^-$, в КМ). Циркулирующие и выделенные из КМ отдельные субпопуляции $PD-1^+$/$TIM-3^+$T-лимофцитов, Р-МСК КМ, а также атипичные ПК и уровни бета-2-микроглобулина сыворотки последовательно увеличивались в группах больных на различных стадиях ММ, от впервые выявленных до пациентов в ремиссии и с прогрессирующим течением. Несмотря на параллельное увеличение изучаемых показателей, не было выявлено каких-либо ассоциаций между маркерами опухолевого роста (атипичные ПК КМ, концентрация бета-2-микроглобулина сыворотки) и исследуемыми популяциями клеток. В образцах КМ больных с ремиссией ПМЯ-МСК обратно коррелировали с относительным

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содержанием CD4⁺Т-лимофцитов, CD4⁺PD-1⁺ и CD8⁺TIM-3⁺ субпопуляциями; также обнаружены позитивные корреляции между резидентными Р-МСК и CD4⁺PD-1⁺TIM-3⁺ клетками и между циркулирующими М-МСК и CD8⁺PD-1⁺ и (в виде тенденции) CD8⁺TIM-3⁺Т-клетками. Не удалось обнаружить каких-либо взаимосвязей между исследуемыми популяциями клеток в образцах КМ и ПК больных с впервые выявленной ММ и у пациентов с прогрессирующим течением. Возможное взаимное влияние атипичных ПК, МСК и PD-1⁺/TIM-3⁺Т-лимфоцитов, по всей видимости, не линейно, в особенности в условиях экстенсивного роста опухоли на момент постановки диагноза и прогрессии ММ. Обнаруженные обратные корреляции между относительным содержанием ПМЯ-МСК и субпопуляциями Т-клеток могли быть ассоциированы с супрессорными эффектами МСК как на преимущественно активированные PD-1⁺ клетки, так и на истощенные TIM-3⁺ субпопуляции. Прямые корреляции между резидентными Р-МСК и CD4⁺PD-1⁺TIM-3⁺ Т-клетками и между циркулирующими М-МСК и PD-1⁺ и TIM-3⁺ CD8⁺Т-клетками могли подтверждать способность МСК индуцировать Т-клеточное истощение.

*Ключевые слова: миелоидные супрессорные клетки, PD-1, TIM-3, Т-лимфоциты, опухолевые плазматические клетки, множественная миелома*

# ATTEMPT TO ASSESS DIRECT INTERACTIONS BETWEEN TUMOR BURDEN, MYELOID-DERIVED SUPPRESSOR CELLS AND PD-1- AND TIM-3-EXPRESSING T CELLS IN MULTIPLE MYELOMA PATIENTS

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**Abstract.** The avoidance of immune surveillance by malignant plasma cells (PCs) in multiple myeloma (MM) is mediated by different mechanisms, among which an induction of T cell exhaustion and expansion of myeloid-derived suppressor cells (MDSCs) appear to play substantial roles, but it is still a lack of data on possible MDSC-mediated induction of T cell exhaustion. The aim of the present work was to evaluate possible relationship between frequencies of MM PCs, MDSCs and phenotypically exhausted PD-1⁺ and TIM-3⁺ T cells in bone marrow (BM) samples and peripheral blood (PB) of MM patients at various disease stages. Peripheral blood (n = 88) and BM samples (n = 56) were obtained from MM patients (newly diagnosed (n = 6), patients in remission (n = 71) and with progressive disease (n = 11)). Frequencies of T cells expressing checkpoint receptors PD-1 and TIM-3, polymorphonuclear MDSCs (PMN-MDSCs, Lin⁻CD14⁻HLA-DR⁻CD33⁺CD15⁺/CD66b⁺), monocyte MDSCs (M-MDSCs, CD14⁺HLA-DR$^{low/-}$), early MDSCs (E-MDSCs, Lin⁻HLA-DR⁻CD33⁺CD15⁻/CD66b⁻), and MM PCs (CD45$^{dim}$CD38⁺CD138⁺CD56⁺CD19⁻CD117⁺CD27⁻CD81⁻) were assessed with flow cytometry. Circulating and BM-resident PD-1⁺/TIM-3⁺T cell subsets, BM E-MDSCs, as soon as MM PCs and serum beta2-microglobulin (B2-M) levels were gradually increased in patients at different stages. Despite that, there were no associations between the markers of tumor load and the studied cell subsets. In patients in remission, BM PMN-MDSCs negatively correlated with CD4⁺T cells, CD4⁺PD-1⁺ and CD8⁺TIM-3⁺T cell subsets; there were positive correlations between BM E-MDSCs and CD4⁺PD-1⁺TIM-3⁺ cells and PB M-MDSCs and CD8⁺PD-1⁺ and (as a trend) CD8⁺TIM-3⁺T cells. We found no associations for the samples of patients at diagnosis and with progression. We can conclude that a possible mutual influence of malignant PCs, MDSCs and PD-1⁺/TIM-3⁺T cells is nonlinear, especially during a manifest tumor growth at diagnosis and progression. The detected negative correlations between resident PMN-MDSCs andT cell subsets might be associated with MDSC suppressive function, affecting both predominantly activated PD-1⁺ cells and exhausted TIM-3⁺ subsets. The positive correlations between BM E-MDSCs and CD4⁺PD-1⁺TIM-3⁺ cell subset and circulating M-MDSCs and PD-1⁺ and TIM-3⁺ CD8⁺T cells might confirm an ability of MDSCs to induce T cell exhaustion.

*Keywords: myeloid-derived suppressor cells, PD-1, TIM-3, T cells, tumor plasma cells, multiple myeloma*

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## Introduction

Multiple myeloma (MM) is an incurable hematological malignancy characterized by the uncontrolled proliferation of a B cell precursor clone that differentiates to plasma cells in the bone marrow (BM). Multiple myeloma pathogenesis involves complex interactions between tumor cells, bone marrow microenvironment, and immune cells with pro- or anti-tumor properties. The avoidance of immune surveillance by malignant B cells in MM is mediated by a variety of mechanisms, among which an induction of T cell exhaustion (TCE) and expansion of myeloid-derived suppressor cells (MDSCs) seem to play crucial roles [10, 14]. T cell exhaustion is characterized by a pronounced decrease in T cell functions and phenotypically recognized by an up-regulation of inhibitory checkpoint receptors (ICRs) PD-1, TIM-3 etc [14]. An increment in T cells expressing ICRs was previously described for MM [1, 2]. Myeloid-derived suppressor cells, which origin and functions are clear from the name, are traditionally divided into three subsets: polymorphonuclear MDSCs (PMN-MDSCs, $Lin^-CD14^-HLA\text{-}DR^-CD33^+CD15^+/CD66b^+$), monocyte MDSCs (M-MDSCs, $CD14^+HLA\text{-}DR^{low/-}$) and early MDSCs (E-MDSCs, $Lin^-HLA\text{-}DR^-CD33^+CD15^-/CD66b^-$) [6].

In MM, an increase in $CD14^+HLA\text{-}DR^{-/low}$ and $CD11b^+CD14^-CD33^+$ MDSC subsets and the absence of quantitative changes compared with healthy individuals have been described [5, 8]. The suppression of the immune response [6, 8], enhancement of angiogenesis [15], support for the survival of tumor cells [8, 11] are currently identified for a tumor-promoting activity of MDSCs. Simultaneously, it is still a lack of data concerning possible MDSC-mediated induction of TCE. The aim of the present work was to evaluate possible relationship between frequencies of MM plasma cells (MMPCs), MDSCs and $PD\text{-}1^+$ and $TIM\text{-}3^+$T cells in BM specimens and peripheral blood (PB) of MM patients during the induction therapy courses.

## Materials and methods

Eighty-eight MM patients who had been treated at the Department of Hematology of Research Institute of Fundamental and Clinical Immunology (Novosibirsk, Russia) were enrolled in the study. All patients gave informed consent in accordance with the Declaration of Helsinki of 1975; the local ethics committee approved the study protocol. The patients were staged according to the Durie-Salmon system (1975). Responses were defined according to the International Myeloma Working Group criteria. Baseline characteristics of patients are described in Table 1.

Peripheral blood (n = 88) and BM (n = 54) samples were obtained during routine diagnostic procedures.

**TABLE 1. BASELINE CHARACTERISTICS OF PATIENTS**

| Characteristic | Value |
|---|---|
| **Age at analysis, years; median (min-max)** | 52 (35-68) |
| **Sex, female/male** | 47/41 |
| **Types** | |
| **IgG** | 53 |
| **IgA** | 18 |
| **Light chain** | 8 |
| **Unknown** | 9 |
| **Durie-Salmon stage** | |
| **II** | 25 |
| **III** | 63 |
| **Disease status at analysis:** | |
| **– newly diagnosed** | 6 |
| **– complete remission, partial response** | 71 |
| **– progressive disease** | 11 |
| **Chemotherapy regimens before analysis** | |
| **1** | 14 |
| **2** | 45 |
| **≥ 3** | 23 |
| **Time from the date of diagnosis to analysis, months; Me ($Q_{0.25}$-$Q_{0.75}$)** | 8.5 (6.1-12.7) |

Mononuclear cells (MNCs) were isolated by density gradient centrifugation and stained with the following mouse anti-human monoclonal antibodies according to the manufacturer`s recommendations: CD3 (FITC, PerCP), CD4 (FITC, PerCP), CD8 (FITC, PE-Cy 7), PD-1 (PE, APC) and TIM-3 (PerCP-Cy 5.5, BV421) – for T cell assessment; Lin (FITC), CD33 (PerCP-Cy 5.5), HLA-DR (FITC, PerCP), CD66b (APC), CD14 (FITC) – for MDSC assessment. Before and after incubation with monoclonal antibodies, samples were washed with PBS 1,500 rpm for 7 min. As controls were used unstained live cells, "fluorescence-minus-one", BD CompBeads Anti-mouse Ig, /Negative Control Compensation Particles Set (BD Biosciences). The evaluated T cell and MDSC subsets were: $CD4^+PD\text{-}1^+$, $CD4^+TIM\text{-}3^+$, $CD4^+PD\text{-}1^+TIM\text{-}3^+$, $CD8^+PD\text{-}1^+$, $CD8^+TIM\text{-}3^+$, $CD8^+PD\text{-}1^+TIM\text{-}3^+$ and $Lin^-HLA\text{-}DR^-CD33^+CD66b^-$ (E-MDSCs), $Lin^-HLA\text{-}DR^-CD33^+CD66b^+$ (PMN-MDSCs), $CD14^+HLA\text{-}DR^{low/-}$ (M-MDSCs), respectively.

Samples were analyzed on FACSCalibur and FACSCanto II flow cytometers (BD Biosciences) using CellQuest Pro and FACSDiva software, respectively. T cell and MDSC subsets were presented as the percentage of lymphocytes and MNCs, respectively. To assess MMPCs, fresh BM samples were stained with the following mouse anti-human monoclonal antibodies: CD38 (FITC or APC), CD56 (PE), CD27 (PerCP-Cy5.5), CD138 (PerCP-Cy5.5 or APC), CD117 (Pe-Cy7), CD81 (APC-H7), CD19 (V450), CD45 (V450), cytIgLambda (FITC), cytIgKappa (PE-Cy7). For intracellular staining, samples were incubated with BD FACS Permeabilizing Solution 2 (BD Biosciences). Samples were analyzed on FACSCanto II flow cytometer using FACSDiva software. Multiple myeloma plasma cells were indicated as $CD45^{dim}CD38^+CD138^+CD56^+CD19^-CD117^+CD27^-CD81^-$ and were presented as the percentage of all nucleated BM cells.

Serum beta2-microglobulin (B2-M) was assessed using latex particle-enhanced turbidimetry kit (Dako Beta-2-Microglobulin PET Kit) on the IMMAGE 800 Immunochemistry System (Beckman Coulter, USA). Statistical analysis was performed using Statistica 6 (StatSoft) package. Data in the text were presented as median and interquartile ranges. The Mann–Whitney U test (two-sided) was used to calculate differences between groups of patients. Spearman's rank correlation was used to evaluate associations for

**TABLE 2. COUNTS OF BONE MARROW PD-1$^+$ AND TIM-3$^+$T CELL SUBSETS AND MYELOID-DERIVED SUPPRESSOR CELLS IN MULTIPLE MYELOMA PATIENTS, Me ($Q_{0.25}$-$Q_{0.75}$)**

| Cell subset | Newly diagnosed (n = 6) | Remission (n = 41) | Progressive disease (n = 7) |
|---|---|---|---|
| **$CD4^+$, %** | 14.4 (11.3-27.5) | 24.3 (17.5-33.4) | 19.4 (16.0-24.1) |
| **$CD4^+PD\text{-}1^+$, %** | 2.4 (2.1-3.1) | 6.2 (3.7-8.7)* | 6.1 (2.0-12.7)* |
| **$CD4^+TIM\text{-}3^+$, %** | 1.1 (0.8-1.7) | 2.1 (1.3-3.4) | 3.0 (1.3-5.1)* |
| **$CD4^+PD\text{-}1^+TIM\text{-}3^+$, %** | 0.2 (0.1-0.3) | 0.7 (0.4-1.2)* | 1.2 (0.6-1.4)* |
| **$CD8^+$, %** | 33.9 (21.6-45.6) | 18.0 (12.7-25.5)* | 15.6 (13.3-29.0)* |
| **$CD8^+PD\text{-}1^+$, %** | 4.3 (2.9-10.9) | 5.1 (3.4-7.2) | 6.4 (4.6-11.4) |
| **$CD8^+TIM\text{-}3^+$, %** | 2.0 (1.4-2.7) | 3.7 (2.6-4.9)* | 6.5 (4.7-7.2)* # |
| **$CD8^+PD\text{-}1^+TIM\text{-}3^+$, %** | 0.2 (0.1-0.7) | 0.5 (0.4-0.8) | 1.5 (1.0-3.4)* # |
| **E-MDSCs, %** | 0.6 (0.4-0.7) | 1.0 (0.6-1.5)* | 1.0 (0.8-1.1)* |
| **PMN-MDSCs, %** | 3.3 (1.5-6.0) | 2.2 (0.7-4.0) | 1.5 (0.8-2.3) |
| **M-MDSCs, %** | 1.6 (1.1-3.9) | 1.9 (1.2-2.9) | 0.3 (0.2-0.4)* # |

Note. Relative counts of T cell subsets and MDSCs are presented as the percentages of lymphocytes and mononuclear cells, respectively. p values are assessed with Mann–Whitney U test. *, $p_U < 0.05$ between newly diagnosed patients and treated patients (in remission or with progression). #, $p_U < 0.05$ between patients in remission and patients with progressive disease. E-, M-, PMN-MDSCs indicate early, monocyte, polymorphonuclear myeloid-derived suppressor cells, respectively.

**TABLE 3. COUNTS OF CIRCULATING PD-1⁺ AND TIM-3⁺T CELL SUBSETS AND MYELOID-DERIVED SUPPRESSOR CELLS IN MULTIPLE MYELOMA PATIENTS, Me ($Q_{0.25}$-$Q_{0.75}$)**

| Cell subset | Newly diagnosed (n = 6) | Remission (n = 71) | Progressive disease (n = 11) |
|---|---|---|---|
| $CD4^+$, % | 33.4 (20.0-51.7) | 47.8 (37.0-59.7) | 41.0 (32.7-58.6) |
| $CD4^+PD\text{-}1^+$, % | 4.6 (3.7-9.9) | 5.1 (3.3-7.7) | 7.0 (4.4-13.2) |
| $CD4^+TIM\text{-}3^+$, % | 1.0 (0.5-1.3) | 1.3 (0.8-3.1) | 2.5 (2.2-5.2)* # |
| $CD4^+PD\text{-}1^+TIM\text{-}3^+$, % | 0.1 (0.1-0.3) | 0.2 (0.1-0.4) | 0.9 (0.4-1.1)* # |
| $CD8^+$, % | 26.1 (16.5-34.8) | 22.9 (12.4-33.7) | 33.7 (14.9-58.3) |
| $CD8^+PD\text{-}1^+$, % | 2.8 (1.5-4.4) | 2.7 (1.4-4.1) | 5.4 (1.6-6.4) |
| $CD8^+TIM\text{-}3^+$, % | 4.4 (3.6-6.3) | 3.2 (1.8-5.5) | 5.8 (2.3-9.6) |
| $CD8^+PD\text{-}1^+TIM\text{-}3^+$, % | 0.2 (0.2-0.4) | 0.2 (0.1-0.3) | 0.8 (0.4-1.4)* # |
| E-MDSCs, % | 0.5 (0.3-0.5) | 0.8 (0.5-1.2) | 0.6 (0.5-0.8) |
| PMN-MDSCs, % | 0.05 (0.02-0.06) | 0.02 (0.01-0.07) | 0.05 (0.02-0.6) |
| M-MDSCs, % | 3.9 (2.2-5.1) | 2.4 (1.2-5.7) | 2.8 (1.7-3.9) |

Note. As for Table 2.

continuous variables. p values presented were two-sided. $p < 0.05$ was considered statistically significant.

## Results and discussion

We first comparatively assessed MMPCs in patients with different stages of the disease. Frequencies of multiple myeloma plasma cells in BM samples of patients in remission were significantly lower compared to both newly diagnosed patients and the individuals with progressive disease: 0.12% (0.027-0.875%) *vs* 21.0% (6.8-38.0%); $p_U = 0.00010$, and 4.2% (1.4-20.6%); $p_U = 0.0021$, respectively.

The percentages of BM $CD4^+PD\text{-}1^+$, $CD4^+PD\text{-}1^+TIM\text{-}3^+$ and $CD8^+TIM\text{-}3^+$T cell subsets, as well as E-MDSCs were significantly higher in both patients in remission and with progressive disease compared with newly diagnosed MM (Table 2). Besides, $CD4^+TIM\text{-}3^+$, $CD8^+TIM\text{-}3^+$ and $CD8^+PD\text{-}1^+TIM\text{-}3^+$ were increased in BM samples of MM patients with progression compared with individuals in remission and/or newly diagnosed patients (Table 2). On the contrary, BM M-MDSCs were significantly lower in patients with progression (Table 2).

Multiple myeloma PCs and the studied cell subsets were evaluated in same BM samples, and we tried to assess possible direct associations between the tumor burden and frequencies of dysfunctional T cells and MDSCs in MM microenvironment. There were no correlations between malignant PCs and these cell compartments in the each group of patients (data not shown).Then we evaluated associations between BM MDSCs and $PD\text{-}1^+/TIM\text{-}3^+$T cells. In specimens of patients in remission, PMN-MDSC frequencies negatively correlated with $CD4^+$T cells ($R_S = -0.39$, $p = 0.030$; $n = 31$), $CD4^+PD\text{-}1^+$ ($R_S = -0.56$, $p = 0.00097$; $n = 31$) and $CD8^+TIM\text{-}3^+$T cells ($R_S = -0.59$, $P = 0.00044$; $n = 31$), while E-MDSCs positively correlated with $CD4^+PD\text{-}1^+TIM\text{-}3^+$ cell subset ($R_S = 0.39$, $p = 0.040$; $n = 31$). We failed to find such associations for the samples of patients at diagnosis and with progression.

Serum B2-M is used as a surrogate marker of tumor burden. Bone marrow MMPC frequencies correlated with serum B2-M levels: $R_S = 0.55$, $p = 0.000014$; $n = 56$. Serum B2-M levels of patients in remission were significantly lower compared to both newly diagnosed patients and the individuals with progressive disease: 2.4 mg/L (1.97-2.99 mg/L; n = 55) *vs* 5.19 mg/L (2.82-15.8 mg/L; n = 6); $p_U = 0.013$, and 6.35 mg/L (3.37-7.75 mg/L; n = 11); $p_U = 0.000029$, respectively. The only association we found was a negative correlation between B2-M and circulating $CD4^+$T cells ($R_S = -0.31$, $p = 0.039$; $n = 45$) in patients with remission.

The percentages of circulating CD4$^+$PD-1$^+$, CD4$^+$PD-1$^+$TIM-3$^+$ and CD8$^+$PD-1$^+$TIM-3$^+$T cell subsets were significantly higher in patients with progressive disease compared with both the newly diagnosed MM patients and the individuals in remission (Table 3). Contrary to the BM samples, there were no differences in PB MDSC subsets depending on the stage of the disease (Table 3).

Further we assessed associations between circulating MDSCs and PD-1$^+$/TIM-3$^+$T cells. In PB samples of patients in remission, only CD14$^+$HLA-DR$^{low/-}$ M-MDSCs negatively correlated with CD4$^+$T cells ($R_S$ = -0.34, p = 0.0064; n = 60), and positively correlated with both CD8$^+$PD-1$^+$ ($R_S$ = 0.28, p = 0.029; n = 60) and – as a trend – CD8$^+$TIM-3$^+$T cells ($R_S$ = 0.22, p = 0.098; n = 60). As in the case of BM samples, we found no associations between circulating MDSCs and PD-1$^+$/TIM-3$^+$T cells of patients at diagnosis and with progression.

## Conclusion

The present work was devoted to an attempt to find possible direct associations between tumor load, MDSCs and PD-1- and TIM-3-expressing T cells in multiple myeloma patients, as these cell compartments appear to play substantial roles in MM escape from immune surveillance and its clinical progression.

The described gradual increase in circulating and BM-resident PD-1$^+$/TIM-3$^+$T cells and E-MDSCs of patients at different stages are in agreement with the previously published data [2, 3, 7, 13]. The only unexpected finding was the decrease in CD14$^+$HLA-DR$^{low/-}$ M-MDSCs in BM samples of patients with progression. Presumably, M-MDSC decrease was only relative, due to increase in other MNC compartments, or might be caused by intensive treatment or tumor growth itself, i.e., due to unfavorable metabolic changes.

Despite the above-described parallel increment in BM MMPCs, PD-1$^+$/TIM-3$^+$T cells and MDSC subsets at MM diagnosis, treatment and progression, we and others [12] found no correlations between tumor load and the studied cell subsets, although it had been obtained from the same samples. Apparently, a possible mutual influence of these cell compartments is nonlinear, especially during a manifest tumor growth at diagnosis and progression.

The detected negative correlations between resident BM PMN-MDSCs and CD4$^+$T cells, CD4$^+$PD-1$^+$ and CD8$^+$TIM-3$^+$T cell subsets might be associated with MDSC suppressive function, affecting both predominantly activated PD-1$^+$ cells and exhausted TIM-3$^+$ subsets [2]. The positive correlations between both BM E-MDSCs and CD4$^+$PD-1$^+$TIM-3$^+$ cell subset and circulating M-MDSCs and CD8$^+$PD-1$^+$ and CD8$^+$TIM-3$^+$T cells might confirm a poorly described ability of MDSCs to induce TCE. Previously the increase in MDSCs and PD-1$^+$T cells was evaluated for high-risk chronic myeloid leukemia [4]. Additionally, the increment in MDSC counts is associated with the resistance to anti-PD-1 therapy for solid tumors [9]. A role of MDSC populations in the development of TCE in MM requires further investigations.

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# СОПРЯЖЕННОСТЬ МИЕЛОИДНЫХ СУПРЕССОРНЫХ КЛЕТОК С ВОССТАНОВЛЕНИЕМ КРОВЕТВОРЕНИЯ ПОСЛЕ ВЫСОКОДОЗНОЙ ХИМИОТЕРАПИИ ПРИ МНОЖЕСТВЕННОЙ МИЕЛОМЕ

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**Резюме.** Супрессорные клетки миелоидного происхождения (МС) играют важную роль в регуляции иммунного ответа при многих патологиях и прежде всего при злокачественных опухолях, однако их роль в приживлении гемопоэтических стволовых клеток и восстановлении кроветворения после высокодозной химиотерапии с аутологичной трансплантацией стволовых клеток остается практически не изученной. Настоящее исследование направлено на изучение зависимости между содержанием субпопуляций МС и показателями крови на этапе восстановления кроветворения после высокодозной химиотерапии с аутологичной трансплантацией гемопоэтических стволовых клеток у пациентов с множественной миеломой (ММ). МС оценивали методом проточной цитометрии в образцах периферической крови на этапе выхода из лейкопении (при количестве лейкоцитов в периферической крови более $1 \times 10^9$/л). Количество трансплантируемых $CD34^+CD45^+$ гемопоэтических стволовых клеток составило $4,38 \times 10^6$/кг (IQR (3,1-5,6) $\times 10^6$/кг). Длительность выхода из лейкопении варьировала от 8 до 18 дней (Me 12 дней). Содержание МС на этапе выхода из лейкопении не было связано с количеством $CD34^+$ клеток/кг в трансплантате. Относительное содержание моноцитарных МС (М-МС, $CD14^+HLA\text{-}DR^{low/-}$) прямо коррелировало с содержанием моноцитов на этапе выхода из лейкопении (R = 0,417, p = 0,002). Для гранулоцитарных МС (Г-МС, $Lin^-HLA\text{-}DR^-CD33^+CD66b^+$) была характерна обратная связь с количеством моноцитов (R = -0,493, p = 0,0003), при этом сопряженность с абсолютным содержанием нейтрофилов была слабо выражена (R = 0,273, p = 0,048). Содержание лимфоцитов на этапе выхода из лейкопении находилось в обратной связи с Г-МС (R = -0,347, p = 0,014) и не коррелировало с М-МС. При анализе длительность лейкопении обратная корреляционная связь с данным показателем была выявлена для процентного и абсолютного содержания М-МС (R = -0,347, p = 0,018 и R = -0,469, p = 0,0008 соответственно). Множественный регрессионный ана-

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лиз показал зависимость длительности лимфопении от доли циркулирующих М-МС (p = 0,014) и количества трансплантированных CD34+ клеток/кг (p = 0,032). Согласно данным многофакторного дисперсионного анализа значимыми факторами для продолжительности лейкопении являлись количество трансплантированных CD34+ клеток/кг и содержание М-МС. При этом такие клинические параметры как глубина ответа и наличие минимальной остаточной болезни перед проведением высокодозной химиотерапии и трансплантации гемопоэтических стволовых клеток, а также стадия ММ не влияли на длительность восстановления кроветворения. Таким образом, полученные результаты свидетельствуют о сопряженности более высокого содержания М-МС с меньшей длительностью лейкопении после высокодозной химиотерапии с аутологичной трансплантацией стволовых клеток и указывают на позитивную роль М-МС в восстановлении кроветворения в раннем посттрансплантационном периоде у пациентов с ММ.

*Ключевые слова: миелоидные супрессорные клетки, множественная миелома, трансплантация, гемопоэтические стволовые клетки, длительность лейкопении, раннее восстановление гемопоэза*

# ASSOCIATION OF MYELOID-DERIVED SUPPRESSOR CELLS WITH HEMATOPOIETIC RECOVERY AFTER HIGH-DOSE CHEMOTHERAPY IN MULTIPLE MYELOMA

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**Abstract.** Myeloid-derived suppressor cells (MDSCs) play an important role in the immune response regulation in many pathologies, primarily in malignant tumors, but their role in the hematopoietic stem cell engraftment and the hematopoietic recovery after high-dose chemotherapy and autologous stem cell transplantation remains practically unexplored. This study is aimed at studying the correlation between the number of MDSC subpopulations and blood parameters at the stage of hematopoietic recovery after high-dose chemotherapy and autologous hematopoietic stem cell transplantation in patients with multiple myeloma (MM). Circulating MDSCs were assessed at the stage of leukopenia recovery (absolute leukocyte count in peripheral blood (PB) > $1 \times 10^9$/L) by flow cytometry. The number of transplanted $CD34^+CD45^+$ hematopoietic stem cells was $4.38 \times 10^6$/kg (IQR $(3.1-5.6) \times 10^6$/kg). The duration of recovery from leukopenia varied from 8 to 18 days (Me 12 days). The number of MDSCs at the engraftment was not associated with the number of $CD34^+$ cells/kg in the graft. The relative number of monocytic MDSCs (M-MDSCs, $CD14^+HLA\text{-}DR^{low/-}$) directly correlated with the number of monocytes at the stage of recovery from leukopenia (R = 0.417, p = 0.002). Granulocytic MDSCs (PMN-MDSCs, $Lin^-HLA\text{-}DR^-CD33^+CD66b^+$) were characterized by an inverse correlation with the number of monocytes (R = -0.493, p = 0.0003) while the association with the absolute number of neutrophils was weak (R = 0.273, p = 0.048). The number of lymphocytes at the stage of recovery from leukopenia had an inverse correlation with PMN-MDSCs (R = -0.347, p = 0.014) and did not correlate with M-MDSCs. When analyzing the duration of leukopenia, an inverse correlation with this indicator was revealed for the percentage and absolute number of M-MDSCs (R = -0.347, p = 0.018 and R = -0.469, p = 0.0008, respectively). Multiple regression analysis showed dependence of the lymphopenia duration on the proportion of circulating M-MDSCs (p = 0.014) and the number of transplanted $CD34^+$ cells/kg (p = 0.032). According to the data of multivariate analysis of variance, the number of transplanted $CD34^+$ cells/kg and the number of M-MDSCs were significant factors for the duration of leukopenia. At the same time, such clinical parameters as the depth of response and minimal residual disease status before high-dose chemotherapy and hematopoietic stem cell transplantation, as well as the MM stage, did not affect the duration of hematopoietic recovery. Thus, the obtained results indicate the association of a higher number of M-MDSCs with a shorter duration of leukopenia after high-dose chemotherapy with autologous stem cell transplantation and indicate a positive role of M-MDSCs in hematopoietic recovery in the early post-transplant period in patients with MM.

*Keywords: myeloid-derived suppressor cells, multiple myeloma, transplantation, hematopoietic stem cells, duration of leukopenia, early hematopoietic recovery*

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## Introduction

Myeloid-derived suppressor cells (MDSCs) play an important role in the immune response regulation in many pathologies, primarily in tumors [6]. MDSCs release a suppressor activity towards innate and adaptive immune cells *in vitro* and *in vivo* with the most pronounced suppressor activity against T cells [5]. In humans, monocytic MDSCs (M-MDSCs, $CD14^{+}HLA\text{-}DR^{low/-}$), granulocytic, or polymorphonuclear, MDSCs with a phenotype similar to neutrophils (PMN-MDSCs, $CD11b^{+}CD33^{+}CD14^{-}CD15^{+}$ ($CD66b^{+}$) $HLA\text{-}DR^{low/-}$) and immature MDSCs (E-MDSCs) with the $Lin^{-}$ (CD3, CD14, CD15, CD19, CD56) HLA-DR$CD33^{+}$) phenotype have been described [1, 2].

In solid tumors, the number of MDSCs increases and correlates with tumor size, stage and poor prognosis [5]. In hemoblastosis, especially in patients with hematopoietic stem cell transplantation (HSCT) the MDSC population is less studied and the role of MDSCs is not clear [11]. MDSCs of donor origin presented in the allograft can suppress the graft-versus-host reaction [4], playing a positive role. There is much less clarity regarding the role of MDSCs in autologous HSCT (auto-HSCT). Since early reconstitution of lymphocytes is provided by homeostatic proliferation of T cells transplanted as a part of the graft, MDSC suppressive activity against T cells can reduce the effectiveness of immune reconstitution after auto-HSCT.

Multiple myeloma (MM) is a malignant lymphoproliferative disease characterized by infiltration of the bone marrow by tumor plasma cells secreting monoclonal immunoglobulin and/or free light chains. Despite advances in therapy, MM still remains an incurable disease with a 5-year survival rate of just over 50% [12]. At the same time, high-dose chemotherapy (HDCT) with auto-HSCT is an important and effective standard of treatment for MM. One of the prognostically favorable factors associated with successful HSCT and greater overall survival after HDCT and auto-HSCT is a faster and more stable hematopoietic recovery [8, 10]. The study of the immune reconstitution mechanisms and the search for prognostic factors affecting the hematopoietic recovery after HSCT attracts special attention in terms of optimizing therapy and predicting the development of infectious complications and MM relapse. However, there is no information about the possible role of MDSCs in the regulation of autologous hematopoietic stem cell (HSC) engrafting after HDCT and the hematopoietic recovery.

**The aim of this study** was to find the relationship between the number of MDSC subpopulations and the hematopoietic recovery after HPCT and auto-HSCT in patients with MM.

## Materials and methods

The study included 55 patients (30 women and 25 men) with MM aged 38 to 72 years (Me 54 years), who underwent HDCT with auto-HSCT. A complete or very good partial response (CR/VGPR) was achieved in 37 patients; a partial response (PR) was achieved in 18 patients at the time of HSC transplantation. Auto-HSCT was performed with a conditioning regimen of melphalan 200 mg/m$^2$, or 140 mg/m$^2$ in patients with impaired renal function. The median number of $CD34^{+}CD45^{+}$ hematopoietic stem cells was $4.38 \times 10^6$/kg (IQR (3.1-5.6) $\times 10^6$/kg).

To assess the number of MDSCs, mononuclear cells (MNCs) at the stage of recovery from leukopenia (number of leukocytes in peripheral blood (PB) $> 1 \times 10^9$/L) were isolated from PB by standard centrifugation of whole heparinized venous blood in a ficoll-verografin density gradient (p = 1.077). Flow cytometry (BD FACSCanto II, USA) was used to study the relative number of PMN-MDSCs ($Lin^{-}HLA\text{-}DR^{-}CD33^{+}CD66b^{+}$), M-MDSCs ($CD14^{+}HLA\text{-}DR^{low/-}$), and P-MDSCs ($Lin^{-}HLA\text{-}DR^{-}CD33^{+}CD66b^{-}$) among MNCs using anti-Lineage Cocktail 1 (CD3, CD14, CD16, CD19, CD20, CD56; FITC, BD Biosciences, USA), anti-CD14 (FITC, BD Biosciences), anti-CD33 ( PerCP-Cy5.5, BD Biosciences), anti-CD66b (APC, BioLegend, USA), anti-HLA-DR (APC-Cy7, PerCP, BD Biosciences). Isotype antibodies conjugated with similar fluorochromes were used as a negative control.

Statistical data processing was performed using the Statistica 6.0 (StatSoft) and GraphPad Prism 8 software packages. Correlation analysis was performed using the Spearman rank correlation test. Additionally, linear regression analysis and multivariate analysis of variance were performed. Differences were considered statistically significant at $p < 0.05$.

## Results and discussion

The duration of recovery from leukopenia (leukocytes in the PB $> 1 \times 10^9$/L, platelets $> 50 \times 10^9$/L) in patients with MM after HDCT and auto-HSCT varied from 8 to 18 days (Me 12 days, IQR 11-14 days). The number of leukocytes and platelets counted at this stage is shown in Table 1.

Since the number of transplanted $CD34^{+}$ HSCs is one of the important factors of immune reconstitution, we conducted a correlation analysis of the transplanted $CD34^{+}$ cells/kg with hematopoietic recovery indicators. The duration of leukopenia was in a reverse association with transplanted $CD34^{+}$ cells/kg (R = -0.305, p = 0.008). A significant de-

TABLE 1. PARAMETERS OF HEMATOPOIETIC RECOVERY AFTER AUTO-HSCT IN MULTIPLE MYELOMA

| Parameter | Me ($Q_{0.25}$-$Q_{0.75}$) | Min-max |
|---|---|---|
| **Neutrophils (× $10^9$/L)** | 1.26 (0.78-2.44) | 0.22-7.95 |
| **Thrombocytes (× $10^9$/L)** | 65 (47-90) | 7-243 |
| **Monocytes (× $10^9$/L)** | 0.12 (0.05-0.25) | 0.005-1.560 |
| **Lymphocytes (× $10^9$/L)** | 0.48 (0.33-0.79) | 0.02-2.68 |
| **M-MDSCs (%)** | 3.4 (2.0-6.9) | 0.3-19.1 |
| **M-MDSCs (× $10^6$/mL)** | 26.8 (14.1-68.8) | 4.4-350.0 |
| **PMN-MDSCs (%)** | 0.32 (0.03-0.90) | 0.001-15.540 |
| **PMN-MDSCs (× $10^6$/mL)** | 2.90 (0.43-7.47) | 0.02-148.40 |
| **E-MDSCs (%)** | 0.63 (0.39-1.19) | 0.09-2.55 |
| **E-MDSCs (× $10^6$/mL)** | 5.5 (3.7-10.5) | 0.6-34.3 |

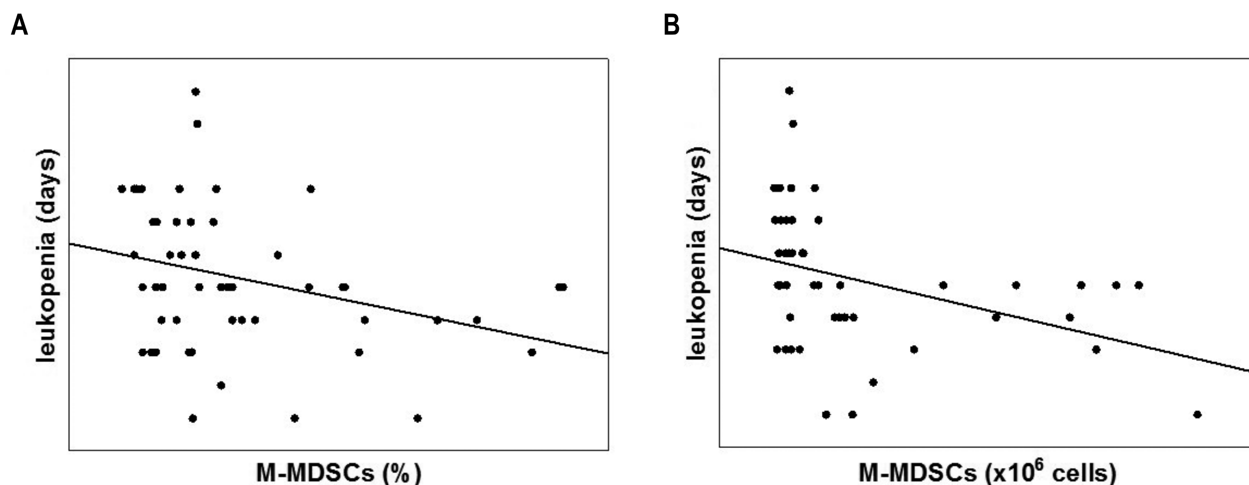


**Figure 1. Association of leukopenia duration and numbers of M-MDSCs at the stage of recovery from leukopenia after high-dose chemotherapy**

TABLE 2. MULTIVARIATE ANALYSIS OF THE CLINICAL PARAMETERS' INFLUENCE ON THE DURATION OF LEUKOPENIA AFTER AUTO-HSCT IN PATIENTS WITH MULTIPLE MYELOMA

| Factorial ANOVA | F-value | p-value |
|---|---|---|
| **$CD34^+$ cells/kg (> Me *vs* < Me)** | 6.95 | 0.01 |
| **M-MDSCs, % (> Me *vs* < Me)** | 7.66 | 0.008 |
| **Response (CR *vs* PR)** | 0.33 | 0.57 |
| **MRD (positive *vs* negative status)** | 0.15 | 0.70 |
| **Stage at diagnosis (II *vs* III Durie–Salmon stage)** | 0.003 | 0.96 |

Note. Response, the depth of the response before the HDCT; CR, complete response; PR, partial response; MRD, minimal residual disease before HDCT

pendence on the transplanted CD34+ cells/kg was found only for the absolute number of platelets (R = 0.378, p = 0.0007) among all the analyzed parameters. A similar relationship with the number of monocytes was weak (R = 0.250, p = 0.03). The number of MDSC subpopulations at the stage of recovery from leukopenia was not associated with the CD34+ cells/kg in the graft.

In order to assess the importance of MDSCs in terms of hematopoietic recovery after HPCT and auto-HSCT, we compared the number of MDSCs with the number of other blood cells populations. The relative number of M-MDSCs directly correlated with the number of monocytes (R = 0.417, p = 0.002). PMN-MDSCs was characterized by an inverse relationship with the number of monocytes (R = -0.493, p = 0.0003). At the same time, PMN-MDSCs and the absolute number of neutrophils were revealed to have weak positive correlation (R = 0.273, p = 0.048). In addition, an inverse significant correlation was found between the proportion of PMN-MDSCs and the number of lymphocytes (R = -0.347, p = 0.014). When analyzing the duration of leukopenia, it turned out that this indicator was associated only with the number of M-MDSCs. The duration of leukopenia had an inverse correlation with the percentage and absolute number of M-MDSCs (R = -0.347, p = 0.018 and R = -0.469, p = 0.0008, respectively; Figure 1), which indicates the association of a higher number of M-MDSCs with earlier recovery of hematopoiesis after HDCT and infusion of autologous HSCs.

Multiple regression analysis confirmed the dependence (determination coefficient $R^2$ 0.241, F-test 3.45, p = 0.015) of the duration of leukopenia on the proportion of circulating M-MDSCs (p = 0.014) and the number of transplanted CD34+ cells/kg (p = 0.032). According to regression analysis, the proportion of circulating PMN-MDSCs and E-MDSCs did not affect the duration of leukopenia (p = 0.14 and p = 0.66, respectively) at the same time. A similar pattern was found for the absolute number of M-MDSCs (p = 0.048).

At the final stage we tried to find the most significant factors associated with a shorter duration of leukopenia using multivariate analysis of variance (Table 2). The number of transplanted CD34+ cells/kg and the number of M-MDSCs were significant factors for the duration of leukopenia. At the same time, such clinical parameters as the depth of response and the presence or absence of minimal residual disease before HDCT, as well as the MM stage, did not affect the duration of hematopoiesis recovery.

In general, the present study showed for the first time that the number of MDSC subpopulations at the stage of recovery from leukopenia after HDCT and auto-HSCT is not linearly related to the number of transplanted CD34+ cells/kg but correlates with the number of other types of leukocytes and platelets during the hematopoietic recovery. In addition, M-MDSCs along with the number of transplanted CD34+ cells/kg are significant factors for an earlier recovery from leukopenia after HSC transplantation. Indeed, the factors affecting the efficiency and duration of hematopoietic recovery in auto-HSCT in patients with MM are the number of CD34+ cells in auto-graft, an early stage of the disease, conditioning regimen, age [7, 8, 9]. Our findings about association between faster recovery from leukopenia and higher number of circulating M-MDSCs may indicate that MDSCs not only have the ability to maintain the stem properties of tumor cells [3], but may also be involved in the microenvironment recovery needed for effective HSC functioning in the bone marrow of patients after HDCT. However, further studies are required to confirm this assumption.

The positive correlation found between M-MDSCs and monocytes, as well as between PMN-MDSCs and neutrophils, indicates the common origin of these cell populations while the inverse correlation between PMN-MDSCs and monocytes indicates competitive lines of cell differentiation from common myeloid progenitors.

## Conclusion

Thus, the revealed relationship between MDSCs and the efficiency of leukocyte recovery in patients with MM after HDCT and auto-HSCT demonstrates a new role for MDSCs. Since the rapid hematopoietic recovery is an important factor in terms of possible complications and transplantation outcomes, it can be assumed that M-MDSCs can play a positive role at the stage of recovery from leukopenia contributing to the rapid engraftment of HSCs and the beginning of hematopoiesis.

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# ОСОБЕННОСТИ ВЗАИМОДЕЙСТВИЯ МЕДИ И КОБАЛЬТА, УЧАСТВУЮЩИХ В КРОВЕТВОРЕНИИ, И ВЛИЯНИЕ ИХ ДЕФИЦИТА НА РАЗВИТИЕ АНЕМИИ

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**Резюме.** Одной из основных задач кроветворения является поддержание постоянного количественного и качественного состава отдельных компонентов и звеньев системы крови, что соответственно болезни крови можно рассматривать как нарушение закона клеточного равновесия. Физиологические механизмы адаптивной перестройки детского организма в экологически неблагоприятных условиях закономерно приводят к сдвигам элементного гомеостаза. Дефицит одного микроэлемента может привести к дисбалансу других микроэлементов.

Целью настоящего исследования является изучение роли и взаимодействия меди, кобальта и железа, участвующих в кроветворении, а также распространенность анемии среди детей, проживающих в регионе Приаралья.

Всего было обследовано 1120 детей и подростков. Был проведен клинический осмотр всех детей, с определением физического развития: выполнены антропометрические измерения и общепринятые лабораторные анализы. Для определения микроэлементного статуса исследованы волосы практически здоровых детей.

По результатам проведенных исследований выявлено, что у 78% детей наблюдается снижение гемоглобина. Показатели анемии у подростков был достоверно выше (в 2,5 раза), чем у детей младших возрастных групп ($p < 0,0001$).

Сравнительный анализ уровня эритроцитов и гемоглобина по основным показателям физического развития показала, что высокий рост положительно коррелирует с уровнем эритроцитов и гемоглобина.

В результате анализа микроэлементного состава волос у детей в регионе Приаралья, выявлены наиболее часто встречаемые гипомикроэлементозы. Общая частота микроэлементозов, обусловлена в основном дефицитом меди в 98,4% случаев (63), дефицитом кобальта в 92,1% (59), дефицитом цинка в 57,8% (37). Также проведено исследование не только содержания элементов, но и их соотношения. Выявлено повышенное соотношение Fe/Cu и Fe/Cu во всех возрастных группах. Дисбаланс микроэлементов, а также дефицит меди, кобальта, цинка способствуют развитию анемии у детей.

Полученные результаты показали, что анемия, выявляемая у детей, проживающих в регионе Приаралья, обусловлена не только снижением железа, но также снижением меди, кобальта, цинка, мар-

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ганца. Анализ волос на микроэлементы имеет свое значение в дифференциальной диагностике и лечении детей. Каждый из изученных микроэлементов, оказывает непосредственное влияние на состояние ребенка и возникновение различных заболеваний, в том числе анемий.

*Ключевые слова: заболевание системы крови, анемия, железодефицитная анемия, дети, микроэлементы, основные микронутриенты гемопоэза*

# INTERPLAY BETWEEN COPPER AND COBALT IN HEMATOPOIESIS AND THE IMPACT OF THEIR DEFICIENCY ON ANEMIA DEVELOPMENT

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**Abstract.** Hematopoiesis is a complex process that requires a specific set of blood components to function properly. Blood diseases can result from imbalances or deficiencies in these components. The body has physiological sensors that respond to environmental changes by maintaining elemental homeostasis. A deficiency in one micronutrient can lead to imbalances in others. The purpose of this study was to investigate the role and interaction of copper, cobalt, and iron in hematopoiesis and to determine the prevalence of anemia in children living in the Aral Sea region.

A total of 1120 children and adolescents were examined, and their physical development was measured using anthropometric measurements and laboratory tests. Hair samples were analyzed to determine the children's micronutrient status. The results revealed that 78% of the children had a decrease in hemoglobin, and anemia was more prevalent in adolescents. A correlation was found between high growth and increased levels of erythrocytes and hemoglobin. The study also identified the most common hypomicroelementoses in the Aral Sea region, including copper deficiency in 98.4% of cases, cobalt deficiency in 92.1%, and zinc deficiency in 57.8%.

The study also analyzed the ratio of trace elements, revealing an increased Fe/Cu and Fe/Cu ratio in all age groups. Imbalances and deficiencies in copper, cobalt, zinc, and manganese were found to contribute to the development of anemia in children. Hair analysis for trace elements was shown to be significant in the differential diagnosis and treatment of children with anemia.

In conclusion, the study highlights the importance of maintaining a proper balance of trace elements in hematopoiesis. Deficiencies in copper, cobalt, zinc, and manganese can contribute to anemia in children, and hair analysis can be used to diagnose and treat the condition. Further research is needed to better understand the role of trace elements in hematopoiesis and their impact on human health.

*Keywords: blood system disease, anemia, iron deficiency anemia, children, trace elements, micronutrients of hematopoiesis*

## Introduction

One of the pivotal goals of hematopoiesis is to maintain the constant quantitative and qualitative composition of individual blood components and subsystems, which is predicated on the principle of kinetic regulation, whereby an equal number of cells are produced and undergo cell death per unit time. As such, hematologic disorders can be conceptualized as aberrations of cellular homeostasis [6].

Hematologic diseases are among the most prevalent maladies worldwide. Approximately one quarter of the global population, including young children and women, suffer from anemia, with iron-deficiency anemia (IDA) accounting for about 90% of all cases [5, 12]. In Karakalpakstan, the prevalence of anemia exceeds 40%, a level that has been identified as a grave health concern for the entire population, particularly children of all ages and women of reproductive age. This issue demands not only a medical approach, but also a social one that involves state-level interventions. Living conditions and health outcomes in the Aral Sea region are inextricably linked to the quantity and quality of available water resources. Research has revealed a correlation between the hardness (r = 0.40) and mineralization (r = 0.53) of drinking water and disease incidence in children. In

this context, the physiological mechanisms that drive adaptive responses in children residing in ecologically adverse conditions inevitably lead to perturbations in elemental homeostasis [8, 9].

According to the available literature, nutritional imbalances among the population of the Aral Sea region may lead to the depletion of essential micronutrient reserves. In addition, the deficiency of one micronutrient may result in imbalances of other micronutrients. Furthermore, the inadequate supply of vital micronutrients can be attributed not only to their insufficient presence in food but also to the antagonistic effects of more toxic micronutrients [7, 8, 9].

Currently, anemia is viewed not only as a symptom of disease but also as a pathogenetic factor that exacerbates systemic damage in the organism. Nonetheless, many etiological and pathogenetic aspects of deficiency anemia remain unresolved to date.

**The objective of the current study** is to investigate the role and interaction of copper, cobalt, and iron in hematopoiesis, as well as the prevalence of anemia among children residing in the Aral Sea region.

## Materials and methods

The study enrolled children and adolescents (from birth to 18 years of age) who underwent regular medical check-ups at their schools and resided in the Aral Sea region. A total of 1120 children were examined, all of whom were native inhabitants residing in the epicenter of the environmental crisis – the Republic of Karakalpakstan – and whose parents provided written consent for their participation in the study.

All children underwent a clinical examination, with assessments of their physical development including anthropometric measurements (height, weight, head and chest circumference) and standard laboratory analyses. The criteria for anemia were based on the reference values of hemoglobin levels proposed by the World Health Organization.

Hair samples were used for the assessment of the children's microelement status through neutron activation analysis. Hair is a convenient and non-invasive biological substrate for medical and biological research and offers several advantages compared to other biosubstrates. Hair analysis enables the evaluation of a child's microelement status, the determination of the state of individual organs and systems of the body based on this assessment, the degree of exposure to environmental factors, nutrition, and the development of methods to correct any identified deficiencies [1, 3, 10].

Hair samples from practically healthy children were examined to determine their micronutrient status. Of the total number of children examined, 64 aged between 1 and 18 years were selected for the study. All children were divided into four age groups: Group I comprised children from birth to 4 years, Group II comprised children from 4 to 8 years, Group III comprised children from 8 to 15 years, and Group IV comprised children from 15 to 18 years.

As a result of the multi-element determination of the composition of children's hair residing in the Aral Sea region, more than 20 elements were identified. This study focuses on the results of the analysis of hair samples for essential micronutrients, namely iron, cobalt, copper, and zinc.

The obtained data on the content of micronutrients in hair samples were compared with reference values for practically healthy children aged 1 to 18 years [1, 9]. Statistical processing of the obtained data was performed using Microsoft Excel 2007 and Statistica 6.0 software packages.

## Results and discussion

According to the results of the conducted research, a decrease in hemoglobin was observed in 78% of the children, with an average of 97.8±1.6 g/L. Based on the obtained data, anemia of varying severity was detected in all age groups of children. Anemia was detected in 33.4% of children in group 1, 42.7% in group 2, 62.4% in group 3, and 72.2% in group 4.

The prevalence of anemia among adolescents was significantly higher (2.5 times) than in younger age groups of children ($p < 0.0001$).

According to the results of our study, the mean value of erythrocyte count in peripheral blood was 3.8±0.4 × 1012 cells/L. This laboratory parameter was significantly higher in children of the 1-2 age groups compared to those of the 3-4 age groups. Additionally, male children demonstrated higher levels of erythrocytes and hemoglobin compared to female children.

Further stratification of erythrocyte and hemoglobin levels by major indicators of physical development revealed a positive correlation between high height and erythrocyte/hemoglobin levels. Additionally, an increase in body mass and mass-height index values also showed a positive correlation with erythrocyte and hemoglobin parameters (Table 1).

However, this trend was observed only among 14-16-year-old children, especially boys. Both boys and girls under 14 years of age did not significantly differ in terms of their height and body weight from the average values for healthy children.

Comparative evaluation of weight and height indicators in children with anemia and healthy children showed that the greatest growth and body weight lag was observed in 14-year-old children. The height of healthy boys was 158.7±0.7 cm and girls 157.9±0.7 cm, while in children with anemia, these indicators did not exceed 153.5±0.7 and 151.4±0.3 cm, respectively ($p < 0.001$). In healthy

**TABLE 1. COMPARATIVE ASSESSMENT OF AGE-RELATED DYNAMICS OF GROWTH AND BODY WEIGHT IN HEALTHY CHILDREN AND THOSE WITH ANEMIA LIVING IN THE ARAL SEA REGION**

| Age | Sex | Height, cm | | | Weight, kg | | |
|---|---|---|---|---|---|---|---|
| | | Children with anemia | Healthy children | p | Children with anemia | Healthy children | p |
| 14 | M | 153.5±0.7 | 158.7±0.7 | < 0.001 | 40.2±0.7 | 45.1±0.6 | < 0.001 |
| | F | 151.4±0.4 | 157.9±0.7 | < 0.001 | 40.1±0.4 | 47.2±0.7 | < 0.001 |
| 15 | M | 162.6±0.2 | 166.1±0.6 | < 0.001 | 46.5±0.2 | 50.3±0.6 | < 0.001 |
| | F | 155.4±0.5 | 158.8±0.6 | < 0.001 | 45.5±0.3 | 48.3±0.6 | < 0.001 |
| 16 | M | 165.9±0.6 | 169.2±0.6 | < 0.001 | 53.4±0.6 | 56.2±0.7 | < 0.01 |
| | F | 156.7±0.4 | 159.5±0.5 | < 0.001 | 48.5±0.7 | 51.1±05 | < 0.01 |

Note. The anthropometric measurements of healthy children were used as a control in this study. p, significance of differences between data for healthy children and children with anemia. M, boys; F, girls.

children of the control group, the body weight in boys averaged 45.1±0.6 kg, and in girls 47.2±0.7 kg, while in children with anemia, it was respectively 40.2±0.7 and 40.1±0.4 kg ($p < 0.001$).

According to the results of recent studies, a correlation has been established between the frequency of verification of a decrease in the level of hemoglobin and erythrocytes and the environmental conditions of the region of residence. This may be explained by the peculiarities of the region's environment, diet, dietary traditions of the population, regional features of iron content in food products, and the absorption of this element in the body of adolescents.

The analysis of hair microelement composition in children living in the region of the Aral Sea revealed the most common hypomicroelementoses. The overall frequency of microelementoses was mainly due to a deficit of copper in 98.4% of cases (63), cobalt deficit in 92.1% (59), and zinc deficit in 57.8% (37). Only 29.6% of the examined children had iron deficiency (19), while the rest had iron levels within the reference values (20-30 μg/g). Table 2 shows the element content values for different age groups of children. As seen in the table, the most commonly encountered hypomicroelementoses are deficits of essential elements: Cu, Zn, Co, and Fe.

According to some authors [2, 5, 11], not only the content of elements but also their ratio should be taken into account. The results of the study showed that the ratios of elements have their peculiarities. The Fe/Cu coefficient was calculated for the examined children. An increased Fe/Cu ratio of more than 3 times was found in all age groups (Table 3).

It is widely known that many physiological and metabolic processes occurring in both children and adults are associated with free radical oxidation of lipids, proteins, and carbohydrates, in which iron plays an important role. According to N. A. Gres, a Fe/Cu ratio exceeding the optimal value of 0.9 indicates an increase in the amount of free radicals [11].

Moreover, the analysis of the Fe/Co coefficient also revealed a 2-4-fold increase in this ratio in each age group. Low values of this coefficient (< 440) indicate a predisposition of the body to thyroid dysfunction. N.A. Gres's data suggests that a decrease in iron content leads to the predominance of cobalt's influence on the metabolism of thyroid hormones, which can lead to disruptions in iodine exchange and the development of diffuse goiter.

Let us delve into the role of each element and its interaction with iron.

One of the essential elements for human health is copper. It is a constituent of vital enzymes involved in crucial respiratory and erythropoietic processes [13]. Copper serves as the primary activator of hemoglobin and participates in iron metabolism, promotes cel-

**TABLE 2. CONTENT OF ELEMENTS IN THE HAIR OF CHILDREN LIVING IN THE ARAL SEA REGION IN ACCORDANCE WITH AGE GROUPS (mcg/g)**

| Element | Examined children | | | | Norm range | |
|---|---|---|---|---|---|---|
| | I gr. 1-3 y. o. n = 16 | II gr. 4-8 y. o. n = 14 | III gr. 9-14 y. o. n = 16 | IV gr. 15-18 y. o. n = 18 | Min | Max |
| **Fe** | 29.0±5.6 | 20.0±1.9 | 21.0±1.5 | 16.0±3.5 | 15 | 30 |
| **Co** | 0.0200±0.0027 | 0.0130±0.0034 | 0.0220±0.0044 | 0.0170±0.0034 | 0.02 | 0.11 |
| **Cu** | 6.90±0.97 | 6.60±0.89 | 7.5±1.0 | 7.2±1.0 | 10 | 15 |
| **Zn** | 83±14 | 120±73 | 120.0±8.1 | 170±18 | 150 | 250 |

**TABLE 3. RATIO OF ESSENTIAL ELEMENTS IN CHILDREN OF DIFFERENT AGE GROUPS IN THE ARAL SEA REGION (mcg/g)**

| Proportion | Optimal | I gr. 1-3 y. o. n = 16 | II gr. 4-8 y. o. n = 14 | III gr. 9-14 y. o. n = 16 | IV gr. 15-18 y. o. n = 18 |
|---|---|---|---|---|---|
| Fe/Cu | 0.9 | 3.50±0.65 | 3.40±0.88 | 3.90±0.61 | 3.00±0.63 |
| Fe/Co | 400 | 1286±133 | 1470±120 | 1620±195 | 1060±205 |

lular membrane stability, and facilitates iron transport from tissues to the bone marrow. Copper deficiency can impair erythro- and granulopoiesis, leading to the development of hypochromic anemia and neutropenia. Additionally, copper plays a crucial role in the functioning of the antioxidant system, being a component of superoxide dismutase. By activating cytochrome oxidase, it participates in the maturation and stimulation of reticulocytes and other hematopoietic cells [4].

The inadequate supply of copper results in poor iron absorption, leading to a reduced iron reserve in the depots and a decline in serum iron levels [11, 13]. Copper deficiency hampers the absorption and utilization of iron, thereby reducing the lifespan of erythrocytes. Previous studies have demonstrated the effectiveness of copper in treating anemia and investigated the potential mechanisms of copper's action on iron metabolism and its absorption in the intestine. These investigations continue today, utilizing modern molecular biology and genetics to further develop our understanding of these questions. In summary, there are copper-dependent factors in the hematopoietic system that promote iron absorption and erythropoiesis.

It is known that cobalt participates in many processes in the body, contributing to the production of erythrocytes in the bone marrow and better absorption of iron. The mass fraction of cobalt is 4.5% in vitamin B12. By activating hematopoiesis, cobalt regulates the synthesis of heme from protoporphyrin and iron, stimulates the production of erythropoietin, activates bone marrow functions, and accelerates the maturation of erythrocytes, preventing the development of anemia. In the liver of animals, more than 40% of cobalt is bound to protein fractions. Cobalt can form compounds with the amino acids histidine and cysteine. In case of cobalt deficiency, animals may develop anemia and hypokobaltosis.

It has been established that zinc deficiency leads to the development of zinc-deficient anemia (ZDA). Zinc affects the absorption of metals in the intestine and competes with copper. Zinc-dependent anemias can lead to taste distortion and muscle hypotonia. By influencing the processes of nucleic acid synthesis, zinc affects hematopoiesis, participates in the transport of carbon dioxide to the lungs, and is part of the enzyme carbonic anhydrase, which is present in erythrocytes [4].

It has been demonstrated that vitamin C facilitates the reduction of trivalent iron to divalent iron through copper-dependent ferrireductase enzymes, which subsequently enter enterocytes via manganese-dependent proteins and are transported into the bloodstream through the ferroportin protein. Iron plays a vital role in immune function, participating in the synthesis of immunoglobulins, collagen, and porphyrins, which can affect the quantity and functional properties of T lymphocytes. In normal conditions, approximately 30% of transferrin is saturated with iron. Iron is also involved in the functioning of non-specific defense factors, cellular immunity, and local immunity. Normal iron levels are necessary for proper phagocytosis and high natural killer activity. Iron deficiency in children can result in increased susceptibility to respiratory and gastrointestinal infections [5]. It has been established that children treated with iron supplements for anemia experience a reduced incidence of respiratory and intestinal infections. Iron maintains normal proliferation and mitotic activity of T lymphocytes through the "ribonucleotide-reductase" system. Iron-containing enzymes regulate the expression of major histocompatibility complex class II surface antigens on T lymphocytes. It has also been found that $Fe2^{+}$ ions, but not $Fe3^{+}$ ions, exhibit cytotoxic effects. In iron homeostasis, 9 copper-containing enzymes and 22 manganese-dependent proteins are involved [12].

The conducted study, analysis, and discussion of the obtained results have shown that anemia detected in children living in the Aral Sea region is caused not only by a decrease in iron but also by a decrease in copper, cobalt, zinc, and manganese. Hair analysis for trace elements has diagnostic and therapeutic value in children. Each of the studied trace elements has a direct impact on the child's health and the development of various diseases, including anemia.

## Conclusion

Thus, the assessment of age-related physical development characteristics in children living in the environmentally disadvantaged region of the Aral Sea showed that it can be one of the sensitive, simplest, and most reliable criteria characterizing the state of

organism development. Environmental factors in the region likely led to a delay in the pubertal age of the children. The slowdown in the pace of physical development in children is due to the general delay in their growth and the development of micronutrient deficiencies resulting from the negative impact of unfavorable environmental factors in the region. The obtained data emphasize the importance of the impact of the environment and the role of elemental status in the development of the child's body.

Imbalances in micronutrients, as well as deficiencies in copper, cobalt, and zinc, contribute to the development of anemia in children. Therefore, investigating the factors that provoke the development of anemia and studying possible ways to correct it is a relevant problem for Karakalpakstan and other countries worldwide.

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# ОПРЕДЕЛЕНИЕ МЕДИАТОРОВ ФИБРОЗИРОВАНИЯ И АНГИОГЕНЕЗА В СЫВОРОТКЕ КРОВИ НЕДОНОШЕННЫХ ДЕТЕЙ С БРОНХОЛЕГОЧНОЙ ДИСПЛАЗИЕЙ

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**Резюме.** При преждевременных родах и послеродовом повреждении развивающегося легкого нарушаются процессы роста легочных сосудов и формирования легочных альвеол, приводящие к формированию бронхолегочной дисплазии (БЛД). БЛД является многофакторным заболеванием, патогенез поражения тканей легкого до сих пор остается не до конца изученным. Исследования биомаркеров ангиогенеза могут быть информативны для оценки развития БЛД. Цель работы – определить уровень биомаркеров ангиогенеза в течении бронхолегочной дисплазии у недоношенных детей для совершенствования ранней диагностики БЛД.

Исследована сыворотка крови 65 недоношенных детей в возрасте от 6 до 180 дней жизни. При рождении гестационный возраст составлял от 23 до 33 недель, масса тела от 480 до 1840 г, оценка по APGAR 5-6. Все дети в раннем неонатальном периоде перенесли респираторный дистресс-синдром, после которого 46 детей сформировали и 19 не сформировали БЛД. Методом иммуноферментного анализа проведено определение концентрации комплекса факторов ангиогенеза и фиброзирования.

Не выявлено значимых различий в уровнях ангиопоэтинов 1 и 2, фактора роста сосудистого эндотелия VEGF-D, трансформирующего фактора роста бета TGF-β, тромбоспондина-1. Отмечена тенденция к повышению уровня фактора роста сосудистого эндотелия VEGF-A, являющегося регулятором ангиогенеза и созревания легких; нарушение его синтеза может привести к долгосрочному повреждению паренхимы легких. Тенденцию к повышению уровня VEGF-A у детей с БЛД мы расцениваем как благоприятный признак положительной динамики заболевания. Выявлены тенденции к повышению концентрации молекулы адгезии эндотелиальных клеток тромбоцитов PECAM-1, интерлейкина-8, фактора роста соединительной ткани CTGF. Экспрессия CTGF усиливается искусственной вентиляцией легких и воздействием высоких концентраций кислорода. Повышение уровня CTGF у детей с БЛД мы считаем неблагоприятным изменением, так как связывание CTGF с VEGF снижает доступность VEGF для его рецепторов, ингибируя индуцированный VEGF ангиогенез. У детей с БЛД отмечено статистически достоверное снижение уровня тромбоцитарного фактора роста PDGF-BB, медиана составила у детей с БЛД 3180 пг/мл против 4782 пг/мл у детей без БЛД ($p = 0,024$). PDGF является важным фактором регенерации тканей, рецепторы к нему имеются на фибробластах

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и гладкомышечные клетках сосудистой стенки. Стимулируя их пролиферацию, PDGF играет важную роль в формировании кровеносных сосудов.

Наиболее выраженным изменением у детей с БЛД явилось снижение уровня PDGF, которое может приводить к нарушению альвеоляризации, необходимой для формирования структуры здоровых легких. Исследования факторов ангиогенеза помогут лучше понять патогенез поражения легких при БЛД.

*Ключевые слова: бронхолегочная дисплазия, медиаторы ангиогенеза, PDGF, CTGF, VEGF, PECAM, недоношенные дети*

# EVALUATION OF MEDIATORS OF FIBROSIS AND ANGIOGENESIS IN THE BLOOD SERUM OF PREMATURE INFANTS WITH BRONCHOPULMONARY DYSPLASIA

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**Abstract.** In premature birth and postpartum damage to the developing lung, the processes of the formation of pulmonary vessels and alveoli are disrupted, leading to bronchopulmonary dysplasia (BPD). BPD is a multifactorial disease and the pathogenesis of lung tissue damage is still not fully understood. Studies of angiogenesis biomarkers can be informative for assessing the development of BPD. In this study we examined the blood serum of 65 premature infants aged 6 to 180 days of life; gestational age at birth was 23-33 weeks, body weight 480-1840 g, APGAR score 5-6. All children in the early neonatal period had respiratory distress syndrome, then 46 children formed and 19 did not form bronchopulmonary dysplasia. The concentration of the factors of angiogenesis and fibrosis was determined in blood serum by ELISA. There were no differences in the levels of angiopoietins 1 and 2, vascular endothelial growth factor VEGF-D, transforming growth factor beta TGF-β, thrombospondin-1. We observed a tendency to increasing the level of VEGF-A, which is a key regulator of angiogenesis and lung maturation; we regard this tendency as a favorable sign of lung formation. We found tendencies to increase of the adhesion molecule of endothelial platelet cells PECAM-1, interleukin 8 and connective tissue growth factor CTGF. CTGF expression is enhanced by artificial lung ventilation and exposure to high oxygen concentrations. We consider an increase of CTGF in BPD to be an unfavorable change, since the binding of CTGF to VEGF inhibits VEGF-induced angiogenesis. In children with BPD, we found a decrease in the level of platelet derived growth factor PDGF-BB, the median concentration was 3180 pg/mL in BPD *versus* 4782 pg/mL without BPD ($p = 0.024$). PDGF is an important factor in tissue regeneration and plays an important role in the formation of blood vessels. We assume the decreasing of PDGF concentration in BPD can lead to a violation of the alveolarization necessary for the formation of the structure of healthy lungs. Studies of angiogenesis factors will help to better understand the pathogenesis of lung damage in BPD.

*Keywords: bronchopulmonary dysplasia, mediators of angiogenesis, PDGF, CTGF, VEGF, PECAM, premature infants*

## Introduction

Bronchopulmonary dysplasia (BPD) is a chronic lung disease that occurs in premature infants who required intensive respiratory support and high oxygen concentration therapy at birth. The main clinical manifestations of BPD are respiratory insufficiency, prolonged need for additional oxygen, intolerance to physical exertion, and the formation of pulmonary hypertension [7]. BPD is a multifactorial disease, the pathogenesis of which is still not fully understood [12]. With premature birth and postpartum damage to the developing lung, the processes of pulmonary vascular growth and the formation of pulmonary alveoli are disrupted [5, 12]. It is assumed that studies of angiogenesis biomarkers can be informative for assessing the development of BPD [13], however, the question of the diagnostic and prognostic significance of early changes in their levels requires further research.

The purpose of the study was to determine the level of some important biomarkers of angiogenesis in the formation and course of bronchopulmonary dysplasia in premature infants to improve the early diagnosis of this disease. Materials and methods of research: the work was carried out in 2022 at the Federal State Autonomous Institution "NMIC of Children's Health" of the Ministry of Health of the Russian Federation in the Laboratory of experimental immunology and virology (head of the department, PhD N.M. Alyabieva), the Laboratory of neonatology

and early childhood health problems (head of the department, PhD, MD I.V. Davydova), and the Department of Pathology of Newborns and infants (head of the department, PhD M.A. Basargina).

The study included 65 premature infants who were hospitalized in the Department of pathology of newborns and infants FSAU "NMIC of Children's Health" and in the department of resuscitation and intensive care of newborns of the GBUZ MO "MOPC" Balashikha. The age at the point of examination was 6 to 180 days of life. There were 23 children (35%) at the stage of respiratory distress syndrome of newborns (up to 28 days of life); and 42 children (65%) older than 28 days were examined. When analyzing the data, 2 groups were formed: children who formed BPD – 46 patients (71%) and those who did not form – 19 children (29%). The gestation period at birth of patients ranged from 23 to 33 weeks, including 32 children (49%) had gestation periods from 23 to 27.6 weeks and 33 children (51%) had gestation periods from 28 to 33 weeks. The birth weight ranged from 480 to 1840 grams (the average 1011±320 g). 43 children (66%) had an extremely low birth weight, 14 children (22%) had a very low birth weight, and 8 children (12%) had a low birth weight. The patients were examined according to clinical indications, taking an acceptable volume of blood (no more than 1 mL, taking into account the child's body weight). To determine the factors of angiogenesis, a residual amount of serum was used after performing a biochemical analysis in the amount of 100-150 μL. The work was approved by the local independent ethical committee of the FSAU "NMIC of Children's Health" of the Ministry of Health of the Russian Federation.

A complex of the most interesting factors from a modern point of view was selected for the study: angiopoietin 1 (ANGPT1), angiopoietin 2 (ANGPT2), vascular endothelial growth factor A (VEGF-A), vascular endothelial growth factor D (VEGF-D), platelet endothelial cell adhesion molecule (PECAM-1), thrombospondin 1 (TSP1), transforming growth factor beta-1 (TGF-β1), platelet derived growth factor BB (PDGF-BB), interleukin-8 (IL-8), and connective tissue growth factor (CTGF). The study was conducted by enzyme immunoassay. Aviscera Bioscience kits were used for CTGF, R&D Systems for thrombospondin, and for all other analytes – manufactured by RayBiotech (all – USA).

## Results and discussion

When analyzing anamnestic factors, we confirmed known patterns: children with BPD compared to children without BPD had lower gestational age at birth (median 27 weeks *versus* 30), lower birth weight (920 g *versus* 1150 g), lower APGAR score (5 *vs* 6 at 1 minute and 6 *vs* 7 at 5 minute), as well as a significantly longer period artificial lung ventilation (13 days *vs* 3 days). There were no diagnostically significant changes in the number of leukocytes and leukocyte formula at the time of the examination; all indicators were within the age norm. At the time of the study, children with BPD had a slightly higher level of C-reactive protein – the median concentration in children with BPD was 0.825 mg/L, without BPD – 0.5 mg/L. This indicates the absence of laboratory signs of inflammatory reactions at the time of examination. The median concentrations of the angiogenesis factors determined are shown in Table 1.

Discussion of the results obtained: To date, it has been established that with premature birth and postpartum damage to the developing lung, the processes of growth of pulmonary vessels and the formation of pulmonary alveoli are disrupted [5, 7, 12]. The interaction of multidirectional factors seems to be important in the pathogenesis of BPD formation [12, 13]. Angiopoietins are important modulators of physiological and pathological neovascularization of the lungs [13]. It is known that experimental management of VEGF and ANGPT1 in laboratory animals stimulated lung growth and vascular maturation more effectively than VEGF therapy alone; the interaction of ANGPT2 with VEGF stimulated angiogenesis in hypoxia, while in the absence of sufficiently strong pro-angiogenic signals, ANGPT2 can cause endothelial cell death and vascular regression [14]. In our study, were no significant differences in the levels of angiopoietin 1 and 2 in children who formed BPD was slightly lower than in those who did not form.

VEGF has been identified as a key regulator of lung angiogenesis and maturation due to the coordination of the branching of the respiratory tract and microvascular bed, a violation of its synthesis can lead to long-term damage to the lung parenchyma. A decrease in VEGF levels in tracheal aspirates has been described in premature newborns born at 28-29 weeks of gestation who later developed BPD [2]. In the cohort we examined, the median concentration of VEGF-D in both groups of children was almost equal. However, the median concentration of VEGF-A in children with BPD was higher than in children without BPD – 109 and 78 pg/mL, respectively; this trend is statistically unreliable, but we can evaluate the data as a trend. A tendency to increase the level of vascular endothelial growth factor VEGF-A can be important for BPD pathogenesis. The violation of VEGF-A synthesis can lead to long-term damage to the lung parenchyma, and we regard the tendency to increase the level of VEGF-A in the examined cohort of children with BPD as a favorable sign of positive dynamics of the disease.

Thrombospondin-1 is an extracellular matrix glycoprotein that has an antiangiogenic effect. It was shown that the level of thrombospondin-1 in the lung tissue of premature newborns who were on a ventilator was 5.5 times higher than in the lungs of children of the same gestational age who did not have a

**TABLE 1. CONCENTRATIONS OF MEDIATORS OF ANGIOGENESIS AND FIBROSIS IN THE BLOOD SERUM OF PREMATURE INFANTS WITH AND WITHOUT BPD, Me ($Q_{0.25}$-$Q_{0.75}$)**

| Mediator | BPD | No BPD | p |
|---|---|---|---|
| **ANGPT1, pg/mL** | 11550 (7172-14037) | 13300 (7537-19004) | p = 0.60 |
| **ANGPT2, pg/mL** | 7019 (5726-8700) | 6624 (5877-9305) | p = 0.81 |
| **VEGF-A, pg/mL** | 109 (48-197) | 78 (48-235) | p = 0.54 |
| **VEGF-D, ng/mL** | 1.8 (1.5-2.4) | 1.9 (1.7-2.2) | p = 0.87 |
| **TSP1, ng/mL** | 25987 (16778-39173) | 21022 (16323-40144) | p = 0.94 |
| **TGF-β1, pg/mL** | 132412 (119206-148262) | 136544 (117180-160888) | p = 0.56 |
| **IL-8, pg/mL** | 62 (38-176) | 48 (23-265) | p = 0.38 |
| **PECAM-1, pg/mL** | 3624 (1413-4845) | 2724 (1171-3565) | p = 0.24 |
| **CTGF, pg/mL** | 167 (107-230) | 117 (72-192) | p = 0.13 |
| **PDGF-BB, pg/mL** | 3180 (2503-5016) | 4783 (3372-6170) | p = 0.024 |

ventilator [11]. In our study, the median concentration of thrombospondin-1 had no significant differences.

One of the well-known regulators of cell growth is TGF-β, which mediates interactions between cells and the extracellular matrix. Its level has been shown to increase with damage and inflammation in lung tissue; at the same time, the authors emphasize that TGF-β has antiangiogenic properties, inhibits inflammatory processes, is a mediator of lung repair and tissue remodeling [8]. There were no differences in the concentration of TGF-β in the groups of children we examined.

The role of inflammatory markers such as interleukin 6, 8, 10, tumor necrosis factor in the development of bronchopulmonary dysplasia has been proven in many studies [4]. Interleukin 8, a neutrophil migration factor, is one of the main pro-inflammatory chemokines produced by macrophages. In a published study in 2022, when assessing the levels of IL-6 and IL-8 in the blood serum of premature infants during the first week of life, it was shown that children with higher levels of these biomarkers were more often diagnosed with BPD in the first week of life [4]. We determined the median concentration of IL-8 in children with BPD of 62 pg/mL *versus* 48 pg/mL in children without BPD, so we can suppose a trend to an elevation in BPD, but the trend has not statistical significance.

PECAM-1 is a glycoprotein, a membrane protein from the immunoglobulin superfamily, belonging to the class of cell adhesion molecules. In infants with BPD, a decrease in the expression of VEGF and PECAM has been described, as well as a decrease in the staining density of alveolar capillaries, which is regarded as evidence of impaired lung development and the development of the pulmonary microcirculatory network [3]. In the groups of children we compared, the median concentrations of PECAM-1 were 3624 pg/mL in children with BPD and 2724 pg/mL without BPD. The differences are statistically unreliable, however, from our point of view, the data can be regarded as a definite trend towards an increase in the level of BPD.

As part of the scientific work carried out at the FSAU “Children’s Health Research Center” of the Ministry of Health of the Russian Federation to determine the clinical and genetic features of the development of a new form of BPD in premature infants, full-exome sequencing was performed in 100 patients with BPD. As a result, 8 genetic variants significant for the pathogenesis of BPD were selected, in particular, the important role of the CTGF gene was established [1]. The molecular structure of CTGF allows it to interact with various growth factors – TGF , VEGF, etc. [10]. The important role of CTGF in the pathogenesis of various forms of pulmonary fibrosis and vascular diseases, including BPD, has been shown in many studies [6, 10, 15].

CTGF expression has been shown to be enhanced by artificial lung ventilation and exposure to high oxygen concentrations. Binding of CTGF to TGF-β provides dimerization of TGF-β with its receptors, thus facilitating the transmission of TGF-β signals [2]. On the contrary, binding of CTGF to VEGF reduces

the availability of VEGF for its receptors, inhibiting VEGF-induced angiogenesis [6]. We obtained data on a higher concentration of CTGF in children with BPD: the median was 167 pg/mL check units with an indicator of 117 pg/mL in children without BPD. The trend was not statistically reliable due to large data spread (p = 0,13); perhaps a larger volume of observations will give more reliable results. We consider an increase in CTGF levels in children with BPD to be an unfavorable change, since the binding of CTGF to VEGF reduces the availability of VEGF for its receptors, inhibiting VEGF-induced angiogenesis.

An important stimulator of tissue repair is PDGF, which is contained in the -granules of platelets. PDGF receptors have fibroblasts and smooth muscle cells of the vascular wall. By stimulating their proliferation, PDGF plays an important role in the formation of blood vessels. There is evidence that the PDGF level decreases with BPD, which leads to a violation of the alveolarization necessary for the formation of the structure of healthy lungs [8]. However, in other studies, when determining the levels of PDGF-AA and PDGF-BB in the aspiration fluid of the trachea, no differences were obtained between aspirates from children who developed BPD, compared with aspirates from children who did not develop BPD [10]. In the children with BPD examined by us, the median concentration of PDGF-BB was 3180 pg/mL, and in children without BPD – 4783 pg/mL (p = 0.024). The diagram of PDGF-BB concentration comparison is shown in Figure 1.

We assume the decreasing of PDGF concentration in infants with bronchopulmonary dysplasia can lead to a violation of alveolarization necessary for the formation of the structure of healthy lungs. Studies of angiogenesis factors will help to better understand the pathogenetic mechanisms of lung damage in BPD.

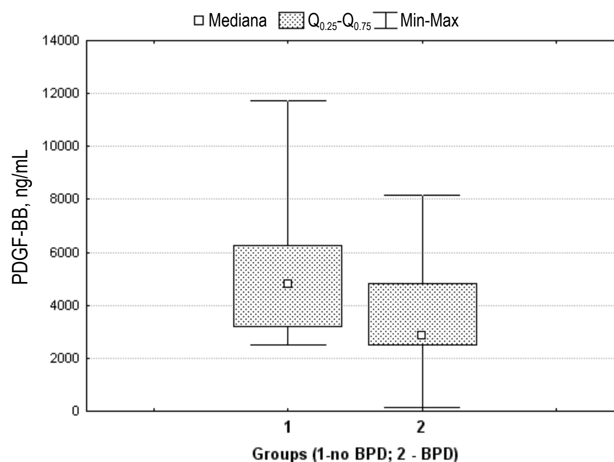


**Figure 1. Medians and spread of serum levels of PDGF-BB in infants with and without BPD**

This study, conducted in limited groups of patients, revealed some trends. In this regard, we consider it appropriate to further study angiogenesis factors on more extensive and more diverse groups of patients, since it is these biomarkers that can help predict respiratory diseases in premature newborns during their initial hospitalization. Understanding the interaction of growth factors, transcription factors and inflammatory processes that regulate the normal development of the parenchyma and microvascular bed of the lungs, as well as their role in the pathogenesis of BPD, can help in the development of new treatment methods aimed at stimulating proper alveologenesis and angiogenesis, as well as the prevention of pulmonary hypertension in premature infants.

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# ЭКСПРЕССИЯ МОЛЕКУЛ CD56 И TIM-3 НА РАЗНЫХ СУБПОПУЛЯЦИЯХ МОНОЦИТОВ ПЕРИФЕРИЧЕСКОЙ КРОВИ ПРИ БЕРЕМЕННОСТИ

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**Резюме.** Моноциты периферической крови играют важную роль в защите организма от патогенов и участвуют в поддержании физиологической беременности. Периферические моноциты мигрируют в децидуальную оболочку и образуют пул децидуальных макрофагов, которые участвуют в формировании и развитии тканей плаценты. Функции моноцитов периферической крови также существенно меняются, что связано с системным изменением иммунореактивности при беременности. Популяция моноцитов периферической крови фенотипически и функционально неоднородна. Выделяют несколько субпопуляций моноцитов в зависимости от экспрессии CD14 и CD16. Также в периферической крови присутствуют CD56-позитивные и Tim-3 (Т-клеточного Ig и белка 3, содержащего домен муцина) – экспрессирующие моноциты. CD56 и Tim-3 играют важную роль в регуляции функциональной активности моноцитов. Однако изменение их экспрессии на разных субпопуляциях моноцитов периферической крови при беременности остается малоизученным. Поэтому целью исследования являлось изучение экспрессии CD56 и Tim-3 разными субпопуляциями моноцитов человека при беременности. Мононуклеарные клетки выделяли из периферической крови беременных женщин (срок беременности 29 недель (28-31) путем центрифугирования в градиенте плотности и анализировали методом проточной цитометрии. Группу сравнения составляли здоровые небеременные женщины (в фолликулярной фазе менструального цикла) фертильного возраста (21-29 лет). Установлено, что беременные женщины имели более низкий процент классических $CD14^{hi}/CD16^{-}$моноцитов в периферической крови по сравнению с небеременными. Процентное содержание промежуточных ($CD14^{hi}/CD16^{+}$) и неклассических ($CD14^{low}/CD16^{+}$) моноцитов не отличалось от небеременных. Экспрессия молекулы CD56 обнаруживалась всех субпопуляциях моноцитов как у беременных, так и у небеременных женщин. Беременные женщины имели более высокий процент CD56-позитивных классических ($CD14^{hi}CD16^{-}$) и неклассических ($CD14^{low}CD16^{+}$) моноцитов, чем небеременные. Процент CD56-позитивных промежуточных моноцитов ($CD14^{hi}CD16^{+}$) не отличался от неберемен-

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ных. У беременных женщин процентное содержание дубльпозитивных $CD56^+Tim\text{-}3^+$ классических ($CD14^{hi}CD16^-$) и неклассических ($CD14^{low}CD16^+$) моноцитов было выше, чем у небеременных. Количество $CD56^+Tim\text{-}3^+$ промежуточных моноцитов ($CD14^{hi}CD16^+$) не отличалось у беременных и небеременных. Таким образом, при физиологической беременности экспрессия молекул CD56 и Tim-3 меняется на разных субпопуляциях моноцитов периферической крови.

*Ключевые слова: классические моноциты, неклассические моноциты, промежуточные моноциты, CD56, Tim-3, периферическая кровь, беременность*

# CD56 AND TIM-3 MOLECULE EXPRESSION IN DIFFERENT MONOCYTE SUBSETS IN PHYSIOLOGICAL PREGNANCY

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**Abstract.** Monocytes play an important role in the systemic immune defense against pathogens and maintaining physiological pregnancy. During pregnancy peripheral monocytes migrate into the decidua and form the pool of decidual macrophages which participate in the formation and development of placental tissues. The population of peripheral blood monocytes is phenotypically and functionally heterogeneous. In humans, there are different monocyte subsets depending on the expression of CD14 and CD16. CD56-positive monocytes are found in healthy women. Their number is positively correlated with body mass index, body fat. Tim-3 (T cell Ig and mucin domain-containing protein 3) expression is observed in peripheral monocytes during pregnancy. It is known that peripheral monocyte functions effectively change at pregnancy to form the immune tolerance at the maternal-fetal interface and the systemic immune defense against pathogens. However, the monocyte phenotype shift during pregnancy remain poorly understood. Therefore, the aim of the study was to evaluate the CD56 and Tim-3 expressions in monocyte subsets in human pregnancy. Peripheral blood mononuclear cells were isolated from peripheral blood of pregnant women (gestational age 29 weeks (28-31) by density gradient centrifugation and analyzed by flow cytometry. Peripheral blood of healthy non-pregnant fertile women (in follicular phase of the menstrual cycle) aged 21-29 years was studied as control. Pregnant women had a lower percentage of classical $CD14^{hi}/CD16^-$ monocytes in comparison with non-pregnant. The percentages of intermediate ($CD14^{hi}/CD16^+$) and non-classical ($CD14^{low}/CD16^+$) monocytes did not change. The CD56 molecule expression was observed in all monocyte subsets in pregnant and non-pregnant women. Pregnant women had a higher percentage of CD56-positive classical ($CD14^{hi}CD16^-$) and non-classical ($CD14^{low}CD16^+$) monocytes than non-pregnant. The percentage of CD56-positive intermediate ($CD14^{hi}CD16^+$) monocytes did not change. The percentages of double-positive $CD56^+Tim\text{-}3^+$ classical ($CD14^{hi}CD16^-$) and non-classical ($CD14^{low}CD16^+$) monocytes were increased in pregnant women. The numbers of double-positive $CD56^+Tim\text{-}3^+$intermediate ($CD14^{hi}CD16^+$) monocytes did not change. Thus, the CD56 and Tim-3 expressions in different monocyte subsets were changed in human pregnancy.

*Keywords: classical monocytes, non-classical monocytes, intermediate monocytes, CD56, Tim-3, peripheral blood, pregnancy*

This study was carried out within the framework of a state task: state topic registration number: AAAA-A19-119112290007-7.

## Introduction

Monocytes play an important role in the systemic immune defense against pathogens and maintaining physiological pregnancy [2]. Monocytes originate in the bone marrow and circulate in the peripheral blood. Monocytes phagocytose, produce cytokines and present antigens to na ve lymphocytes [2, 7]. During pregnancy peripheral monocytes migrate into the decidua and form the pool of decidual macrophages which since with natural killer cells participate in the

formation and development of placental tissues [2, 6]. In humans, there are two main monocyte subpopulations depending on the expression of CD14 and CD16: classical ($CD14^{hi}CD16^{-}$), non-classical ($CD14^{low}CD16^{+}$), and intermediate subpopulation ($CD14^{hi}CD16^{+}$) [7, 10]. CD14 is a pattern recognition receptor and was first identified as a marker of monocytes to initiate intracellular responses to bacterial antigens [2]. CD16 is the Fc RIII receptor responsible for antibody-dependent phagocytic activity [2].

Monocytes are able to differentiate into many cell types. Classical monocytes are the main sources of the macrophage pool in tissues [7, 10]. Only a minor proportion of classical monocytes differentiates into intermediate, and most of the intermediate monocytes finally mature into non-classical monocytes [7, 10]. Classical monocytes are considered mature; they show pronounced phagocytic activity and are capable of producing reactive oxygen species and cytokines through activation of toll like receptors signaling pathway [7, 10]. Non-classical monocytes do not produce reactive oxygen species but are better at production of pro-inflammatory cytokines [7, 10]. Non-classical monocytes patrol the surface of the endothelium and infiltrate tissues under normal state and during inflammation [7, 10]. Non-classical monocytes are involved in resolving inflammation and restoring the tissue and releasing cytokines [7, 10]. The intermediate monocyte role is poorly understood, but given the high expression level of MHC-II they probably participate in antigen presentation and activation of T lymphocytes [5, 7, 10]. It is known that peripheral monocyte functions effectively change at pregnancy to form the immune tolerance at the maternal-fetal interface and the systemic immune defense against pathogens [11]. However, the monocyte phenotype shift during pregnancy remains poorly understood.

CD56-positive monocytes are found in low frequencies in the peripheral blood of healthy individuals [3, 4]. Their number is expanded in obesity, autoimmune diseases and correlated positively with body mass index, body fat, C-reactive protein [3]. The $CD56^{+}$ monocyte characteristics are controversial now. Some authors note effective production of reactive oxygen intermediates and pro-inflammatory cytokines by $CD56^{+}$ monocytes, and are more efficient antigen-presenting function or dysregulated cytokine response to inflammatory stimuli [3, 4]. There are not $CD56^{+}$ monocyte characteristics at physiological pregnancy.

Tim-3 (T cell Ig and mucin domain-containing protein 3) molecule plays critical role in function regulation of innate and adaptive immune cells during pregnancy [11]. Tim-3 expressions are observed in peripheral monocytes during pregnancy [11]. However, the Tim-3 expression in different peripheral blood monocyte subsets during physiological pregnancy are not elucidated. The aim of the study was to evaluate the occurrence of CD56 and Tim-3 expression in monocyte subsets in human pregnancy.

## Materials and methods

Peripheral blood of healthy pregnant women in third trimester (gestational age 29 weeks (28-31) aged 21-29 years was studied (n = 7). Peripheral blood of healthy non-pregnant fertile women (in follicular phase of the menstrual cycle) aged 21-29 years was studied as control (n = 7). The inclusion criteria were the absence of acute and chronic somatic, endocrine, autoimmune, genetic diseases; compliance with diet, treatment with contraceptive and hormonal, anti-inflammatory or antibacterial drugs. This study was approved by the local ethics committee of the Institute of Ecology and Genetics of Microorganisms of the Ural Branch of the Russian Academy of Sciences in accordance with the Helsinki Declaration. Written informed consent was received from all participants.

Peripheral blood samples were collected in sodium heparin vacutainer tubes. Peripheral blood mononuclear cells (PBMC) were obtained from peripheral blood by ficoll-verografin (1.077 $g/cm^3$) density gradient centrifugation. PBMC was collected for further flow cytometry analysis.

Monocytes were harvested for flow cytometry using the following antibodies: CD14 (PE anti-human CD14, clone ME5E2, “BioLegend”, UK), CD16 (FITC anti-human CD16, clone 3G8, “BioLegend”, UK), CD3 (APC/Cy7 anti-human CD3, clone UCHT1, “BioLegend”, UK), CD56 (Brilliant Violet 605™ anti-human CD56 (NCAM), clone HCD56, “BioLegend”, UK), CD366 (APC anti-human CD366 (Tim-3), clone F38-2E2, “BioLegend”, UK), isotype controls (APC Mouse IgG1, Isotype Ctrl, “BioLegend”, UK; Brilliant Violet 605™ Mouse IgG1, Isotype Ctrl “BioLegend”, UK). Cells were labeled with Zombie (Zombie UV™ Fixable Viability Kit, BioLegend) to assess viability. Gating strategy was presented in Figure 1 (see 2nd page of cover). Flow cytometry was performed on a CytoFlex S flow cytometer using CytExpert and Kaluza 1.5 software (Beckman Coulter, USA).

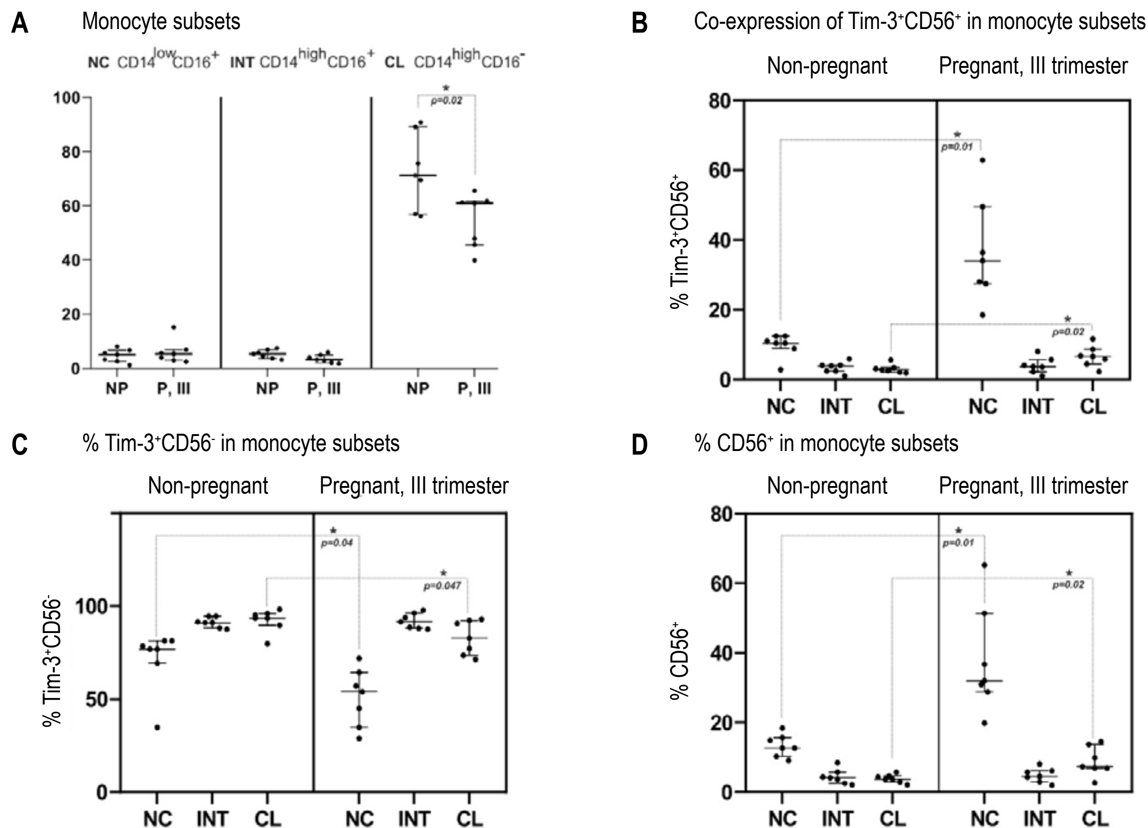


**Figure 2. Assessment of monocyte subsets and Tim-3 and CD56 expression**

Note. (A) Assessment of monocyte subsets (NC, INT, CL) in non-pregnant (NP) and pregnant women, 3rd trimester (P, III). (B) Percentage of co-expressions of Tim-3 and CD56 (Tim-3+CD56+) (C) (Tim-3+CD56-) and (D) (CD56-) in monocyte subsets in non-pregnant (NP) and pregnant women, 3rd trimester (P, III). Data are presented as median and the lower and upper quartiles, Me ($Q_{0.25}$-$Q_{0.75}$); *, p value by two-tailed unpaired t-test in corresponding subsets in NP and (P, III) groups.

The data were presented as median and the lower and upper quartiles, Me ($Q_{0.25}$-$Q_{0.75}$). Statistical analyses were performed using “GraphPad Prism version 8.01” (StatSoft, USA). The Kolmogorov-Smirnov test was used for verifying normal distribution. The significance of the difference between two groups was determined using the two-tailed unpaired t-test. The differences were considered as significant at $p < 0.05$.

## Results and discussion

To investigate the subsets of monocytes in peripheral blood of pregnant women, PBMC were isolated from peripheral blood and analyzed by flow cytometry. Three subpopulations of monocytes in peripheral blood of pregnant and non-pregnant women: classical ($CD14^{hi}CD16^-$), non-classical ($CD14^{low}CD16^+$), and intermediate subpopulation ($CD14^{hi}CD16^+$) were identified according to the literature [7, 10]. Classical monocytes were the predominant subpopulation in both pregnant and non-pregnant women (Figure 1 (see 2nd page of cover), 2A). Pregnant women had a lower percentage of classical $CD14^{hi}/CD16^-$ monocytes in comparison with non-pregnant (Figure 2A). The percentages of intermediate ($CD14^{hi}/CD16^+$) and non-classical ($CD14^{low}/CD16^+$) monocytes did not change in pregnant women in comparison with non-pregnant (Figure 2A).

The obtained results are in accordance with the data of other authors [2]. It is known that a minor proportion of classical monocytes matures into intermediate monocytes and subsequently into non-classical monocytes [7]. The majority of classical monocytes transform in tissue macrophages [2]. Therefore, the decrease in the number of classical monocytes can be explained by their migration into tissues at pregnancy and maturation in macrophages [2]. The data about monocyte subset changes in peripheral blood at pregnancy are controversial, which may reflect the

influence of methods used for monocyte isolation, gating strategy, gestational ages [5].

The CD56 molecule expression was observed in all monocyte subsets in pregnant and non-pregnant women (Figure 2D). The obtained results are in accordance with the data of other authors [3, 4]. Pregnant women had a higher percentage of CD56-positive classical ($CD14^{hi}CD16^{-}$) and non-classical ($CD14^{low/-}CD16^{+}$) monocytes than non-pregnant. The percentage of CD56-positive intermediate ($CD14^{hi}CD16^{+}$) monocytes did not changed compared non-pregnant women. It is established that monocytes have intensive adhesion to endothelium due to high expression of adhesion molecules (CD11a, CD11b, CD11c, CD29) during physiological pregnancy [6]. CD56 (neural cell adhesion molecule) plays an important role in the recruitment of monocytes into the tissues [3]. Therefore, it may be supposed that CD56 high expression in monocytes explained the mechanism of transendothelial migration of monocytes during physiological pregnancy. Additionally, there were strong associations between the number of $CD56^{+}$ classical monocytes and fat mass increase in human [3], which is also associated with late pregnancy.

The coexpression of CD56 and Tim-3 molecules were determined in all monocyte subsets in pregnant and non-pregnant women (Figure 2B). It was shown that the percentages of double-positive $CD56^{+}Tim\text{-}3^{+}$ classical ($CD14^{hi}CD16^{-}$) and non-classical ($CD14^{low}CD16^{+}$) monocytes were increased at third trimester of pregnancy. The numbers of double-positive $CD56^{+}Tim\text{-}3^{+}$intermediate ($CD14^{hi}CD16^{+}$) monocytes did not change. The percentages of Tim-3-positive classical ($CD56^{-}CD14^{hi}CD16^{-}$) and non-classical ($CD56^{-}CD14^{low}CD16^{+}$) monocytes was decreased at third trimester of pregnancy (Figure 2C). The numbers of Tim-3-positive intermediate ($CD56^{-}CD14^{hi}CD16^{+}$) monocytes did not change. According to the literature, Tim-3 signaling effectively stimulates the functional activity of innate immune cells to maintain the systemic immune defense against pathogens [1, 11]. Some authors had reported the participation of Tim-3 signaling in monocyte phagocytic activity stimulation [1]. There are no studies about Tim-3 expression on different monocyte subsets during physiological pregnancy. It may be supposed that the changes in CD56 and Tim-3 expression in different monocyte subsets occurring in the third trimester of physiological pregnancy are important in their function regulation.

## Conclusion

Thus, the CD56 and Tim-3 expressions in different monocyte subsets were changed in human pregnancy. The obtained results are important for understanding the underlying mechanism of immune dysfunctions during pregnancy and could have significant value in treatment of reproductive disorders associated with monocyte dysfunctions.

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# АНАЛИЗ ФАКТОРОВ, ОТРАЖАЮЩИХ РАЗВИТИЕ СТЕРИЛЬНОГО ВОСПАЛЕНИЯ, НА ФОНЕ РАЗЛИЧНЫХ СХЕМ ГИПОТЕНЗИВНОЙ ТЕРАПИИ У БЕРЕМЕННЫХ С ПРЕЭКЛАМПСИЕЙ

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**Резюме.** Считается, что системное воспаление и эндотелиальная дисфункция играют значимую роль в патогенезе ПЭ. Оценить эндотелиальную дисфункцию возможно по степени поражения эндотелиального гликокаликса (эГК). эГК представляет собой поверхностный слой клеток, связанных с эндотелиальной мембраной, и обеспечивает все функции эндотелиальной клетки. Его повреждение можно оценить по уровню циркулирующих его компонентов в материнской крови. Пациентки с ПЭ в основном получают антигипертензивную терапию в объеме только метилдопы («Допегит») или в комбинации с нифедипином («Кордафлекс»). И до сих пор нет данных о влиянии данных препаратов на провоспалительный фон сосудов. Целью нашего исследования было определение уровней IL-6, IL-18, TNFα, галектина-3, гомоцистеина и синдекана-1 (структурного компонента эГК), отражающих развитие системного воспалительного ответа и эндотелиальной дисфункции в крови женщин с ранней и поздней ПЭ, получающих разные схемы антигипертензивной терапии. В данное интервенционное продольное пилотное исследование вошли 82 пациентки. Все пациентки были подобраны методом подбора пар с учетом срока беременности и ИМТ. Группу сравнения составили 15 пациенток до 34 недель и 15 – после 34 недель беременности. Опытная подгруппа 1 состояла из 12 пациенток с ранней ПЭ, получающих только «Допегит», и 16 пациенток с ранней ПЭ, получающих «Допегит» совместно с «Кордафлексом». Опытная подгруппа 2 включала 12 пациенток с поздней ПЭ, получающих «Допегит», и 12 пациенток с поздней ПЭ на комбинированной терапии. В результате исследования оказалось, что уровень только IL-6 был статистически значимо выше у пациенток с ранней ПЭ вне зависимости от типа лечения. Провоспалительный фон был более выражен при поздней ПЭ. Уровень IL-6 был значимо повышен у пациенток с поздней ПЭ на монотерапии «Допегитом». Уровни IL-6 и TNFα были значимо выше у пациенток, получающих «Допегит» + «Кордафлекс» в сравнении

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с контролем. Уровень синдекана-1 был значимо повышен у пациенток с ранней ПЭ, получающих только «Допегит». Не было выявлено статистически значимых различий в уровне синдекана-1 между группами при поздней ПЭ несмотря на его статистически незначимо повышенные уровни у данных пациенток. Уровни галектина-3 и гомоцистеина также значимо не различались между группами, что свидетельствует об отсутствии выраженной воспалительной реакции и эндотелиальной дисфункции у пациенток с ПЭ.

*Ключевые слова: преэклампсия, эндотелиальный гликокаликс, эндотелиальная дисфункция, воспаление, антигипертензивные препараты*

# ANALYSIS OF FACTORS ASSOCIATED WITH STERILE INFLAMMATION IN WOMEN WITH PE RECEIVING DIFFERENT ANTIHYPERTENSIVE TREATMENT STRATEGIES

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**Abstract.** Systemic inflammation alongside endothelial dysfunction is considered to play a crucial role in PE pathogenesis. Endothelial dysfunction can be assessed by endothelial glycocalyx (eGC) damage. eGC is a superficial layer of cells associated with endothelial membrane that provides all endothelial cells functions. Its damage can be evaluated by the levels of its circulating components in blood. Patients with PE generally receive methyldopa (Dopegyt) solely or in combination with nifedipine (Cordaflex), and there is no understanding of their effect on proinflammatory state of blood vessels. Our study aimed to assess levels of IL-6, IL-18, TNFα, galektin-3 and homocysteine as well as levels of syndecan-1, eCG structural component, representing system inflammatory response and endothelial dysfunction development in blood of women with early- and late-onset PE receiving different antihypertensive treatment strategies. Eighty-two patients were enrolled into this interventional longitudinal pilot study. The comparison group included 15 patients before 34 gestational weeks and 15 patients after 34 weeks. Study subgroup 1 included 12 patients with early-onset PE receiving Dopegyt solely and 16 patients with early-onset PE receiving Dopegyt together with Cordaflex. Study subgroup 2 included 12 patients with late-onset PE receiving Dopegyt solely and 12 patients with late-onset PE receiving combined therapy. As for early-onset PE, only IL-6 demonstrated statistically significant differences in patients receiving both treatment strategies compared to control. Proinflammatory state was more profound in late-onset PE. IL-6 levels were significantly increased in late-onset PE treated with Dopegyt. IL-6 and TNFα levels were significantly higher in late-onset PE patients treated with Dopegyt + Cordaflex compared to control. Syndecan-1 levels were statistically significantly higher in patients with early-onset PE treated with Dopegyt solely. There were no statistically significant differences between the groups despite elevated mean values of syndecan-1 in late-onset PE. Galectin-3 and homocysteine levels did not differ significantly between the groups, representing lack of pronounced inflammatory response and endothelial dysfunction.

*Keywords: preeclampsia, endothelial glycocalyx, endothelial dysfunction, sterile inflammation, antihypertensive drugs*

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## Introduction

Preeclampsia (PE) remains one of the leading causes of maternal and perinatal morbidity and mortality worldwide. Alongside with risks that face the mother and fetus, there is proof that women with the history of PE have long-term risk of cardiovascular diseases. Prolonged system inflammation and as a result endothelial dysfunction cause changes in cardiovascular system in PE. Sterile inflammation in contrast to infectious etiological factor is considered to be one of the leading triggers of PE [1, 11].

According to present beliefs, there are two clinical phenotypes of PE, i.e. early-onset PE that manifest before 34 gestational weeks and late-onset PE with its debut after 34 weeks. Generally healthy pregnant women develop early-onset PE with impaired placentation being the main cause leading to formation of proinflammatory milieu in placental tissues [11]. Since extragenital maternal pathology is a risk factor of late-onset PE its rate is higher than that of early-onset PE, i.e. chronic arterial hypertension, inherited and acquired thrombophilia, diabetes mellitus ant other diseases accompanied by proinflammatory state and endothelial activation. Placental stress as a result of hypoxia-ischemic changes of placental tissues [12] leads to formation of molecular patterns associated with damage (DAMPs). DAMPs activate maternal immune system. Thus, vicious circle forms leading to permanent endothelial activation transforming into endothelial dysfunction. Clinically these processes manifest with loss of the ability to hold physiological blood pressure (BP), "capillary leakage" syndrome of different severity and proadgesive, thrombogenic state. According to recent findings, these symptoms are a result of endothelial dysfunction that starts with dysfunction of superficial layer of cells associated with endothelial membrane, endothelial glycocalix (eGC) [16].

Antihypertensive therapy in pregnancy is limited by a small number of drugs that are safe for the mother and the fetus and do not affect feto-placental blood flow. Standard antihypertensive treatment strategies represented by monotherapy with methyldopa (Dopegyt) or alongside with nifedipine (Cordaflex) have demonstrated stabilizing effect on cardiovascular eGC state. However, eGC destruction evaluated by the level of circulating structural components of eGC did not occur only in late-onset PE [8]. It should be noted that effect of these medicines on eGC has not been fully studied yet [14]. In particular, there is no information on their action on proinflammatory state in vessels, especially in terms of evaluation of complex of parameters produced by activated immune cells (cytokines), endothelium (cytokines and homocysteine), eGC components (syndecan-1) and associated molecules (galectin-3).

Aim of this study was an assessment of immune factors and biochemical parameters representing system inflammatory response and endothelial dysfunction development in blood of women with early- and late-onset PE receiving antihypertensive monotherapy and combined treatment.

## Materials and methods

Eighty-two patients were enrolled into this interventional longitudinal pilot study. The comparison group included 30 pregnant women: i. 15 patients before 34 gestational weeks, and ii. 15 patients after 34 weeks (NP1 and NP2 groups, respectively). Study group comprised 52 patients that were stratified by gestational age and type of antihypertensive treatment. Study subgroup 1 included 28 patients before 34 weeks: 12 patients with early-onset PE receiving Dopegyt solely (subgroup PE 1); and 16 patients with early-onset PE receiving Dopegyt together with Cordaflex (subgroup PE 2). Study subgroup 2 included 24 patients after 34 weeks: 12 patients with late-onset PE receiving Dopegyt solely (subgroup PE 3); and 12 patients with late-onset PE receiving combined therapy (subgroup PE 4). The study was conducted on the basis of NSBI "National Medical research center for obstetrics, gynecology and perinatology named after academician V.I. Kulakov" (further in the text Center) in accordance with the principles of WMA Declaration of Helsinki. Study design was approved at local ethical committee (protocol № 5, May, 27$^{th}$, 2021). Preeclampsia was diagnosed according to the clinical recommendations of Ministry of Health of Russian Federation criteria [11]. Inclusion criterion for the study group was PE and for the comparison group – healthy pregnancy. Non-inclusion criteria were as follows: ART pregnancy, severe extragenital disease, history of organ transplantation, immunotherapy in pregnancy. Exclusion criteria were HELLP-syndrome, acute viral and infectious diseases during pregnancy. Patients were matched by age, BMI, gestational term. All patients signed informed consent. Antihypertensive therapy comprised of drug of central action, Dopegyt (mean daily dose 1500 mg). When there was insufficient hypotensive effect, additional Ca-channel blocker, Cordaflex, was prescribed (mean daily dose 40 mg). Mean therapy duration was at least 11 days. In order to assess effectiveness of given therapy 24-hor BP monitoring was performed (BPLab® device, Peter Telegin, Nizhniy Novgorod, Russia).

Fasting blood samples were collected into vacuum test-tubes. Preparation of blood serum was performed according to standard operation procedure of Center Biobank where probes were stored at -80°C till factors analysis. Humoral factors were studied by ELISA using commercial test-systems. We used test-systems for identification of desquamated forms of eGC structural component, syndecan-1 (SEB966Hu, Cloud-Clone Corp., USA); identification of cytokines – IL-18 (BMS267-2, Bender MedSystem, Austria), IL-6 (A-8768, Vector-Best, Russia), TNFα (BMS223-4, Bender MedSystem, Austria), galektin-3 (BMS279-4, Bender MedSystem, Austria) and homocysteine (FHCY100, Axis-Shield, United Kingdom).

We used the program MedCalc version 16.4 (MedCalc, Belgium) for statistical analysis of obtained data. The normality of distribution of studied characteristics was assessed using Shapiro–Wilk test. The nonparametric Mann–Whitney U test was used. Data are represented as median and interquartile range for continuous variables. Differences were considered significant when p-value was less than 0.5. We included data with p-value less than 0.1 considered as a trend in changes into our pilot study.

## Results and discussion

Typical feature of inflammatory response, including that caused by non-infectious agents, is an increase in proinflammatory cytokines and factors, thus proving endothelium activation. An increase in gene expression of cytokines in placenta and in blood levels of IL-18, IL-6, and TNFα was reported in PE [1, 6, 7, 10]. Proinflammatory milieu induces eGC destabilization which in turn reduces protective layer of endothelium proteoglycans and destroys composition of their carbohydrate chains [16]. Meanwhile, blood levels of circulating proteoglycans and molecules normally bound to glycocalyx rise.

An elevation of shed forms of eGC proteoglycans in blood of patients with PE was demonstrated [15], as well as an increase in galectin-3, secreted car-

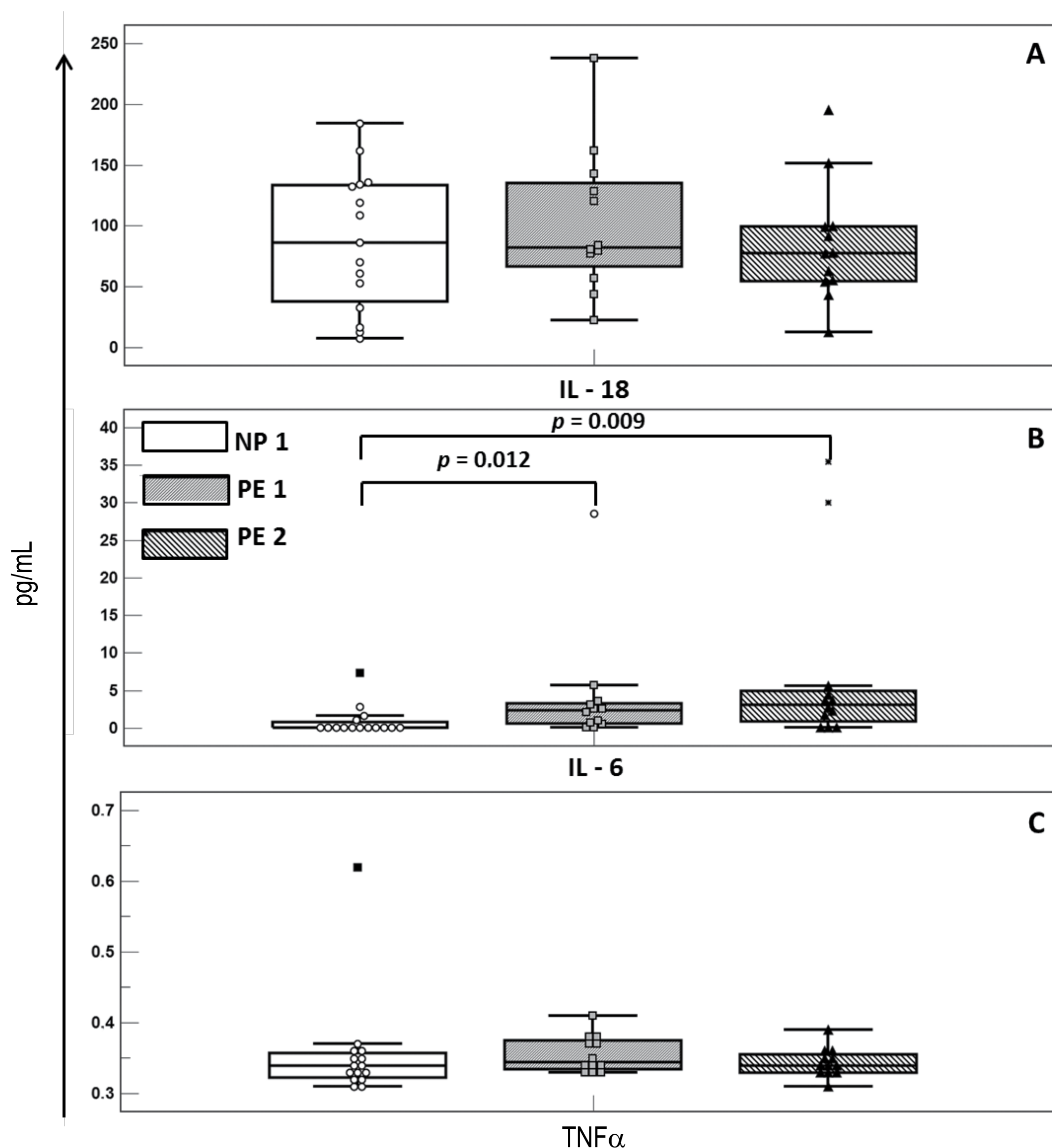


**Figure 1. IL-18 (A), IL-6 (B), and TNFα (C) levels in peripheral blood of patients with early-onset PE, receiving Dopegyt and Dopegyt + Cordaflex**

Note. Differences are given at p < 0.1.

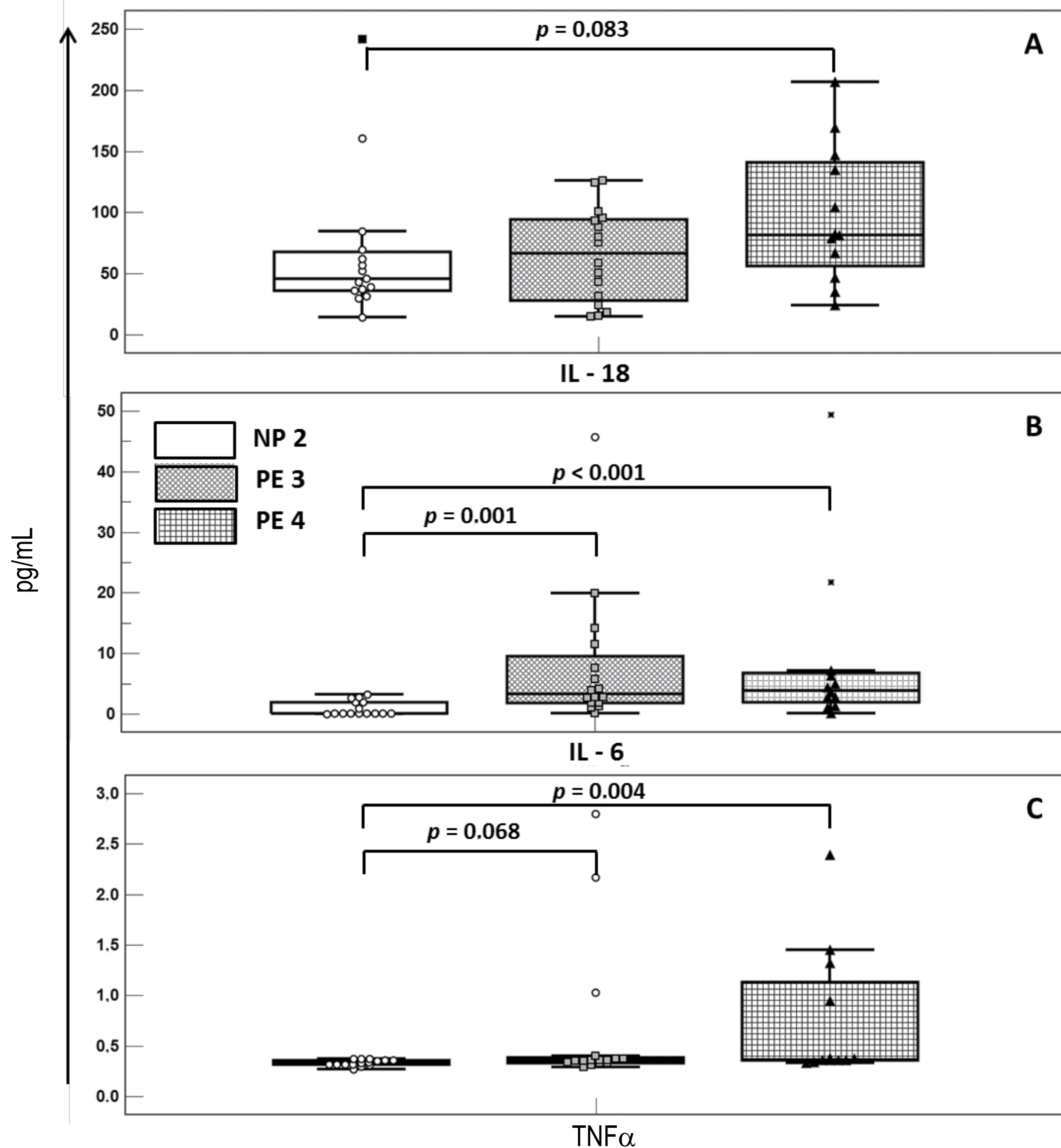


**Figure 2. Cytokines IL-18 (A), IL-6 (B), and TNFα (C) levels in peripheral blood of patients with late-onset PE receivng Dopegyt and Dopegyt + Cordaflex**

Note. Differences are given at p < 0.1.

**TABLE 1. COMPARATIVE ANALYSIS OF SYNDECAN-1, GALECTIN-3, AND HOMOCYSTEINE LEVELS IN PATIENTS WITH PE RECEIVING DOPEGYT AND DOPEGYT + CORDAFLEX**

| Parameter | NP 1 (n = 15) | PE 1 (n = 12) | PE 2 (n = 16) | NP 2 (n = 15) | PE 3 (n = 12) | PE 4 (n = 12) |
|---|---|---|---|---|---|---|
| SDC-1, pg/mL | 930 (753-3640) | 3065# (1595-5385) | 1715## (1130-5265) | 1290 (820-11288) | 5070 (2095-9835) | 2570 (1475-3490) |
| Galectin-3, ng/mL | 11.96 (10.91-19.17) | 17.04 (9.49-29.26) | 12.97 (11.56-16.90) | 15.17 (10.52-32.00) | 13.09 (9.74-15.63) | 14.82 (11.48-20.16) |
| Hcy, umol/L | 10.00 (8.84-11.15) | 9.16 (6.18-10.79) | 8.67 (6.62-9.69) | 8.61 (6.90-10.04) | 8.99 (7.05-9.91) | 9.67 (7.84-11.89) |

Note. #, p = 0.019 comparing groups NP1 and PE1; ##, p = 0.075 comparing groups NP1 and PE2.

bohydrate-binding protein bound to glycocalyx [9]. Amino acid homocysteine (Hcy) is a factor associated with inflammation. Higher levels of Hcy were demonstrated in PE (above 10 mkMol/L), being considered as hyperhomocysteinemia associated with elevated risk of cardiovascular, cerebral, and peripheral arteries pathology leading to endothelial dysfunction [5].

Comparative analysis revealed elevated levels of proinflammatory factors in patients with PE receiving Dopegyt and Dopegyt + Cordaflex compared to control. However, only IL-6 demonstrated statistically significant differences reflecting increased proinflammatory state in blood vessels of patients with early-onset PE receiving both treatment strategies (Figure 1B).

Proinflammatory state was more profound in late-onset PE. In particular, IL-6 levels were significantly increased in patients treated with Dopegyt ($p = 0.001$), as well as a trend to increase in TNFα levels, $p = 0.068$ (Figure 2B, C). Generally proinflammatory state was seen in patients treated with Dopegyt + Cordaflex: IL-6 (Figure 2B) and TNFα (Figure 2C) levels were significantly higher compared to control ($p < 0.001$ and $p = 0.004$, respectively). Same trend was seen for IL-18, $p = 0.083$ (Figure 2A).

Syndecan-1 levels were statistically significantly higher in patients with early-onset PE treated with Dopegyt solely, $p = 0.019$, and non-significantly higher in those receiving combined therapy, $p = 0.075$ (Table 1). There were no statistically significant differences between the groups despite elevated mean values of syndecan-1 in late-onset PE. Galectin-3 and homocysteine levels did not differ significantly between the groups (Table 1), representing lack of pronounced inflammatory response and endothelial dysfunction.

Symptoms of system inflammatory response are known to be seen in healthy pregnant women, however, as pregnancy progresses those symptoms become more prominent due to penetration of allogenic fetal cells, debris and fetal DNA into mother blood as well as physiological “placenta aging” [3]. More pronounced proinflammatory state is observed in patients of elder age because of blood vessel aging due to cardiovascular and metabolic, i.e. obesity, diseases [8]. Given this fact we enrolled patients matched by age, BMI and gestational age to eliminate confounders that intervene with study results.

IL-6 plays vital role in chronic inflammatory response and TNFα controls its production. Our results demonstrate that despite antihypertensive therapy key proinflammatory cytokines levels are increased, especially in late-onset PE and in those receiving combined therapy. What is more, not only IL-6 and TNFα levels are elevated in patients treated with Dopegyt + Cordaflex but strong direct correlation between their blood levels was observed ($r_s = 0.72$, $p = 0.003$). This finding proves their pathogenic role and demonstrates absence of positive effect of therapy on inflammatory response in this group of patients. Strong direct correlation between IL-6 and galectin-3 levels was revealed in patients with early-onset PE receiving Dopegyt + Cordaflex ($r_s = 0.75$, $p = 0.005$). Though galectin-3 levels did not differ significantly between these patients and control group, identified correlation may imply its potential pathogenic role in long-term changes of vessels as galectin-3 is a biomarker of heart failure in cardiology [2]. It is believed that main source of galectin-3 in maternal blood is placenta and not endothelium [9]. Nevertheless, vessels molecular changes as a result of PE may bear long-term consequences and galectin-3 is a promising candidate for long-term prognosis of risk of cardiovascular diseases.

It is known that antihypertensive drugs affect cytokine production by immune cells of placenta and peripheral blood. In particular, dose-dependent effect, i.e. inhibition of cytokine production by placental cells and mononuclear cells in preeclamptic patients, was demonstrated *in vitro* for clonidine, diazoxide, hydralazine and furosemide [13]. Combined antihypertensive treatment affects cytokines state in patients with arterial hypertension. Therapy with enalapril + hydrochlorothiazide and enalapril + indapamide significantly decreased IL-6 and TNFα levels as well as mean blood pressure. These effects were accompanied by clinical improvement of heart failure parameters according to scale of patient’s clinical state assessment [4]. As for Dopegyt and Cordaflex, we have not found similar articles. Limitation of our study is having no data on baseline levels of studied molecules before the start of therapy, sometimes because of urgent need for delivery of some patients with severe PE. This point limits therapy duration and explains retrospective analysis of antihypertensive treatment. However, obtained data allow us to evaluate proinflammatory state in blood vessels of patients with early- and late-onset PE receiving different treatment options.

## Conclusion

Immune and biochemical factors reflecting development of system inflammatory response and

endothelial dysfunction in patients with early- and late-onset PE receiving Dopegyt solely and Dopegyt + Cordaflex were studied. It was demonstrated that proinflammatory state was more profound in late-onset PE patients treated with combined therapy. This finding may be explained by the presence of comorbidities that require additional prescription of Cordaflex. In early-onset PE more prominent eGC destruction alongside with higher IL-6 levels was observed. Our results demonstrate necessity for differential approach to therapy of different PE clinical phenotypes and perspectives of anti-inflammatory drugs prescription in patients with late-onset PE to negate symptoms of sterile inflammation.

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# ИММУНОЛОГИЧЕСКИЕ ФАКТОРЫ РАЗВИТИЯ НАРУЖНОГО ГЕНИТАЛЬНОГО ЭНДОМЕТРИОЗА

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**Резюме.** Наружный генитальный эндометриоз (НГЭ) – одно из распространенных гинекологических заболеваний женщин репродуктивного возраста с рецидивирующим, прогрессирующим течением, ухудшающим качество жизни пациенток из-за болевого синдрома, эмоциональной неуравновешенности, страха рецидива и возможного оперативного вмешательства. В настоящее время эндометриоз признан одним из наиболее распространенных заболеваний, связанных с бесплодием. Так, среди фертильных женщин с сохраненной детородной функцией заболевание в целом диагностируется примерно у 6-7%, тогда как среди пациенток, страдающих бесплодием, его частота может достигать 20-48%.

Однако причины, определяющие репродуктивную дисфункцию у больных с НГЭ, изучены недостаточно. Большое внимание в настоящее время уделяется роли иммунитета в формировании эндометриоза. У больных с НГЭ отмечаются изменения как факторов местного иммунитета, так и иммунологических компонентов циркулирующей крови.

Цель исследования – изучение факторов врожденного и адаптивного иммунитета у пациенток репродуктивного возраста с наружным генитальным эндометриозом (НГЭ).

В исследование была включена 71 пациентка с различными стадиями наружного генитального эндометриоза, в контрольную группу вошли 24 пациентки, без эндометриоза. Определение популяционного состава лимфоцитов периферической крови, уровня моноцитов, экспрессирующих TLR, активационных маркеров, проводили методом лазерной проточной цитометрии – Immunotex (Франция), Caltag (США), FITC (изотиоцинат флуоресцеина) – меченые CD3, CD4, CD8, CD16, CD19, HLA-DR, CD282, CD284 и PE(фикоэритрин) – меченые CD25, CD69, CD95, CD107a, CD14.

Наружный генитальный эндометриоз характеризуется: при I-II стадии заболевания – нарушением ранних этапов врожденного иммунного ответа (повышение количества моноцитов, экспрессирующих TLR-4, нарушением процессов активации и дифференцировки иммунокомпетентных клеток, что отражается в снижении экспрессии CD16, CD8, $CD16^{+}HLA\text{-}DR^{+}$, $CD16^{+}CD107a^{+}$, $CD8^{+}CD107a^{+}$,

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а при III-IV стадии заболевания отмечается снижении уровня CD16 и маркеров активации CD69, HLA-DR, CD107a на их поверхности, что сочетается со снижением экспрессии CD8, CD16, HLADR и CD107a на их поверхности. Повышение $CD16^+CD95^+$ и $CD8^+CD95^+$ выявлено при различных стадиях НГЭ.

Полученные результаты позволяют понять особенности функционирования врожденного и адаптивного иммунитета на различных стадиях наружного генитального эндометриоза, а изученные иммунологические показатели могут быть использованы в качестве диагностических критериев формирования различных стадий НГЭ. Эти данные могут служить теоретической основой для дальнейшей идентификации маркеров прогрессирования НГЭ, а также механизмов, лежащих в основе иммунного воспаления.

*Ключевые слова: врожденный иммунитет, адаптивный иммунитет, TLR-рецепторы, цитотоксичность, маркеры активации, наружный генитальный эндометриоз*

# IMMUNOLOGICAL FACTOR DEVELOPMENT OF EXTERNAL GENITAL ENDOMETRIOSIS

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**Abstract.** External genital endometriosis (EGE) is one of the common gynecological diseases of women of reproductive age with a relapsing, progressive course that worsens the quality of life of patients due to pain, emotional imbalance, fear of relapse and possible surgical intervention. Currently, endometriosis is recognized as one of the most common diseases associated with infertility. Thus, among fertile women with preserved childbearing function, the disease is generally diagnosed in approximately 6-7%, while among patients suffering from infertility, its frequency can reach 20-48%.

However, the causes that determine reproductive dysfunction in patients with EGE are not well understood. Much attention is currently paid to the role of immunity in the formation of endometriosis. Patients with EGE show changes in both local immunity factors and immunological components of circulating blood.

Purpose of the study: the study of factors of innate and adaptive immunity in patients of reproductive age with external genital endometriosis (EGE).

The study included 71 patients with various stages of external genital endometriosis, the control group included 24 patients without endometriosis. Determination of the population composition of peripheral blood lymphocytes, the level of monocytes expressing TLR, activation markers, was carried out by laser flow cytometry – Immunotex (France), Caltag (USA), FITC (fluorescein isothiocynate) – labeled CD3, CD4, CD8, CD16, CD19, HLA -DR, CD282, CD284 and PE (phycoerythrin) – labeled with CD25, CD69, CD95, CD107a, CD14.

External genital endometriosis is characterized by: at stages I-II of the disease – a violation of the early stages of the innate immune response (an increase in the number of monocytes expressing TLR-4, a violation of the activation and differentiation processes of immunocompetent cells, which is reflected in a decrease in the expression of CD16, CD8, $CD16^+HLA\text{-}DR^+$, $CD16^+CD107a^+$, $CD8^+CD107a^+$, at III-IV stages of the disease, there is a decrease in the level of CD16 and activation markers CD69, HLA-DR, CD107a on their surface, which is combined with a decrease in the expression of CD8, CD16, HLADR and CD107a on their surface. $CD95^+$ and $CD8^+CD95^+$ were found at various stages of EGE.

The results obtained allow us to understand the features of the functioning of innate and adaptive immunity at various stages of external genital endometriosis, and the studied immunological parameters can be used as diagnostic criteria for the formation of various stages of EGE. These data can serve as a theoretical basis for further identification of markers of EGE progression, as well as the mechanisms underlying immune inflammation.

*Keywords: innate immunity, adaptive immunity, TLR receptors, cytotoxicity, activation markers, external genital endometriosis*

## Introduction

External genital endometriosis (EGE) is one of the common gynecological diseases of women of reproductive age with a relapsing, progressive course that worsens the patient's quality of life due to pain, emotional imbalance, fear of relapse and possible surgical intervention. Currently, endometriosis is recognized as one of the most common diseases associated with infertility. Thus, among fertile women with preserved childbearing function, the disease is generally diagnosed in approximately 6-7%, while among patients suffering from infertility, its frequency can reach 20-48% [1].

However, the causes that determine reproductive dysfunction in patients with EGE are not well understood. Much attention is currently paid to the role of immunity in the formation of endometriosis [3, 4]. Patients with EGE show changes in both local immunity factors and immunological components in the circulating blood [2, 5].

To date, it is obvious that immune system disorders play an important role in the development of EGE, however, despite research in this area, there is practically no information on markers of positive and negative activation, cytotoxicity of T lymphocytes, NK cells. Insufficiently studied are the mechanisms of innate immunity, in particular, the level of monocytes expressing TLR-2, TLR-4.

In this regard, **the purpose of our study** was to study the factors of innate and adaptive immunity in patients of reproductive age with external genital endometriosis.

## Materials and methods

Under observation there were 71 patients with EGE, who were divided into two groups: group 1 – women with I-II stages of EGE (n = 31); Group 2 – patients with III-IV stages of EGE (n = 40). The control group included 24 patients who were examined in the Department of Operative Gynecology, who underwent therapeutic and diagnostic laparoscopy. In this group of patients, neither EGE, nor acute or chronic inflammatory diseases of the pelvic organs, nor benign neoplasms were detected intraoperatively. Criteria for inclusion of patients in the study: the reproductive age of patients 18-49 years, the presence of complaints of infertility and/or pain syndrome, body mass index 18.5-25 kg/m$^2$, normal body temperature, laparo- and hysteroscopy. with morphological verification of the diagnosis. The exclusion criteria from the study were: pubertal and perimenopausal age of patients, malignant neoplasms, severe extragenital pathology at the stage of decompensation, acute infectious diseases or exacerbation of their chronic forms. Determination of the population composition of peripheral blood lymphocytes, the level of monocytes expressing Toll receptors (TLR), activation markers was carried out by laser flow cytometry – Immunotex (France), Caltag (USA), FITC (fluoresceinisothiocyanate) – labeled CD3, CD4, CD8, CD16, CD19, HLA-DR, CD282, CD284 and PE (phycoerythrin) labeled with CD25, CD69, CD95, CD107a, CD14. The results were taken into account on a BECKMAN COULTER EPICS XL-II laser flow cytometer (USA). To form a database and conduct a statistical study, the capabilities of the Excel 2003 spreadsheet processor and application packages ("Megastat" and Statistica 6.0) were used. Descriptive statistics was carried out with the determination of the values of the median and interquantile range: Me ($Q_{0.25}$-$Q_{0.75}$). A p value < 0.05 was considered statistically significant.

## Results and discussion

Analysis of clinical data in observed women with EGE showed a high percentage of primary infertility – 69%. In patients with stages I-II of the disease – in 67.7% of cases, in patients with stages III-IV – in 70%. Secondary infertility occurs in 31% of patients with EGE, while with minimal forms of the disease – in 32.3%, and with severe forms of endometriosis – in 30%. In women with EGE, chronic inflammatory processes were detected in the same percentage of cases (67.7%), and sexually transmitted infections in patients with stage I-II EGE were detected in 32.2% of women, while in patients with EGE III-IV stages are 2 times less.

Our work showed that the formation of external genital endometriosis was accompanied by significant changes in immunity parameters. When studying the parameters of the immune status in the 1st and 2nd groups, compared with the control group, a statistically significant decrease in CD8 was revealed, the most pronounced deviations were noted in the 2nd group, the indicators were 1.7 times lower than in the control group ($p < 0.05$).

According to current data, NK cells are divided into two types: typical CD56bright CD16-NK cells producing cytokines, and CD56dimCD16$^+$ NK cells characterized by high cytotoxicity. Analyzing the indicators of the content of natural killers (CD16$^+$) in the 1st and 2nd groups, a statistically significant decrease in their relative number was noted compared to the control group (by 2.9 and 1.3 times, ($p < 0.05$); indicators in the 2nd group were lower than in the

1st ($p < 0.05$). The role of B lymphocytes in the development of endometriosis and their relationship with clinical symptoms and disease severity are not well understood. In the 2nd group, the CD19 values were higher than in the control group ($p < 0.05$), which indicates the activation of the B cell link of immunity.

When analyzing the functional state of immunocompetent cells, it was found that the level of $CD16^+CD69^+$ in group 2 was lower than the values in the control group and in group 1 by 2.0 times ($p < 0.05$). The content of $CD8^+CD25^+$ in the 2nd group was statistically significantly lower than in the control group ($p < 0.05$). When studying the expression of markers of late activation, a decrease in $CD16^+HLADR^+$ was revealed at various stages of EGE, the most pronounced deviations were noted in the 2nd group, the indicators were 2 times lower control ($p < 0.05$). The study of the content of $CD8^+$, expressing HLA-DR, showed their decrease in the 2nd group compared with the control group and the 1st group (1.7 and 1.89 times ($p < 0.05$).

$CD107a^+$ is a marker for degranulation of $CD8^+$ and $CD16^+$ lymphocytes.

The study of the number of CD16 lymphocytes expressing the CD107a molecule involved in immune inflammation through the mechanism of cellular cytotoxicity revealed that in the 2nd group the content of $CD16^+CD107a^+$ was 2.6 times lower than in the control group ($p < 0.05$), and 1 – 1.8 times ($p < 0.05$). Similar data were found for the content of $CD8^+CD107a^+$, the indicators in the 2nd group were lower than the control level and group 1 by 1.7 and 1.1 times, respectively, ($p < 0.05$), which indicates a decrease in functional activity natural killer cells and cytotoxic T lymphocytes. The study of the content of cytotoxic lymphocytes and NK cells expressing the Fas receptor $CD95^+$ revealed an increase in $CD16^+CD95^+$ at various stages of ege. Similar data were found for $CD8^+CD95^+$, their level exceeded the control values in the 1st group by 1.2 times, in the 2nd – by 1.46 times ($p < 0.05$).

It is known that the proliferation of endometrioid lesions is regulated by the innate immune system. Important components of the innate immune system are Toll-like receptors (TLRs), which are type 1 transmembrane proteins with an N-terminal extracellular domain. TLRs can be activated by endogenous ligands, including lipopolysaccharide (LPS), heat shock protein, S100, fibronectin, fatty acids, neutrophil elastase, etc. In our study, groups 1 and 2 showed a statistically significant increase in the number of peripheral blood monocytes expressing TLR-4 ($CD14^+CD284^+$), compared with the control group. The most significant deviations were noted in the 2nd group.

Thus, external genital endometriosis is characterized by: at stages I-II of the disease, a violation of the early stages of the innate immune response (an increase in the number of monocytes expressing TLR-4, a violation of the activation processes of immunocompetent cells, which is reflected in a decrease in the expression of CD16, CD8, $CD16^+HLA\text{-}DR^+$, $CD16^+CD107a^+$, $CD8^+CD107a^+$, at III-IV stages of the disease, there is a decrease in the level of CD16 and activation markers CD69, HLA-DR, CD107a on their surface, which is combined with a decrease in the expression of CD8 and CD16 lymphocytes, $HLADR^+$, CD107a on their surface. An increase in $CD16^+CD95^+$ and $CD8^+CD95^+$ was found at various stages of EGE.

Aberrant immune responses of cytotoxic T lymphocytes and NK cells are trigger factors for the development of endometriosis. Violation of the work of innate immunity mediated by natural killers leads to disruption of the functioning of adaptive immunity, the development and progression of endometriosis and infertility. Immune dysfunction in endometriosis is probably formed both due to a decrease in the number of cytotoxic T lymphocytes and NK cells, and due to a functional defect [7]. The decrease in cytotoxicity mediated by T lymphocytes and NK cells in women with EGE may also be associated with the induction of apoptosis along the Fas-FasL pathway and contributes to the formation of endometrial heterotopias.

The revealed increase in the content of $CD14^+CD284^+$ in women with EGE suggests that TLR-4-dependent signaling can lead to an increase in the synthesis of pro-inflammatory cytokines and chemokines, stimulates the proliferation of endometrial cells, activates macrophages, DC, natural killer cells, forming an inflammatory microenvironment, contributing to the progression EGE.

## Conclusion

The results obtained allow us to understand the features of the functioning of innate and adaptive immunity at various stages of external genital endometriosis, and the studied immunological parameters can be used as diagnostic criteria for the formation of various stages of EGE. These data may provide a theoretical basis for further identification of markers of EGE progression as well as underlying immune inflammation. Further research is needed to explore the role of cytotoxic T lymphocytes, NK cells, and TLR signaling pathways in the pathogenesis and molecular mechanisms of EGE.

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# КОЛИЧЕСТВЕННОЕ ОПРЕДЕЛЕНИЕ С1-ЭСТЕРАЗНОГО ИНГИБИТОРА В СЫВОРОТКЕ КРОВИ ЧЕЛОВЕКА МЕТОДОМ ИММУНОФЕРМЕНТНОГО АНАЛИЗА: КОРРЕЛЯЦИЯ С ТУРБИДИМЕТРИЧЕСКИМ МЕТОДОМ

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**Резюме.** С1-ингибитор сериновых протеаз (C1-INH) выполняет регуляторную функцию в системе комплемента и проницаемости сосудов. Дефицит C1-INH приводит к различным формам ангиоотеков, в том числе наследственному ангионевротическому отеку (НАО). Причиной НАО является генетически обусловленное нарушение синтеза C1-INH. Снижение уровня C1-INH до 50% относительно нормы приводит к увеличению продукции брадикинина, что является основанием для постановки диагноза «НАО». Разработка доступных иммуноферментных тест-систем (ИФА) для количественного определения C1-INH является востребованным направлением для клиницистов. В процессе разработки нового набора для количественного определения C1-INH были получены два моноклональных антитела мыши (МАТ), обладающих разной эпитопной специфичностью. На их основе была разработана ИФА тест-система по типу сэндвич. Специфичность полученных МАТ была подтверждена с использованием коммерческого препарата «Беринерт». Для приготовления калибраторов C1-INH был аффинноочищен из плазмы крови человека с использованием сорбента с иммбилизованными МАТ. Идентичность белку C1-INH подтверждали при помощи электрофореза в ПААГ, иммуноблоттинга и масс-спектрометрии на MALDI-TOF/TOF масс-спектрометре UltrafleXtreme. Для оценки показате-

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лей качества разработанного набора реагентов были проведены исследования в соответствии с ГОСТ Р 51352-2013 и ТУ 21.20.23-041-01967164-2022. Значения показателей качества: точность (правильность) – 93,53%; интервал линейности измерений – 22,00-176,07 нг/мл. Присутствие интерферирующих агентов (гемоглобина, билирубина, триглицеридов) не влияло на эффективность измерений. С помощью разработанной ИФА тест-системы нами были исследованы 28 сывороток крови здоровых доноров и 7 сывороток крови пациентов с подтвержденным диагнозом «НАО». В этих же образцах определяли содержание C1-INH при помощи турбидиметрического метода, используя медицинское изделие «Реагенты диагностические для иммунохимических исследований *in vitro* специфических белков крови. Модель: С1-ингибитор эстеразы (C1 EsteraseInhibitor)» (Aptec, Бельгия). Коэффициент корреляция составил 0,94 (p < 0,05). Было установлено, что диагностическая чувствительность и специфичность разработанной тест-системы составляет 100%. В результате проведенного исследования разработана оригинальная ИФА тест-система для количественного определения C1-INH «Набор реагентов для иммуноферментного количественного определения С1-ингибитора человека (C1-inh PS)».

*Ключевые слова: С1-ингибитор, моноклональное антитело, иммуноферментный анализ, турбидиметрия, калибратор, корелляционный анализ*

# QUANTIFICATION OF C1 ESTERASE INHIBITOR IN HUMAN SERUM BY ENZYME-LINKED IMMUNOSORBENT ASSAY: CORRELATION WITH TURBIDIMETRIC IMMUNOASSAY

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**Abstract.** C1 inhibitor of serine proteases (C1-INH) performs a regulatory function in the complement system and vascular permeability. Deficiency of C1-INH leads to various forms of angioedema, including hereditary angioedema (HAE). The cause of HAE is a genetically determined violation of the synthesis of C1-INH. A decrease in the level of C1-INH to 50% relative to the norm leads to an increase in the production of bradykinin, which is the basis for the diagnosis of HAE. The development of affordable ELISA for the quantitative determination of C1-INH is a popular direction for clinicians. During the development of a new kit for quantitative determination of C1-INH, two mouse monoclonal antibodies (mAb) with different epitope specificities were obtained. On their basis, a sandwich-type ELISA was developed. The specificity of the obtained mAb's was confirmed using the medical device “Berinert”. To prepare calibrators, C1-INH was affinity purified from human blood plasma using a sorbent with immobilized mAbs. The identity of the C1-INH protein was confirmed by PAGE electrophoresis, immunoblotting, and mass spectrometry on MALDI-TOF/TOF UltrafleXtreme mass spectrometer. To assess the quality indicators of developed reagents kit, studies were carried out in accordance with GOST R 51352-2013 and TU 21.20.23-041-01967164-2022. Values of quality indicators: accuracy – 93.53%; measurement linearity interval – 22.00-176.07 ng/mL. Using the developed ELISA test system, we examined 28 blood sera from healthy donors and 7 blood sera from patients with confirmed HAE. In the same samples, the content of C1-INH was determined by turbidimetric method, using the "Diagnostic reagents for *in vitro* immunochemical studies of specific blood proteins. Model: C1-esterase inhibitor (C1 EsteraseInhibitor)" (Aptec, Belgium). The correlation coefficient was 0.94 (p < 0.05). It was found that the diagnostic sensitivity and specificity of the developed ELISA is 100%. As a result of the study, an original ELISA test system for the quantitative determination of C1-INH was developed "Reagent kit for enzyme-linked immunosorbent assay of human C1-inhibitor (C1-inh PS)".

*Keywords: C1 inhibitor, monoclonal antibody, enzyme immunoassay, turbidimetry, calibrator, correlation analysis*

## Introduction

C1 esterase inhibitor (C1-INH) belongs to the superfamily of serine protease inhibitors (serpins) and is a single-chain highly glycosylated protein. The main function of C1-INH is to control the inflammatory response by inhibiting spontaneous complement activation via the classical and lectin pathways, as well as regulate vascular permeability by inactivating proteases of the fibrinolytic, coagulation, and kinin pathways, preventing unregulated production of bradykinin [2, 3].

Deficiency of the C1-INH inhibitor leads to increased generation of complement anaphylatoxins, which stimulate the release of vasoactive amines by mast cells and eosinophils and cause a strong inflammatory response. The weakening of control over the activation of contact systems leads to enhanced production of the powerful vasodilator bradykinin. In both cases, this leads to systemic inflammation, which manifests in the form of angioedema (AE) [2, 4].

The most severe manifestation of AE is hereditary angioedema (HAE), which can occur in presence or absence of urticaria, accompanied by repeated, self-limiting relapses and not reversed by antihistamines [1].

The cause of HAE disease is a rare dominant mutation in the human autosome, which leads to genetically determined defects in the genes encoding C1-INH synthesis. The incidence of HAE ranges from 1:20,000 to 1:100,000 [1, 6].

The symptoms of AE are similar to those of other diseases (allergies, asthma, inflammatory processes, etc.), which causes difficulties in determining of its cause, diagnostics and choosing a therapy strategy by clinicians. A decrease in the level of C1-INH to a value below 50% of normal (20-40 mg/L) is strictly associated with the diagnosis of HAE, which makes it relevant to determine its concentration in the blood of patients [5, 6].

In this regard, **the aim of the work** was to develop an available ELISA test system for the quantitative determination of C1-INH, which can compete with other registered more expensive test systems.

## Materials and methods

**Human serum samples**

The panel of serum samples from healthy donors and confirmed type I HAE patients was provided by the Pasteur Institute Medical Center (St. Petersburg, Russia).

**Obtaining mouse monoclonal antibodies (mAbs) against human C1-INH**

Monoclonal Abs against human C1-INH used to develop human C1-INH enzyme-linked immunosorbent assay (ELISA) test system were obtained according to Köhler and Milstein hybridoma technology. To immunize animals (Balb/c mice), commercial intravenous human C1-INH «Berinert» (Aptec) was used. Immunization was carried out in three stages with 31 day interval. At the first stage, 20 μg of protein together with Freund's complete adjuvant (FCA) was administered subcutaneously by footpad injection. Re-immunization was carried out with the same dose of antigen with Incomplete Freund's Adjuvant (IFA), followed by booster dose of 100 mg protein in saline.

Six days after boosting, lymphocytes were harvested and fused with SP 2/0 mouse myeloma cells. The cells were cultured in RPMI-1640 (Sigma) supplemented with 10% fetal bovine serum (Sigma) and HAT selection agent (Sigma) containing hypoxanthine, aminopterin, thymidine. Thus obtained hybridoma clones were screened by ELISA. The antigen was immobilized on the plate well surface, then the supernatants from hybridoma cells were added to the wells, and the resulting immune complexes were detected using commercial goat anti-mouse IgG conjugated with horseradish peroxidase (A4416, Sigma). mAbs were produced by intraperitoneal injection of hybridoma cells into experimental animals, followed by collecting of ascitic fluid once ascites was developed. To purify the mAbs, ascitic fluid was preliminarily precipitated with ammonium sulfate and applied to a column filled with a protein A resin (MabSelectXtra, GE), elution was carried out according to the manufacturer's instructions, having regard to the antibody isotype, previously determined using Mouse Monoclonal Antibody Isotyping Reagents (ISO 2, Sigma).

**Monoclonal antibodies labeling with horseradish peroxidase**

Monoclonal Ab conjugates with horseradish peroxidase were obtained by the periodate-oxidation method.

**Development of an enzyme-linked immunosorbent assay test system for determination of human C1-INH**

To create a sandwich-type ELISA test system for determination of human C1-INH, a selection of mAbs with different epitope specificity was made. For this purpose, each of the obtained mAbs was tested as both a capturing (immobilized on the wells of the plate) and detecting (conjugated with horseradish peroxidase) antibody with respect to other mAbs against C1-INH. Concentration of C1-INH in the calibration samples was selected by successive dilutions of the initial sample. The sample was diluted until no saturation of capture antibodies with C1-INH was observed. The titer of capturing antibodies was

selected in order to ensure optimal characteristics of the ELISA test system, i.e., the consistency of the following parameters: low background values, the calibration sample signal level providing a relatively linear relationship between C1-INH concentration and optical density values on the calibration curve. The titer of the conjugate was determined by ELISA. The antigen was immobilized on the plate well surface, then serial dilutions of the tested mAb HRP conjugate were added into the wells, and the first dilution after which a decrease in the signal level was observed relative to the previous dilution was determined.

**Preparation and characterization of human C1-INH for calibration samples**

Human C1-INH was obtained by chromatography purification from blood plasma using an affinity sorbent with immobilized monoclonal antibodies. Phosphate-buffered saline, pH 7.5 was used as an equilibration buffer, and 3M $MgCl_2$ solution was used as an eluent. The matrix for affinity sorbent was BrCN-activated Sepharose 6B (GE), antibodies were immobilized according to the manufacturer's instructions.

The physicochemical and immunological properties of the obtained human C1-INH were compared with human C1-INH from "Berinert" drug (Aptec) using such methods as SDS-PAGE, immunoblotting, ELISA, and mass spectrometry on UltrafleXtreme MALDI-TOF/TOF mass spectrometer.

**Study of the analytical characteristics of an experimental test system for the quantitative determination of human C1-inhibitor**

Such parameters as the accuracy (correctness) of measurements, assessment of control samples $K1^{+}$ and $K2^{+}$, precision under reproducibility conditions; sensitivity, detection limit, linearity, analytical measurement range; possible influence of interfering substances in blood serum samples; concordance with the reference medical device (MD), diagnostic significance were determined.

To evaluate these parameters, the components of the developed ELISA test system (calibration and control samples) were used, as well as 2 panels of biological samples: the first comprising 24 sera from healthy donors, and 11 sera from patients with confirmed HAE; the second comprising 28 sera from healthy donors, and 7 sera from patients with confirmed type I HAE. As a reference MD, a commercial C1 Esterase Inhibitor assay kit (Aptec) was used, designed for the analysis of C1-INH by the turbidimetric method on a FURONO 270 Clinical Chemistry Analyzer.

To assess the possible influence of interfering substances in blood serum samples, solutions of hemoglobin 1000 mg/dL, bilirubin 60 mg/dL, triglycerides 50 mg/dL were used.

## Results and discussion

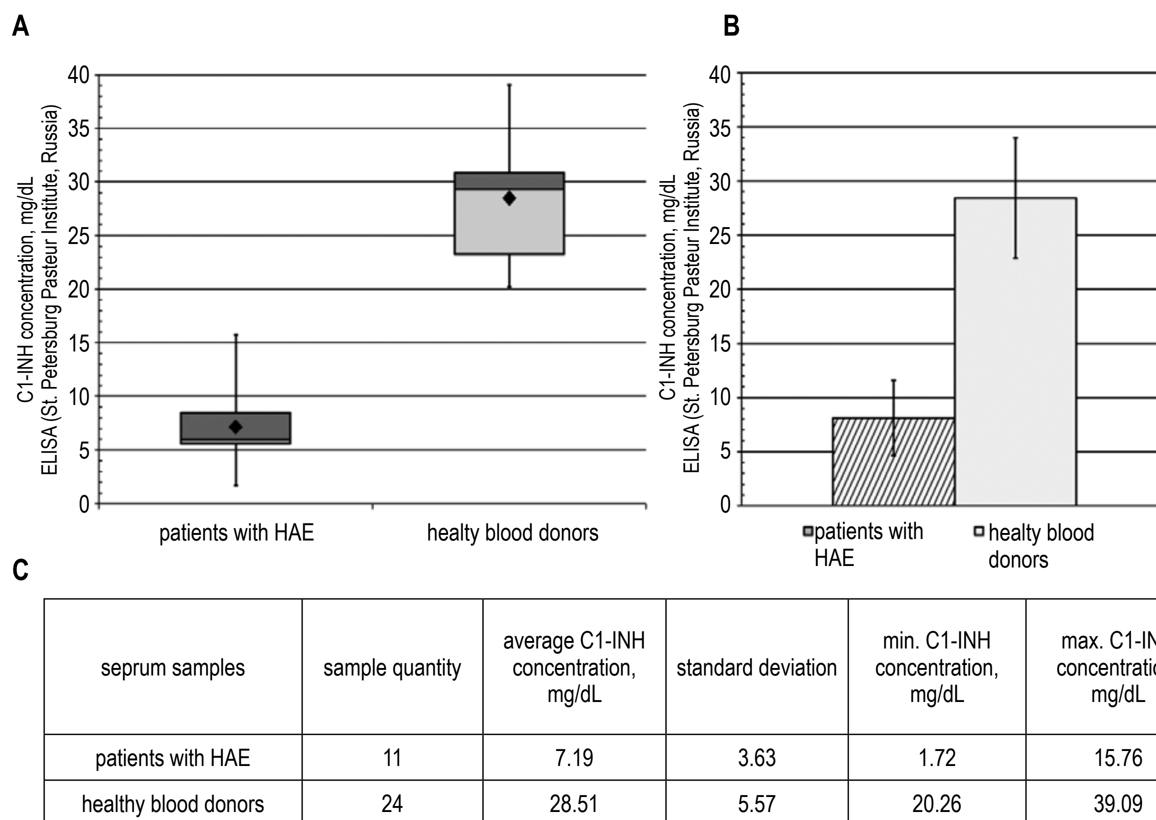
As a result, two mouse monoclonal antibodies, C1-1i and C1-2i, against human C1-INH were obtained.

The antibodies were characterized by ELISA and immunoblotting. The specificity of the obtained antibodies was confirmed by detection in the above assay of the immune complex of target antibodies with the C1-INH protein contained in the commercial "Berinert" drug (control protein), a similar complex was also detected in the presence of human blood serum. One band was stained on the nitrocellulose membrane, corresponding to the molecular weight of C1-INH, 105 kDa, when analyzing a sample loaded to the gel without addition of β-mercaptoethanol. It was determined that the mAbs have different epitope specificity, which made it possible to implement the developed ELISA test system as a sandwich-type assay, where one of the antibodies is capturing and immobilized in the wells of the plate, and the second one is detecting and conjugated with horseradish peroxidase.

A combination of mAbs was determined for the developed ELISA test system, where mAb C1-2i is capturing, and mAb C1-1i is detecting.

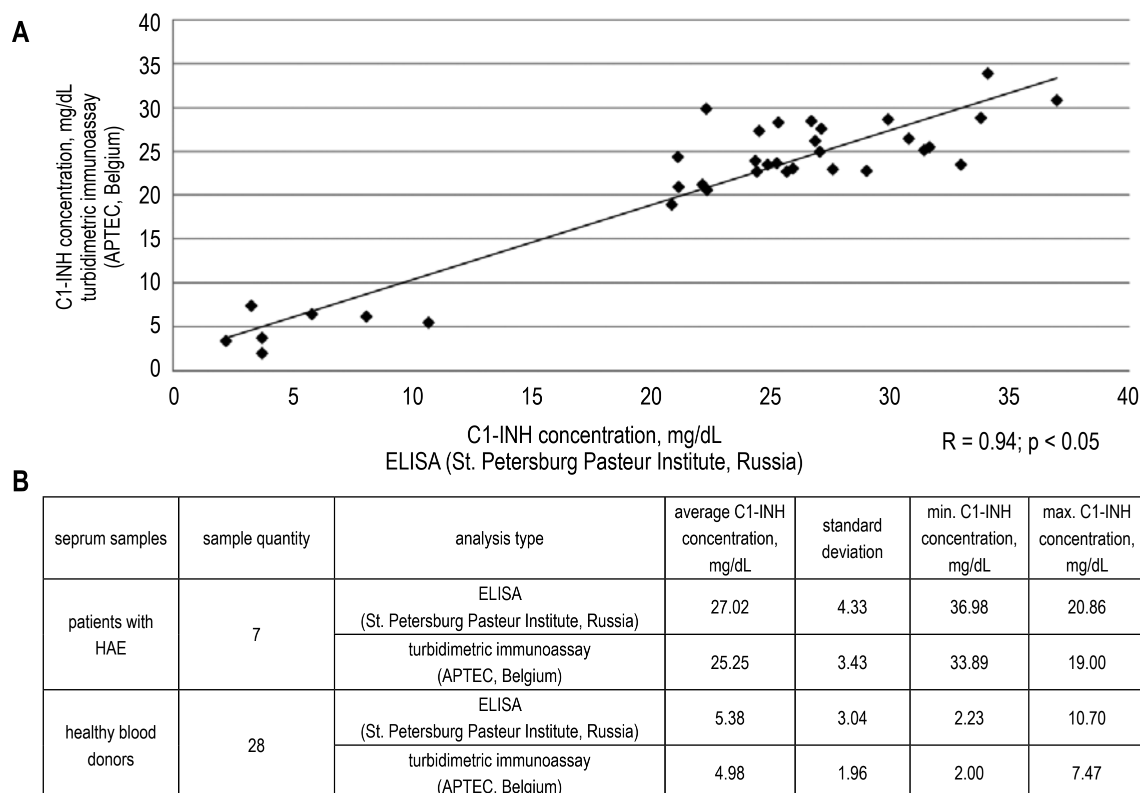
A sample of human C1-INH was obtained and characterized, intended for the preparation of calibration samples. This sample was analyzed using various analytical methods. According to SDS-PAGE, the molecular weight of the protein was 105 kDa, which corresponded to the molecular weight of the control protein. According to the results of immunoblotting using a conjugate of the obtained specific mAb against C1-INH with horseradish peroxidase (C1-1i-HRP), one band was observed on the nitrocellulose membrane, corresponding to the molecular weight of the control protein. MALDI-TOF/TOF mass spectrometric analysis on UltrafleXtreme mass spectrometer was also carried out. The processed spectra were used to identify proteins by accessing the NCBI or SwissProt databases using MASCOT. The mass determination error was limited to 20 ppm. According to the analysis, the studied protein was reliably identified as "complement C1-inh [Homo sapiens]" (NP_000055.2); the Score value was 491 with a threshold value of 68.

Studies of the developed original ELISA test system for the quantitative determination of C1-INH were carried out. In the study of the diagnostic significance, sera from the first panel of biological samples were




| seprum samples | sample quantity | average C1-INH concentration, mg/dL | standard deviation | min. C1-INH concentration, mg/dL | max. C1-INH concentration, mg/dL |
|---|---|---|---|---|---|
| patients with HAE | 11 | 7.19 | 3.63 | 1.72 | 15.76 |
| healthy blood donors | 24 | 28.51 | 5.57 | 20.26 | 39.09 |

**Figure 1. Results obtained when studying the diagnostic significance for the developed original ELISA test system for the quantitative determination of C1-INH**




| seprum samples | sample quantity | analysis type | average C1-INH concentration, mg/dL | standard deviation | min. C1-INH concentration, mg/dL | max. C1-INH concentration, mg/dL |
|---|---|---|---|---|---|---|
| patients with HAE | 7 | ELISA (St. Petersburg Pasteur Institute, Russia) | 27.02 | 4.33 | 36.98 | 20.86 |
| | | turbidimetric immunoassay (APTEC, Belgium) | 25.25 | 3.43 | 33.89 | 19.00 |
| healthy blood donors | 28 | ELISA (St. Petersburg Pasteur Institute, Russia) | 5.38 | 3.04 | 2.23 | 10.70 |
| | | turbidimetric immunoassay (APTEC, Belgium) | 4.98 | 1.96 | 2.00 | 7.47 |

**Figure 2. Results obtained when studying concordance with reference MD for the developed original ELISA test system for the quantitative determination of C1-INH**

**TABLE 1. CHARACTERISTICS OF THE DEVELOPED ORIGINAL ELISA TEST SYSTEM FOR THE QUANTITATIVE DETERMINATION OF C1-INH, OBTAINED DURING THE STUDY**

| Parameter | Value |
|---|---|
| **Accuracy** | within 90-110% |
| **Limit of detection** | 2.00±0.60 ng/mL |
| **Analytical measuring range** | 22.00 to 175.00 ng/mL |
| **Linearity** | within 90% to 110%<br>in the range from 22.00 to 175.00 ng/mL |
| **Repeatability and reproducibility** | the coefficient of variation (CV) does not exceed 15% |
| **Concordance with reference MD** | corresponds |
| **Diagnostic specificity** | 100% |
| **Diagnostic sensitivity** | 100% |

analyzed. The value of diagnostic sensitivity and diagnostic specificity was determined to be at the level of 100%, which indicates the absence of both false negative and false positive results. Based on the data, a graph (Figure 1A) and a histogram (Figure 1B) were plotted, reflecting statistically significant differences between the studied groups of sera, and Figure 1C shows the data used to plot the above graphs.

In the study of concordance with the reference MD, a correlation graph was plotted reflecting the relationship between the values of C1-INH, mg/dL, determined using the developed test system, and the values of C1-INH, mg/dL, determined using the reference MD (Figure 2). For this study, a second panel of biological samples was used. The coefficient R was determined to be 0.940, $p < 0.05$. The value of this coefficient indicates a strong positive correlation of data on the level of C1-INH in the studied samples analyzed using the two test systems. The graph of the correlation is presented in Figure 2A, Figure 2B shows the data used to plot the graph.

Also, such parameters as the accuracy of measurements, assessment of control samples $K1^+$ and $K2^+$, precision under reproducibility conditions; sensitivity, detection limit, linearity, analytical measurement range; possible influence of interfering substances in blood serum samples were determined. All the studied parameters corresponded to the declared ones. The results of the study of these parameters are shown in Table 1.

## Conclusion

As a result of the study, an original ELISA test system for the quantitative determination of C1-INH was developed. “Reagent kit for enzyme-linked immunosorbent assay of human C1-inhibitor (C1-inh PS)”.

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# АКТИВНОСТЬ ЯДЕРНОГО ФАКТОРА ТРАНСКРИПЦИИ κB (NF-κB) В ПОПУЛЯЦИЯХ ЛИМФОЦИТОВ У ДЕТЕЙ С БОЛЕЗНЬЮ ВИЛЬСОНА–КОНОВАЛОВА

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**Резюме.** Болезнь Вильсона (БВ) – редкое наследственное заболевание, вызванное дефицитом транспортера АТФ7В. Накопление меди может вызывать повреждение органов и клеток, преимущественно печени. Воздействие меди может модулировать синтез цитокинов посредством молекулярных и клеточных сигнальных путей, включая путь ядерного фактора транскрипции NF-κB. NF-κB является главным регулятором воспаления и гибели клеток, действует как центральное звено между повреждением печени, фиброзом и гепатоцеллюлярной карциномы. Избыток NF-κB-зависимого цитокинового ответа стимулирует воспалительные реакции, но и чрезмерное ингибирование NF-κB может негативно влиять на жизнеспособность гепатоцитов. Метод проточной цитометрии с визуализацией – Amnis ImageStream$^X$ позволяет оценить активность NF-κB (% активированных клеток, в популяциях клеток). Цель работы – оценить активность NF-κB в популяциях лимфоцитов у детей с болезнью Вильсона–Коновалова. Иммунофенотипирование лимфоцитов и оценку уровня транслокации NF-κB проводили у 52 ребенка с БВ и у 25 детей группы сравнения. Массовую концентрацию меди в суточной моче определяли атомно-абсорбционным методом с помощью спектрометра AAnalyst 800. У детей с БВ содержание клеток с транслокацией NF-κB варьировало от 5 до 90% в зависимости от популяции лимфоцитов, наибольший уровень выявлен в В-клетках и составил 57,5 (37-68) %. Показана достоверная разница в распределениях количества клеток с транслокацией NF-κB в популяциях лимфоцитов между БВ и здоровыми детьми (F-критерий, $p < 0,01$). Для детей с БВ в большинстве случаях характерно снижение активности фактора транскрипции NF-κB в популяциях В-клеток (в 43% случаев), Т-хелперов (48%), Т-цитотоксических (44%) и Th17-лимфоцитов (41%). У детей с БВ

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концентрация меди варьировала от 9,7 до 2582 мкг/сут, Med = 616 (210-1173). Получена прямая зависимость между содержанием меди в моче и уровнем транслокации NF-κB в В-лимфоцитах, r = 0,34, p = 0,016. Активность фактора NF-κB коррелирует с биохимическими маркерами тяжести поражения печени (АЛТ, АСТ, ГГТ) и с содержанием меди в моче. Исследование сигнального пути NF-κB представляется перспективным для большего понимания патогенетических механизмов формирования процессов воспаления и фиброза печени у детей с БВ.

*Ключевые слова: дети, болезнь Вильсона–Коновалова, лимфоциты, Th17, Tc17, проточная цитометрия с визуализацией, NF-κB*

# NUCLEAR TRANSCRIPTION FACTOR κB (NF-κB) ACTIVITY IN LYMPHOCYTE POPULATIONS IN CHILDREN WITH WILSON–KONOVALOV DISEASE

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**Abstract.** Wilson's disease (WD) is a rare hereditary disease caused by a deficiency of the ATF7B transporter. The accumulation of copper can cause damage to organs and cells, mainly the liver. Copper exposure can modulate cytokine synthesis through molecular and cellular signaling pathways, including the nuclear transcription factor NF-κB pathway. NF-κB is the main regulator of inflammation and cell death, acts as a central link between liver damage, fibrosis and hepatocellular carcinoma. An excess of NF-κB-dependent cytokine response stimulates inflammatory reactions, but excessive inhibition of NF-κB can negatively affect the viability of hepatocytes. Method of flow cytometry with visualization – Amnis ImageStreamX allows to evaluate the activity of NF-κB (% of activated cells in cell populations). The aim: to evaluate the activity of NF-κB in lymphocyte populations in children with WD disease. Immunophenotyping of lymphocytes and assessment of the level of translocation of NF-κB were performed in 52 children with WD and in 25 children of comparison group. The mass concentration of copper in daily urine was determined by atomic absorption method using the AAnalyst 800 spectrometer. In children with WD, the content of cells with NF-κB translocation varied from 5 to 90% depending on the lymphocyte population; the highest level was detected in B cells – 57.5 (37-68) %. A significant difference in distributions of the number of cells with NF-κB translocation between WD and healthy children was shown (F-criterion, $p < 0.01$). In most cases, children with WD are characterized by a decrease in the activity of NF-κB in populations of B cells (in 43% of cases), T helper cells (48%), T cytotoxic (44%) and Th17 lymphocytes (41%). In children with WD, the concentration of copper varied from 9.7 to 2582 mcg/day, Me = 616 (210-1173). A direct relationship was obtained between the copper content in urine and the level of translocation of NF-κB in B lymphocytes, r = 0.34, p = 0.016. The activity of the NF-κB correlates with biochemical markers of the severity of liver damage (ALT, AST, GGT) and with copper content in urine. The study of the NF-κB signaling pathway seems promising for a better understanding of the pathogenetic mechanisms of the formation of inflammation and liver fibrosis in children with WD.

*Keywords: children, Wilson–Konovalov disease, lymphocytes, Th17, Tc17, flow cytometry with visualization, NF-κB*

## Introduction

Wilson's disease (WD) is a rare hereditary disease caused by a deficiency of the ATP7B transporter. The protein encoded by this gene promotes the incorporation of copper into the copper-containing protein, ceruloplasmin. In WD, copper accumulates primarily in the liver and secondarily in other organs, such as the central nervous system. WD in some patients is asymptomatic, while others develop acute liver failure. Verification of the diagnosis requires a combination of clinical signs and diagnostic tests such as decreased serum ceruloplasmin levels, increased

urinary copper excretion, liver biopsy or genetic testing [3, 6].

Copper (Cu) is an essential micronutrient, however, excessive accumulation of copper can cause damage to organs and cells, catalyze the formation of free radicals and trigger lipid peroxidation. Malondialdehyde formed as a result of lipid peroxidation stimulates collagen synthesis, promoting fibrogenesis. The toxic effect of copper on the liver tissue can manifest itself in the form of fatty degeneration of hepatocytes, hepatitis, fibrosis and cirrhosis of the liver [12]. The toxicological and inflammatory effects of Cu have been investigated in various animal models and cells [2]. It has been shown that excessive exposure to Cu can modulate cytokine synthesis through various molecular and cellular signaling pathways, including the NF-κB nuclear transcription factor pathway, the MAPKs pathway, the JAK-STAT pathway, and NLRP3 pathway [2].

Disturbances in the immune system in patients with WD have been described, progressing with an increase in the stage of liver fibrosis and with an increase in the concentration of copper in daily urine [5].

The transcription factor NF-κB is the main regulator of inflammation and cell death, in the development of hepatocellular damage, liver fibrosis and hepatocellular carcinoma, acts as a central link between liver damage, fibrosis and hepatocellular carcinoma, however, inhibition of NF-κB can not only provide beneficial influence, but also negatively affect the viability of hepatocytes, especially with pronounced inhibition of NF-κB [7]. The p50 NF-κB subunit plays a critical protective role in damaged liver by limiting TNFα expression and inflammatory cell recruitment. In an experimental model that mimics chronic liver disease, NF-κB-mice developed more severe neutrophilic inflammation and fibrosis compared to NF-κB$^+$ mice [9]. However, an excess of NF-κB-dependent cytokine response can stimulate inflammatory responses. Therefore, NF-κB activity should be kept under control to maintain immune balance [8].

The modern method of flow cytometry with visualization – Amnis Image Stream X allows to evaluate the activity of the transcription factor NF-κB (% of activated cells in which NF-κB passes from the cytoplasm to the cell nucleus) in various cell populations [1]. NF-κB activity has not been studied in children with WD.

**The aim:** to evaluate the activity of NF-κB in the populations of lymphocytes in children with WD.

## Materials and methods

We examined 52 children with WD aged 6 to 18 years, Me 13.6 (11.0-16.4). The comparison group consisted of 25 healthy children (HC) without somatic, autoimmune, oncological pathology, comparable in age. Immunophenotyping of major and minor populations of lymphocytes in peripheral blood was performed by flow cytometry (Novocyte, ACEA Biosciences, USA). The level of NF-κB translocation was assessed on pre-isolated peripheral blood mononuclear cells (PBMCs) by flow cytometry with imaging (Amnis ImageStreamX Mk II) using the Amnis NF-κB Translocation Kit (Luminex, USA). We used monoclonal antibodies labeled with fluorochromes: CD19-PE, CD4-PE, CD8-PE, CD(16/56)-PE, CD161-PE, CD3-ECD, CD4-PB (Beckman Coulter, USA). The following cell populations were studied: $CD3^-CD19^+$ (B lymphocytes); $CD3^-CD16^+/CD56^+$ (NK cells); $CD3^+CD4^+$ (T helpers); $CD3^+CD8^+$ (cytotoxic T lymphocytes); $CD3^+CD161^+CD4^+$ (Th17 lymphocytes); $CD3^+CD161^+CD8^+$ (cytotoxic T17 lymphocytes – Tc17).

The mass concentration of copper in daily urine was determined by the atomic absorption method using an AAnalyst 800 spectrometer (N.F. Izmerov Research Institute of Occupational Medicine).

Statistical processing of the obtained data was carried out using the Statistica 10.0, descriptive statistics of the indicators are presented in the form: Me ($Q_{0,25}$-$Q_{0,75}$). To assess the significance of differences between groups, the nonparametric Mann–Whitney test, the Wilcoxon test for conjugated pairs, and the Fisher test were used. Differences were considered statistically significant at $p < 0.05$.

## Results and discussion

The content of cells with NF-κB translocation in different populations differed significantly both in the group of children with WD and in the comparison group (Wilcoxon test for conjugated pairs, p = 0.000). The highest percentage was characteristic of the B cell population (WD – 57.5 (37-68) %; HC – 58.4 (43-79) %), the smallest for T cells (WD – 16.4 (13-24)) %; HC – 17.6 (16-21) %), intermediate values were found in the NK cell population (WD – 27.1 (19-43) %; HC – 27.8 (20-34) %). The Mann–Whitney test did not reveal significant differences between the groups in terms of the content of cells with NF-κB translocation, however, due to the large scatter of indicators in the group with WD, a significant difference was shown in the distribution of indicators in groups according to the F-criterion (Figure 1A, p < 0.01). The distributions of NF-κB activity in the studied populations differ, with the exception of B cells.

In children with WD, NF-κB activity in Tc17 lymphocytes was 20.8% (12.5-32.5) and exceeded NF-κB activity in T helpers (p = 0.024), Th17 (p = 0.000) and cytotoxic T – lymphocytes (p = 0.001) (Figure 1B). In children of the control group, NF-κB activity in Th17 lymphocytes was 19.3% (17-21) and

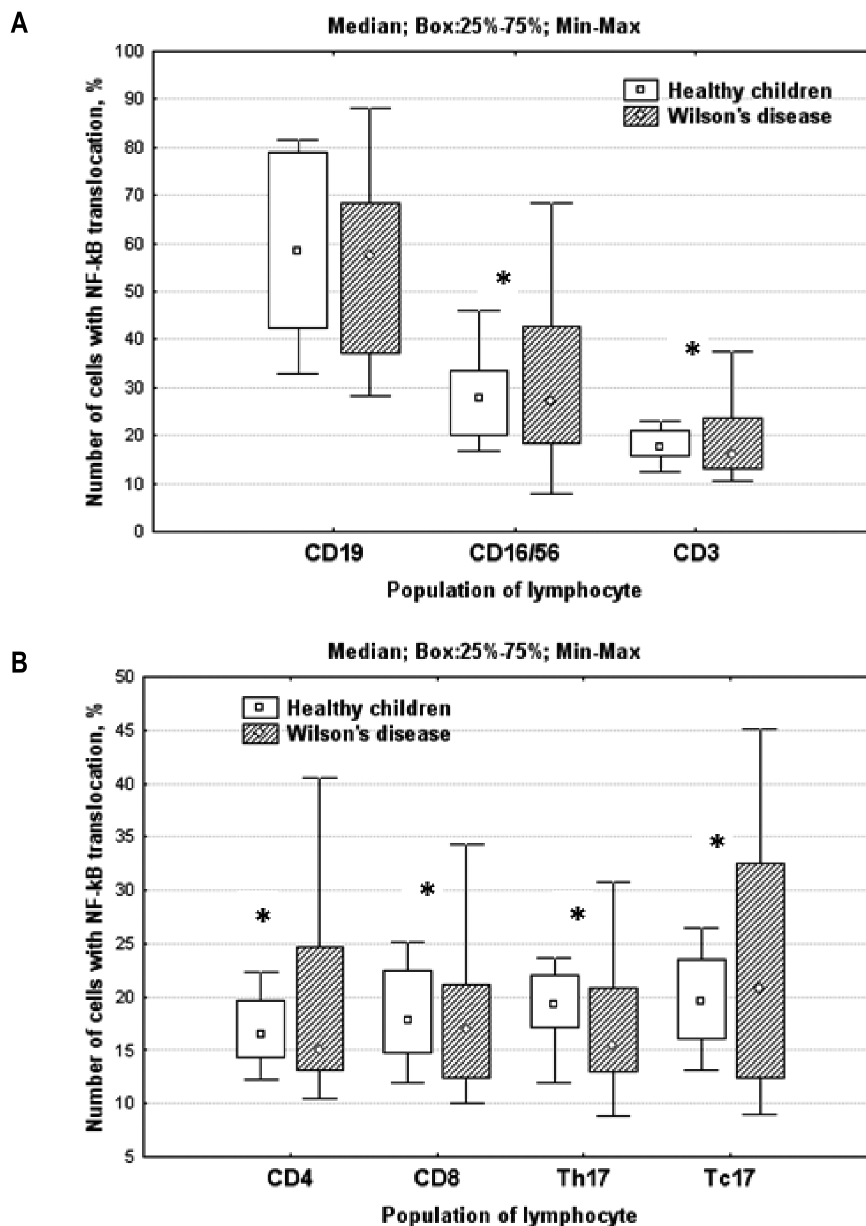


**Figure 1. Content of the main and small populations of lymphocytes with NF-κB translocation in children with wd and healthy children**

Note. *, F-test, $p < 0.01$.

exceeded NF-κB activity in T helpers ($p = 0.033$) (Figure 1C).

To identify the features of NF-κB activity in the group of children with WD, we performed a frequency analysis of the distribution of indicators in all the studied populations of lymphocytes. It was found that the content of cells with NF-κB translocation in B lymphocytes varied from 25 to 90%. At the same time, only in 45% of patients with WD, the content of cells with NF-κB translocation in B lymphocytes corresponded to the level of the comparison group, in 43% of patients this indicator was reduced, and in 12% of patients it was increased (Figure 2A).

The content of cells with NF-κB translocation into NK cells varied from 5 to 70% of activated cells, only in 29% of patients with WD the content of NF-κB in NK cells corresponded to the level of the control group, in 42% of patients this indicator was higher than the control group, decreased in 29% of patients (Figure 2B). The content of cells with NF-κB translocation in the T helper ($CD4^+$) population was characterized by less variability – from 10 to 50% of activated cells, and in most children with WD (48%) a decrease in this indicator was noted, and corresponded to the level of the comparison group only in 21% of patients (Figure 2C). A similar distribution was also characteristic of the T-cytotoxic lymphocyte

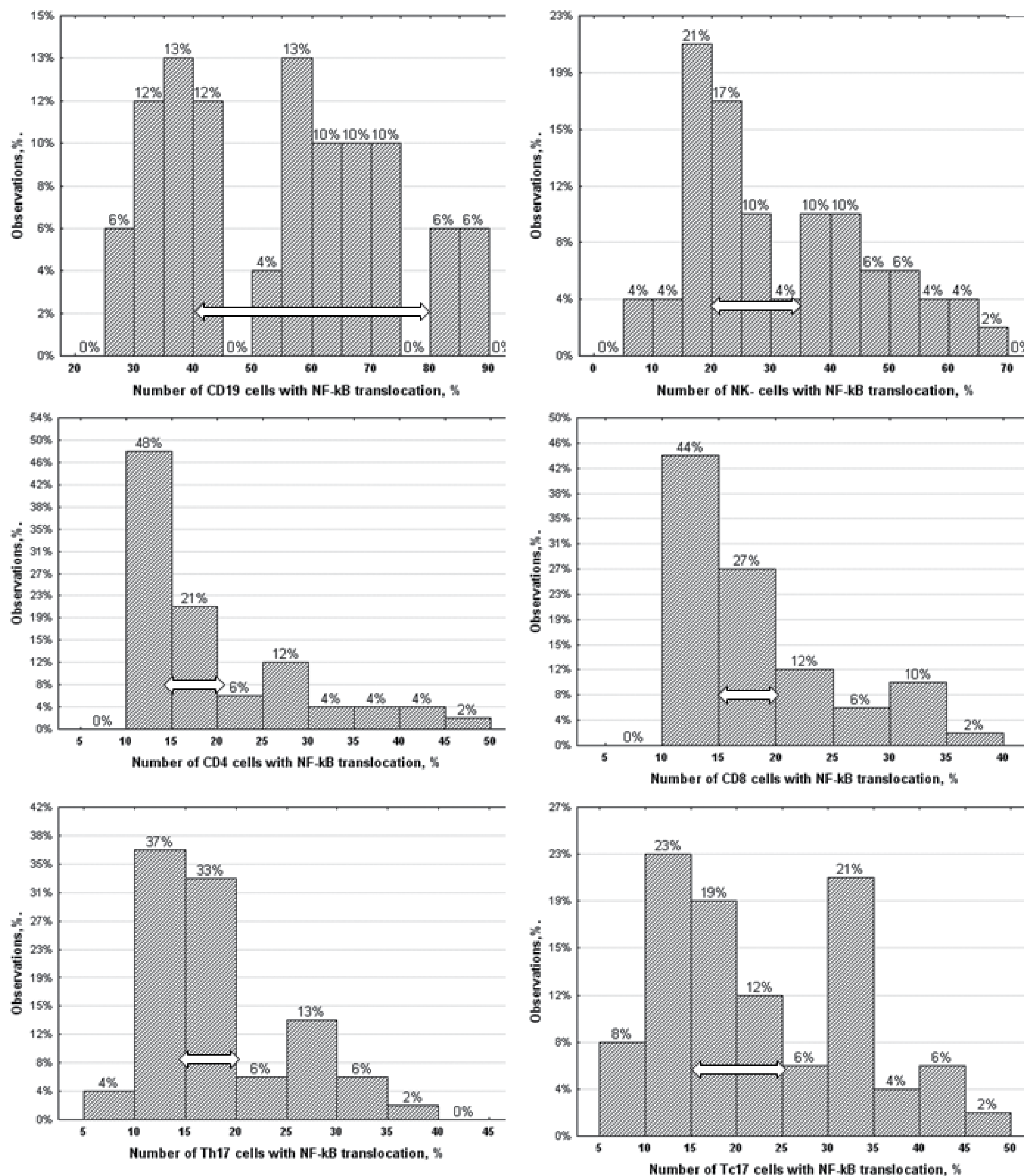


**Figure 2. Distribution of the number of cells with NF-κB translocation in different populations of lymphocytes in children with WD**

population: a decrease in 44% and an increase in 29% of patients (Figure 2D). The content of cells with NF-κB translocation in the Th17 lymphocyte population was reduced in 41%, increased in 26% of patients (Figure 2E). In the Tc17 lymphocyte population, a decrease was found in 31% and an increase in 38% of patients (Figure 2F). An increase in NF-κB activity was most characteristic of the NK cell population (42% of observations), and the increase in the content of cells with NF-κB translocation in children with WD reached 70%.

It is interesting to note that in children with WD, the translocation level indicators are comparable with those of the comparison group (medians do

not differ significantly), in contrast to children with autoimmune diseases (IBD, psoriasis), who have an increased level of NF-κB activity [4, 10, 11].

The number of lymphocytes with reduced NF-κB activity in the studied populations ranged from 29 to 48% of patients. This fact may be explained by the fact that lymphocytes reflect the intensity of inflammatory processes in the liver, which is consistent with the data of Luedde T. that complete blockade of NF-κB in hepatocytes enhances liver damage [7].

At the next stage of the study, we analyzed the correlations between the content of major and minor populations of lymphocytes and the percentage of activated cells in these populations. An analysis of cellular immunity parameters in this cohort of children with WD confirms the previously published data on the presence of an increase in the content of T helpers, regulatory T cells, Th17 lymphocytes and activated T helpers against the background of a decrease in cytotoxic T lymphocytes and NK cells relative to the comparison group [5].

An inverse correlation was found between the relative number of B cells and the number of B lymphocytes with NF-κB translocation, r = -0.347, p = 0.012. Correlation analysis between the content of other populations of lymphocytes and activated cells with NF-κB translocation in these populations did not reveal statistically significant relationships. The level of NF-κB translocation in the lymphocyte populations of children with WD also did not depend on age.

Significant sensitive markers of liver damage and the proportion of affected cells with NF-κB translocation were identified. Thus, the concentration of alanine aminotransferase (ALT) increased with an increase in the number of activated cells with NF-κB translocation in the populations of T helpers (r = 0.53, p = 0.000), T lymphocytes (r = 0.5, p = 0.000), cytotoxic T lymphocytes (r = 0.48, p = 0.000), Th17 lymphocytes (r = 0.41, p = 0.003) and NK cells (r = 0.32, p = 0.023). The concentration of aspartate aminotransferase (AST) increased with an increase in the number of activated cells with NF-κB translocation in the populations of T helpers (r = 0.55, p = 0.000), cytotoxic T lymphocytes (r = 0.53, p = 0.000), T lymphocytes (r = 0.52, p = 0.000), Th17 lymphocytes (r = 0.28, p = 0.041).

The concentration of gamma-glutamine transferase (GGT) increased with an increase in the number of activated cells with NF-κB translocation in the populations of T helper cells (r = 0.54, p = 0.000), T lymphocytes (r = 0.52, p = 0.000), cytotoxic T lymphocytes (r = 0.5, p = 0.000), Th17 lymphocytes (r = 0.42, p = 0.0021). Thus, an increase in the number of cells with NF-κB translocation in populations of T cell immunity may reflect the severity of the patient's condition, possibly by activating the inflammatory pathway involving NF-κB [13].

Considering the toxicological and inflammatory effects of copper described by Deng H. [2] and the ability of copper to influence the NF-κB signaling pathway, we investigated the relationship between urinary copper levels and NF-κB activity in lymphocyte populations. In children with WD, the copper concentration ranged from 9.7 to 2582 μg/day, Me = 616 (210-1173). We obtained a statistically significant direct relationship between the copper content in urine and the level of NF-κB translocation in B lymphocytes, r = 0.34, p = 0.016.

## Conclusion

Thus, in most cases, children with WD are characterized by a decrease in the activity of the transcription factor NF-κB in populations of B cells, T helpers, T cytotoxic and Th17 lymphocytes. The activity of the factor correlates with biochemical markers of the severity of liver damage, as well as with the content of copper in the urine in children with WD. The study of the NF-κB signaling pathway seems to be promising for a better understanding of the pathogenetic mechanisms of the formation of liver inflammation and fibrosis in children with Wilson's disease.

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# ЦИТОКИНЫ СЛЮНЫ ПРОТИВ ПЛАЗМЫ КАК ВОЗМОЖНЫЕ ПРЕДИКТОРЫ ТЯЖЕСТИ АУТИЗМА У ДЕТЕЙ

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**Резюме.** Расстройства аутистического спектра (РАС) являются широко распространенными, гетерогенными нарушениями нейроразвития с множественной этиологией, подтипами и траекториями развития, для которых отсутствуют доступные и эффективные биомаркеры. Важным фактором риска, способствующим дефициту развития нервной системы, наблюдаемому при РАС, является иммунная дисфункция, которая проявляется в том числе дисбалансом цитокинов в мозге и на периферии. В последние годы в качестве биологического материала для диагностики РАС предложена слюна, что обусловлено доступностью и неинвазивностью метода ее получения. Вместе с тем, вопрос о том, могут ли уровни цитокинов в слюне быть использованы в качестве эффективных ранних биомаркеров аутизма требует дополнительных исследований, в том числе сравнений: слюна против плазмы/сыворотки крови.

Цель – провести сравнительный анализ уровней цитокинов: IL-6, IFNγ, TNFα, IL-1β, IL-4, IL-10, в слюне и плазме крови для выделения возможных маркеров РАС и их тяжести у детей.

В исследование было включено 11 детей с типичным нейроразвитием (ТРД) и 55 детей с диагнозом РАС, среди которых 37 человек имели легкую или умеренную степень тяжести аутизма (по CARS), а 18 детей – тяжелую. У всех детей одномоментно были собраны образцы не стимулированной смешанной слюны и венозной крови. Концентрации цитокинов: IL-6, IFNγ, TNFα, IL-1β, IL-4, IL-10, слюне определяли multiplex Luminex™ analysis. Плазменные уровни тех же цитокинов оценивали с помощью ELISA. Различия между группами проверяли с помощью U-критерия Краскела–Уоллиса, с апостериорными попарными сравнениями по Коноверу–Инману, между образцами (плазма/слюна) – парного критерия Уилкоксона. Наличие зависимости между концентрациями цитокинов в плазме и слюне определяли с помощью линейной регрессии методом RMA.

Во всех обследуемых группах уровни IL-6, IFNγ и IL-10 в слюне были ниже, а TNFα, IL-1β и IL-4 – выше, чем соответствующие уровни тех же цитокинов в плазме. Не зависимо от состояния здоровье/болезнь, значимых корреляций между уровнями цитокинов в слюне и плазме у детей не обнаружено. Значимых различий в концентрациях цитокинов слюны между детьми с легкой и тяжелой степенью РАС не обнаружено, однако уровни IL-1β были значимо ниже, а IL-10 – выше, в слюне обеих групп детей с РАС, по сравнению с ТРД.

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Таким образом, цитокины слюны могут быть использованы в качестве маркеров РАС у детей, но не тяжести состояния. Отсутствие корреляций в уровнях некоторых про-/противовоспалительных цитокинов между слюной и плазмой крови, вероятно, может свидетельствовать об особом иммунологическом статусе экологической ниши – ротовая полость.

*Ключевые слова: цитокины, слюна, плазма, расстройства аутистического спектра, дети, диагностика*

# SALIVA VERSUS PLASMA CYTOKINES AS POSSIBLE PREDICTORS OF AUTISM SEVERITY

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**Abstract.** The autism spectrum disorders (ASD) are now widely accepted as a pervasive, complex, heterogeneous neurodevelopmental disorders with multiple etiologies, subtypes, and developmental trajectories. There are no available and effective biomarkers for them. Immune dysfunction is seen as an important risk factor contributing to the neurodevelopmental deficit in ASD, and is signified, among other things, by an imbalance of cytokines in the brain and on the periphery. In recent years, saliva has been proposed as a biological material for diagnosing ASD, due to the accessibility and non-invasiveness of the method for its production. However, the question of whether salivary cytokine levels may be used as effective early biomarkers for autism requires further research, including saliva versus plasma/serum comparisons.

Aim: a comparative analysis of the levels of cytokines: IL-6, IFNγ, TNFα, IL-1β, IL-4, IL-10, in saliva and blood plasma to identify possible markers of ASD and their severity in children.

The study included 11 children with typical neurodevelopment (TDC) and 55 children with ASD, among whom 37 children had mild or moderate autism (according to CARS), and 18 children had severe autism. Samples of unstimulated mixed saliva and venous blood were simultaneously collected from all children. Salivary concentrations of cytokines: IL-6, IFNγ, TNFα, IL-1β, IL-4, IL-10 were determined by multiplex Luminex™ analysis. Plasma levels of cytokines were assessed by ELISA. Differences between groups were tested using the Kruskal–Wallis U-test with post-hoc Conover–Inman comparisons, between samples (saliva/plasma) are using the Wilcoxon signed-rank test. The correlation between the concentrations of cytokines in plasma and saliva was determined using linear regression by the RMA method.

In all examined groups, the levels of IL-6, IFNγ and IL-10 in saliva were significantly lower, and TNFα, IL-1β and IL-4 were higher than the corresponding levels of the same cytokines in plasma. Regardless of health/disease status, no significant correlations were found between salivary and plasma cytokine levels in children. IL-1β levels were significantly lower and IL-10 levels were higher in the saliva of both groups of children with ASD compared with TDC. No significant differences in salivary cytokine concentrations were found between children with mild and severe ASD.

Thus, salivary cytokines can be used as markers of ASD in children, but not the severity of the condition. The absence of correlations in the levels of some pro/anti-inflammatory cytokines between saliva and blood plasma may probably indicate a special immunological status of an ecological niche, the oral cavity.

*Keywords: cytokines, saliva, blood plasma, autism spectrum disorders, children, diagnosis*

## Introduction

The autism spectrum disorders (ASD) is now widely accepted as a pervasive, complex, heterogeneous neurodevelopmental disorders with multiple etiologies, subtypes, and developmental trajectories [8]. Currently there are no available diagnostic biomarkers and the diagnosis of ASD is based on typical features that include repetitive behaviors, and impaired social communication and interaction [11]. The etiology of ASD is largely unknown but, in most cases, likely due to a combination of genetic and environmental factors [1]. Compounding evidence supports the role of maternal immune system activation (MIA), particularly due to infection, at the specific periods of gestation as a risk factor for autism [10]. The exact molecular pathways that lead from MIA to ASD are not clear, however, a fair amount of research indicates that cytokines and chemokines may be the key elements in this process [13]. Normally, cytokines regulate growth, cell proliferation and synaptogenesis in nervous tissue. Cytokines also modulate host responses

to infection, injury, inflammation and diseases of uncertain etiology [7]. However, the results of the studies addressing serum or plasma levels of cytokines in autism appear to be inconsistent, probably due to these inconsistencies reflect the heterogeneity of the ASD diagnosis [12].

In addition, the invasiveness and painfulness of the procedure combined with the neuropsychological features of children with ASD provides difficulties in obtaining plasma samples. In recent years, a new direction has been developing – the use of saliva as a biological material for the diagnosis of a number of pathological conditions, including autism [3, 5], which is due to the accessibility of biological material and the non-invasive method of obtaining it.

According to the current protocol for assessing the immunity of saliva, it is a biological fluid with a high potential of antibacterial and antiviral molecules. Saliva contains a large number of immune cells, among which 95% are heterogeneous neutrophils, and the rest are lymphocytes and myeloid cells. The antigen-presenting cells, neutrophils in the network of cells of the innate immune system, T- and B-lymphocytes are predominant in the oral mucosa. Moreover, saliva plays an important role in the regulation of the oral microbiome. Salivary mucin, antimicrobial peptides and proteins, produced by salivary neutrophils, help innate selection of bacterial colonization and biofilm formation [4]. However, the question of whether salivary cytokine levels may be used as effective early biomarkers of neurodevelopmental diseases requires further research, including saliva versus plasma/serum comparisons.

**Aim:** a comparative analysis of the levels of cytokines: IL-6, IFNγ, TNFα, IL-1β, IL-4, IL-10, in saliva and plasma to identify possible markers of ASD and their severity in children.

## Materials and methods

A total of 66 children participated in the study. The study comprised 55 children diagnosed with autism spectrum disorders (ASD, male / female ratio 43/13, age range 3-13 years) and 11 neurotypical children (TDC) (male/ female ratio 9/2, age range 4-13 years). The ASD children met the International Classification of Diseases $10^{th}$ Revision (ICD-10) criteria for Childhood autism (F84.0) and Atypical autism (F84.1), which were determined a child psychiatrist and a psychologist. Using the Childhood Autism Rating Scale (CARS), children with ASD were divided into 2 groups: 37 people with mild or moderate autism (mean CARS score 32.0±1.5) and 18 people with severe autism (mean CARS score 39.0±3.4). The protocol for this study was approved by the Bioethics Committee of Chelyabinsk State University (2/2019). Written informed consent was obtained from the parents of each child before any study procedure was carried out.

Unstimulated mixed saliva was collected from all children between 8:00 and 9:00 a. m., by passive flow in SaliCaps tubes (IBL International, Germany). One hour before sampling, ASD and TDC children refrained from eating, drinking, mouth rinsing, and teeth brushing. Venous blood samples in a volume of 4 mL were collected at the same time in vacuette tubes with $K_3EDTA$. Blood was centrifuged for 10 min at 3000 rpm to obtain plasma. Saliva and plasma samples were stored frozen at -70 °C until assayed.

The concentrations of the cytokines: IL-6, IFNγ, TNFα, IL-1β, IL-4, IL-10 in saliva were determined by multiplex Luminex™ analysis, using a 41-plex Human Cytokine/Chemokine Magnetic Bead Panel (Merck, Millipore) and a MAGPIX analyzer (Luminex). Plasma levels of the cytokines were measured using the commercial ELISA kit (JSC Vector-Best, Novosibirsk, Russia) on a MultiscanEX plate analyzer.

The statistical analysis was carried out with PAST software (v. 4.03). Data are presented as medians and interquartile ranges (IQR). The differences between groups were checked using the Kruskal–Wallis test with post-hoc Conover–Inman comparisons, between samples (plasma/saliva) were using the Wilcoxon signed-rank test. The correlation between the concentrations of cytokines in plasma and saliva was determined using linear regression by the RMA method.

## Results and discussion

The results of the assessment of the concentration of cytokines: IL-6, IFNγ, TNFα, IL-1β, IL-4, IL-10 in the saliva and plasma of children with ASD and TDC are summarized in the Table 1.

We found that in all the examined groups of children (regardless of the state of health/disease), the levels of IL-6, IFNγ and IL-10 in saliva were lower, and the levels of TNFα, IL-1β and IL-4 were higher than the corresponding levels of the same cytokines in plasma. All differences were statistically significant except for IL-6 concentrations in the TDC group (Table 1). This may indicate the determination of the immune response by the humoral type in saliva and by the cellular type in plasma.

Analysis of the levels of some pro/anti-inflammatory cytokines in saliva depending on the presence/absence of ASD and its severity showed that the concentrations of IL-1β were significantly lower, and the concentrations of IL-10 were significantly higher in the saliva of children with ASD, compared with TDC (Table 1). Thus, in the saliva of children with ASD, we showed the activation of the anti-inflammatory potential, whose indicators may be used as markers of autism, but not of the severity of the condition.

In plasma, in contrast to saliva, children with severe ASD have reduced concentrations of early

**TABLE 1. CYTOKINE LEVELS IN PLASMA AND SALIVA OF TDC AND CHILDREN WITH MILD / SEVERE ASD, Me ($Q_{0.25}$-$Q_{0.75}$)**

| Parameters (pg /mL) / source | TDC | mild ASD | severe ASD |
|---|---|---|---|
| **IL-6 / saliva** | 0.89<br>(0.67-1.57) | 1.28<br>(0.92-1.69) | 1.45<br>(0.71-2.57) |
| **IL-6 / plasma** | 2.29<br>(0.89-3.00)<br>$W_{(11)}$ = 54.0; p = 0.060 | 1.89<br>(0.67-3.06)<br>$W_{(37)}$ = 507.0; p = 0.019 | 3.83* **<br>(2.31-5.78)<br>$W_{(18)}$ = 146.0; p ≤ 0.001 |
| **IFNγ / saliva** | 0.49<br>(0.28-0.80) | 0.83<br>(0.59-1.44) | 0.59<br>(0.44-1.36) |
| **IFNγ / plasma** | 11.13<br>(8.91-11.15)<br>$W_{(11)}$ = 66.0; p = 0.003 | 10.48<br>(9.00-11.48)<br>$W_{(37)}$ = 703.0; p = 0.001 | 14.58* **<br>(11.99-15.93)<br>$W_{(18)}$ = 171.0; p ≤ 0.001 |
| **TNFα / saliva** | 6.58<br>(4.10-7.83) | 7.27<br>(3.37-8.99) | 7.0<br>(3.59-12.94) |
| **TNFα / plasma** | 2.24<br>(1.53-3.40)<br>$W_{(11)}$ = 66.0; p = 0.030 | 2.69<br>(2.36-3.60)<br>$W_{(37)}$ = 637.0; p = 0.001 | 1.29* **<br>(1.17-1.69)<br>$W_{(18)}$ = 165.0; p ≤ 0.001 |
| **IL-1β / saliva** | 60.85<br>(43.43-101.64) | 5.60*<br>(2.04-14.06) | 8.18*<br>(3.77-46.55) |
| **IL-1β / plasma** | 3.07<br>(1.59-3.55)<br>$W_{(11)}$ = 65.0; p = 0.004 | 3.20<br>(2.54-4.32)<br>$W_{(37)}$ = 532.0; p = 0.006 | 1.86* **<br>(1.62-2.09)<br>$W_{(18)}$ = 165.0; p ≤ 0.001 |
| **IL-4 / saliva** | 10.89<br>(6.67-31.36) | 9.46<br>(5.95-13.67) | 10.89<br>(6.67-30.01) |
| **IL-4 / plasma** | 2.22<br>(1.80-2.35)<br>$W_{(11)}$ = 66.0; p = 0.003 | 2.72*<br>(2.40-3.07)<br>$W_{(37)}$ = 690.0; p = 0.001 | 2.21**<br>(2.00-2.46)<br>$W_{(18)}$ = 170.0; p ≤ 0.001 |
| **IL-10 / saliva** | 1.15<br>(0.69-1.50) | 2.33*<br>(1.61-3.57) | 2.94*<br>(1.20-6.54) |
| **IL-10 / plasma** | 9.68<br>(6.47-11.84)<br>$W_{(11)}$ = 66.0; p = 0.004 | 7.75<br>(6.38-10.42)<br>$W_{(37)}$ = 636.0; p = 0.001 | 5.91* **<br>(3.20-8.50)<br>$W_{(18)}$ = 136.0; p = 0.028 |

Note. ASD, autism spectrum disorders; TDC, typically developing children. *, statistically significant differences between the indicators of the groups “TDC” and “mild ASD” / “severe ASD” (p ≤ 0.05); **, statistically significant differences between the indicators of the groups “mild ASD” and “severe ASD” (p ≤ 0.05). W is the value of the Wilcoxon test, p is the level of significance of differences between the cytokine values in plasma and saliva.

inflammatory response cytokines – TNFα, IL-1β and anti-inflammatory – IL-10, against the background of an increased concentration of late inflammatory response cytokines – IL-6 and IFNγ, possibly supporting systemic low-grade inflammation (Table 1). Thus, plasma cytokines are relevant predictors of ASD severity in children.

An assessment of the links between the concentrations of cytokines in saliva and plasma showed no significant correlations, regardless of the presence/absence of ASD and its severity (Figure 1).

Different concentrations of cytokines and the lack of correlations between salivary and plasma parameters suggest that salivary cytokine levels are not representative of plasma concentrations in children, regardless of the presence/absence of neurodevelopmental disorders. Although 30% of salivary proteins are filtered out of plasma [6], cytokines are too large to enter saliva via either diffusion or ultra-filtration. Therefore, these molecules enter saliva via leaky patches, such as tissue damage sites and inflammation, as well as crevicular fluid. Maintenance of the basic level of cytokines in saliva occurs with the help of the immune components of the mucous membranes of the oral cavity [2].

It is known that the oral cavity is the entrance gate to the human body. Commensal microorganisms, airborne antigens/allergens, and foodstuffs initially enter the oral mucosa before entering the gastrointestinal tract and respiratory tract. For the normal functioning of the body, the local immune system of the oral cavity provides a delicate balance: it provides effective immune surveillance without an excessive inflammatory response, and at the same time tolerance to commensals and harmless antigens [9]. Violation of this balance towards inflammation can lead to serious consequences for the body as a whole. Therefore, the anti-inflammatory potential of saliva in children with ASD and the absence of correlations in the levels of some pro/anti-inflammatory cytokines between saliva

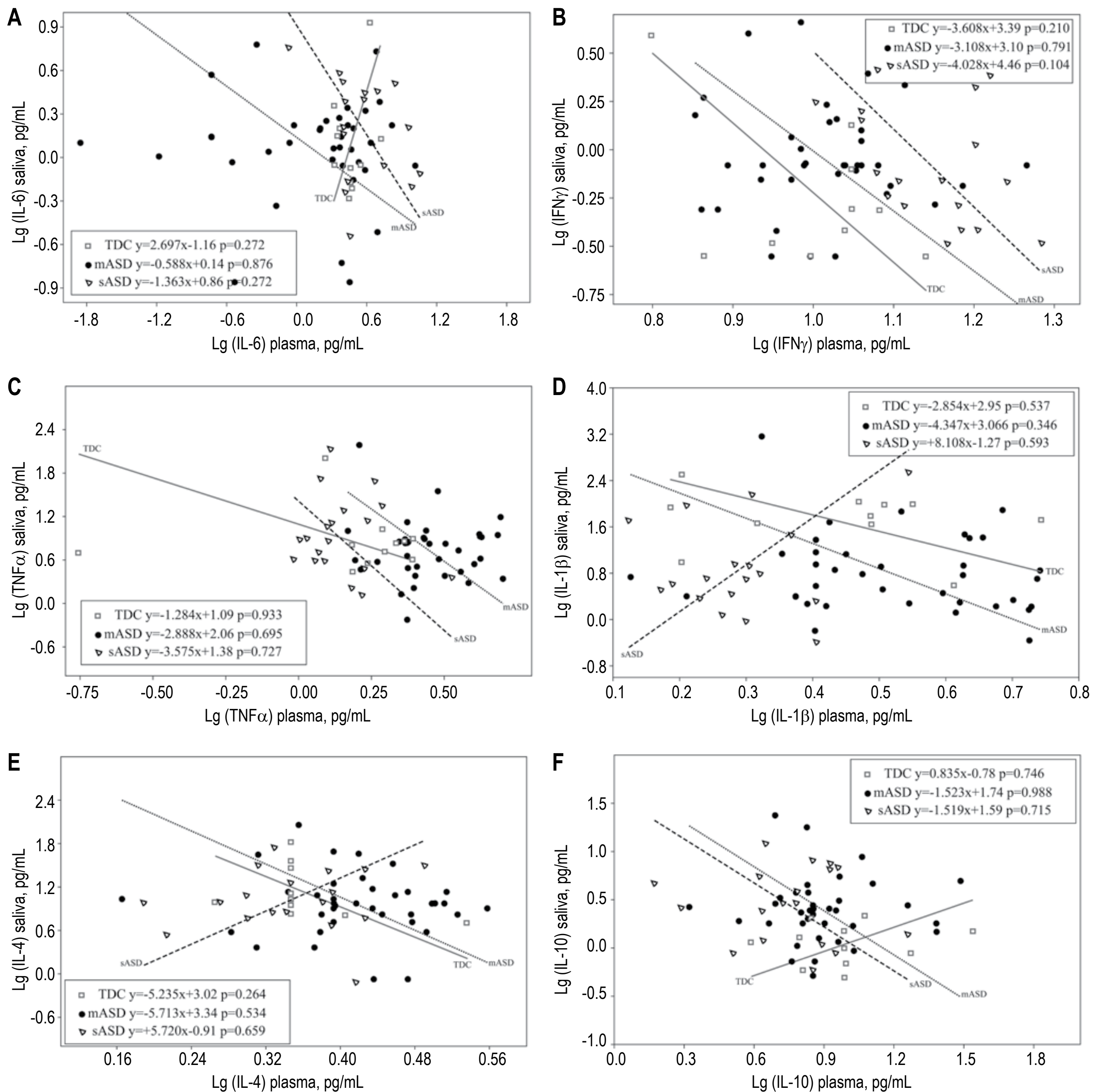


**Figure 1. Linear regression between salivary and plasmatic levels of cytokines in typically developing children (TDC) and children with mild/severe autism (mASD/sASD)**

Note. Linear regression saliva versus plasma cytokines: (A) IL-6, (B) IFNγ, (C) TNFα, (D) IL-1β, (E) IL-4, (F) IL-10 are shown. TDC, typically developing children; mASD, childen with mild autism; sASD, childen with severe autism Cytokine concentrations are converted to base logarithm 10. The lines show the regression equations for the TRD – solid line, midl ASD – dots line, severy ASD – long dashes line. Values cytokines for TDC are shown by squares, mild ASD by dots, severe ASD by triangles. y = ax+b – regression equation. p, the level of significance of the corelations between saliva and plasma.

and blood plasma, shown in our pilot study, can probably indicate the effectiveness of the regulatory functions of the ecological niche, the oral cavity.

## Conclusion

Thus, saliva and plasma are two completely different systems, with their own development programs, structural components and functions. Changes of salivary cytokine concentrations do not correlate with plasma cytokine levels and do not reflect ASD severity in children. It is probably necessary to talk about the study of salivary immunome, which reflects the homeostasis/destabilization of a unique local niche, its tissue immunity, which, like any tissue immunity, is a component of the axis of the immune-neuroendocrine systems and the microbiome of the digestive tract.

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# РОЛЬ IL-6 В ИММУНОПАТОГЕНЕЗЕ УШИБА ГОЛОВНОГО МОЗГА РАЗЛИЧНОЙ СТЕПЕНИ ТЯЖЕСТИ

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**Резюме.** Иммунная система имеет ключевую роль в патогенезе черепно-мозговой травмы. Травматические повреждение головного мозга обуславливает высвобождение молекул «опасности» с последующем вовлечением клеток врожденного иммунного ответа, инициирующих развитие нейровоспаления. Возникающие нарушения в иммунной системе при черепно-мозговой травме являются проявлением защитной реакции организма, при этом исход черепно-мозговой травмы обусловлен не только тяжестью первичного поражения головного мозга, но и вторичными реакциями. Нейровоспаление – это иммунный ответ на поражение мозга, в ходе которого происходит высвобождение молекул, связанных с повреждением, дальнейшей активацией и пролиферацией клеток микроглии и астроглии, миграцией в зону повреждения Т-лимфоцитов, обладающих как протективным, так и деструктивным действием в отношении мозговой ткани. Управляющую роль в данных процессах играют цитокины – белки, продуцируемые резидентными клетками глии, опосредующие межклеточные взаимодействия при различных патологических состояниях. Уже на ранних стадиях развития в ответ на травму клетками микроглии синтезируются провоспалительные цитокины, которые дополнительно стимулируют активацию микроглии и блокируют процессы ремиелинзации. Показано, что после тяжелой травмы головного мозга высокие концентрации IL-6 в цереброспинальной жидкости демонстрируют прямую корреляцию со степенью тяжести и исходом заболевания. Целью данного исследования явилось изучение особенностей уровня IL-6 в цереброспинальной жидкости пациентов с ушибом головного мозга легкой, средней и тяжелой степени тяжести. Методом мультиплексного анализа по технологии xMAP определяли концентрацию IL-6 в цереброспинальной жидкости. Контролем служили образцы цереброспинальной жидкости пациентов с сотрясением головного мозга. Обнаружено достоверно повышенное содержание у всех пациентов с ушибом головного мозга: 19,59 пг/мл в группе с ушибом легкой степени тяжести, 103,6 пг/мл в группе с ушибом средней степени тяжести и 2225 пг/мл в группе с ушибом тяжелой степени тяжести против 2,58 пг/мл в контрольной группе. Установлена прямая корреляционная взаимосвязь с содержанием основного белка миелина в цере-

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броспинальной жидкости, что отражает степень воспаления и процессов нейродегенерации. Выявление особенностей содержания IL-6 у больных с ушибом головного мозга может свидетельствовать об его важной роли в течения заболевания. А также требует дополнительного более детального изучения, сопоставления с результатами содержания IL-6 в периферической крови.

*Ключевые слова: черепно-мозговая травма, биомаркер, провоспалительные цитокины, нейровоспаление, IL-6, основной белок миелина, мультиплексный анализ*

# ROLE OF IL-6 IN THE IMMUNOPATHOGENESIS OF MILD, MODERATE AND SEVERE TBI

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**Abstract.** Traumatic brain injury (TBI) results in a significant inflammatory burden that increase the production of inflammatory mediators and biomarkers. The immune system plays a key role in the pathogenesis of traumatic brain injury. Neuroinflammatory mediators released from resident glia (activated microglia and astrocytes) inside the brain recruit immune cells where cytokines are small soluble proteins that confer instructions and mediate communication among immune and non-immune cells. Interleukin-6 (IL-6) is a proinflammatory cytokine known to be elevated after trauma, and a major contributor to the inflammatory response following TBI. Previous studies have investigated associations between IL-6 and outcome following TBI, but to date, studies have been inconsistent in their conclusions. The purpose of the current study was to assessment of cerebrospinal fluid (CSF) interleukin-6 (IL-6) and MBP levels in patients with TBI. Samples of cerebrospinal fluid of 85 patients with TBI were examined. Concentrations IL-6 were measured via xMAP multiplexing technology. The control was the course of CSF in patients with concussion. An increased content was found in all patients with traumatic brain injury: 19.59 pg/mL in the group with mild traumatic brain injury; 103.6 pg/mL in the group with moderate traumatic brain injury; and 2225 pg/mL in the group with severe traumatic brain injury load versus 2.58 pg/mL in the control group. A direct correlation was found with the presence of basic myelin proteins in the cerebrospinal fluid, which indicates the degree of damage and neurodegeneration processes. Identification of the features of IL-6 content in patients with brain injury may indicate its important role in the course of disease. It also requires additional more detailed study, including comparison with IL-6 content in peripheral blood.

*Keywords: traumatic brain injury, biomarker, inflammatory cytokines neuroinflammation, IL-6, myelin basic protein, multiplex analysis*

## Introduction

Traumatic brain injury (TBI) is defined as intracranial injury when a direct and indirect external mechanical force transmitted to the head or body results in structure of the brain damage and dysfunction, change in its functioning [5]. Extensive progress has been made in understanding the immunopathogenesis over the last 10 years. Many of the issues that TBI patients face are thought to be mediated by the immune system. TBI results in a significant inflammatory burden that increases the production of inflammatory mediators and biomarkers. It is a disease with a wide variety of injury mechanisms and tissue pathologies.

The clinical presentation and prognosis TBI depend on both the type and severity of the exposure termed the "primary injury" which leads to primary structural and functional brain damage of varying degrees and prevalence at the molecular, subcellular, cellular, tissue and organ levels with abnormalities of the central regulation of body system. When the brain is injured, it can cause disturbance of cerebral circulation, fluid circulation, hypothalamic-pituitary-adrenal system and blood-brain barrier (BBB) [3]. Following the primary injury extensive and lasting damage is sustained through a complex cascade of events referred to as "secondary injury". Secondary injury includes BBB-disturbance, excitotoxicity, mitochondrial dysfunction, oxidative stress and neuroinflammation [10]. Thus, the pathogenesis of brain injury may be divided into two injury-mechanisms: primary and secondary [12].

There is increasing interest in the role the immune system in TBI pathogenesis because neuroinflammation caused by detrimental or beneficial outcomes. In addition, neuroinflammation leads to appear CSF Myelin basic protein (MBP). MBP is a constituent of the sheath, is essential for normal myelination and axonal signal conduction, and mediates adhesion

between cytoplasmic surfaces of individual myelin layers. Brain injury contributes to increased MBP in blood and CSF. Neuroinflammation instigated by TBI is a complex immune process resulting from a mechanical compression insult and depending on the degree of the insult and leads to axon damage occurs due to direct cytotoxic intercellular interaction or due to the synthesis of pro-inflammatory cytokines and chemokines [2].

Already at the early stages of development in response to damage microglial cells synthesize IL-1β, IFNγ, IL-6, IL-12, IL-18, which can subsequently induce the synthesis of GM-CSF and CCL2 by astrocytes, additionally stimulating microglial activation and blocking remyelination processes [4, 6]. Elevated levels of many cytokines have been noted in peripheral blood plasma and cerebrospinal fluid (CSF), but the clinical results of their determination are often contradictory [12]. It has been shown that after TBI high concentrations CSF IL-6 directly correlate with the severity and outcome [7]. IL-6 together with TGFβ stimulates the maturation clones of T-lymphocytes into of Th17 and suppresses the development of Treg, which provides aggravating the course of the disease [8].

One of the factors in the development of the inflammatory process induced by trauma and accompanied by a structural impairment of the brain and BBB permeability is immune dysfunction the main regulators of which are cytokines and their synthesis have provided by resident neuronal and glial cells of the brain that secrete pro- and anti-inflammatory cytokines [1]. In addition, the source of cytokines in the CNS is the cells of the immune system recruited to the focus of inflammation due to a violation of the BBB [4]. The inflammatory process that occurs during traumatic damage to brain tissues accompanied by hypersecretion of proinflammatory and inhibitory cytokines is essential during and after TBI. Canonically, a shift in cytokine profile towards anti-inflammatory mediator predominance can increase neuroprotection and regeneration of the CNS after injury [7, 11]. However, the role of IL-6 in TBI pathogenesis remains insufficiently studied and literature reviews are often contradictory, debatable and require further study.

**The purpose of the current study** was to assessment of CSF Interleukin-6 (IL-6) and MBP levels in patients with TBI.

## Materials and methods

Informed consent was obtained from patients for sample collection. The study included 85 TBI patients (aged 18-55 years (mean: 42.3±11.3). Cerebrospinal fluid (CSF) samples were obtained from patients with the diagnosis of TBI at the time of the patient's admission to the hospital. Control CSF samples were obtained from twenty-five age-matched controls who underwent lumbar puncture.

According to the international classification, TBI is classified as mild, moderate or severe, typically based on the Glasgow Coma Scale (GCS) score:

1 group – control (n = 25);

2 group – mild brain injury (n = 30);

3 group – moderate brain injury (n = 31);

4 group – severe brain injury (n = 24);

CSF was obtained by lumbar puncture. All samples were collected in tubes and they were centrifuged (1100 g, 10 min, room temperature), aliquoted into several portions and frozen at -80 °C until assayed.

The CSF IL-6 (pg/mL) level were measured using Luminex xMAP technology for multiplexed quantification. The samples were analyzed using the "Milliplex MAP" (Millipore) (USA) with magnetic microspheres "Milliplex Mag" (USA), according to the manufacturer's instructions. Registration and analysis of data on the device "Luminex MAGPIX" (Luminex) (USA). All samples were assayed in duplicate wells and the mean of the ensuing results was used.

Statistical analysis of the data was performed on commercially available software (GraphPad Prism 5.00 for Mac). The nonparametric Mann–Whitney U test was used for analysis of data. A P value less than 0.05 was considered to be statistically significant. Data is presented as the median (Me) and interquartile range ($Q_{0.25}$-$Q_{0.75}$). Spearman's correlation coefficient was used for assesses how well the relationship between MBP and IL-6.

## Results and discussion

In order to investigate the cytokine level patients with brain injury of varying severity underwent lumbar puncture according to indications. A comparative analysis was carried out with samples of the cerebrospinal fluid of patients with concussion, which was due to the absence of structural changes in them, on the one hand, as well as the difficulties of collecting cerebrospinal fluid (CSF) from apparently healthy individuals, as a group that does not have indications for this medical manipulation, on the other hand.

Our study was showed that TBI patients had a significantly higher CSF level of IL-6 as compared to the 1st group (Control) (Table 1).

IL-6 in all groups with TBI: 19.59 pg/mL (8.4-46.5) at p = 0.0117 in the 2nd group (Mild TBI), 103.6 pg/mL (27.4-138.7) at $p < 0.0001$ in the 3rd group (Moderate TBI) and 2225 pg/mL (872.3-3739) in the 4th group (Severe TBI) at $p < 0.0001$ as compared to the 1st group (2.58 pg / mL (1.1-3.7)).

In order to investigate the diagnostic values of the IL-6 for TBI we performed receiver operating characteristic (ROC) curve analysis. Using ROC curves, sensitivity and specificity were calculated for each possible threshold value. The ROC characteristic significant increased were IL-6 (Figure 1): sensitivity – 87.5%; specificity -100%; AUC = 0.906; Cutoff = 11.4 pg/mL, p = 0.0117 for the 2nd group (Mild TBI); sensitivity – 94%; specificity -100%; AUC = 0.941; Cutoff = 17.33 pg/mL, $p < 0.0001$ for the 3rd group (Moderate TBI); sensitivity -100%; specificity – 87.5%; AUC = 0.961; Cutoff = 241 pg/mL, $p < 0.0001$ for the 4th group (Severe TBI). The AUC of IL-6 all greater than 0.7 which indicated that they represented a high diagnostic value.

Additionally, to identify IL-6 characteristics of TBI with different severity we performed receiver operating characteristic (ROC) curve analysis. The optimal cut-off for CSF IL-6 application in the 3rd group was 46.14 pg/mL; the sensitivity and specificity were 69% and 75%, respectively (Figure 2).

The optimal cut-off for CSF IL-6 application in the 4th group was 240.9 pg/mL; the sensitivity and specificity were 100% and 88%, respectively. The 4th group appeared to have the best discriminatory ability with an AUC of 0.981.

In this paper, we report the optimal cut-off for CSF IL-6 the 2nd group was 11.4 pg/mL.

Spearman's test showed there was clinically relevant correlation between IL-6 and MBP level (Figure 3) in TBI patients ($r = 0.465$; $p = 0.017$). These findings reflect the degree of inflammation and the process of neurodegeneration.

TBI activates microglia, the endogenous brain immune cells and a major source of pro-inflammatory cytokines in the central nervous system. In recent years, the functions and role of IL-6 have been actively studied in the in the pathogenesis of inflammation from brain injury.

Some studies have identified associations between IL-6 levels and outcome following TBI. Inflammation in the context of TBI can be contributed to CNS damage, including astrogliosis and disruption of the BBB. Obviously, neuroinflammation contribute to breakdown of the BBB and infiltration of immune cells. So, in the study by Monsour M. [9] an increase in IL-6 is considered as an unfavorable marker of outcomes. According to our results, an increased concentration of IL-6 was found in all patients with TBI and had significantly significant informative indicators ($p < 0,05$).

**TABLE 1. CEREBROSPINAL FLUID LEVELS OF CYTOKINES (PG/ML) IN PATIENTS WITH TBI, Me ($Q_{0.25}$-$Q_{0.75}$)**

| Cytokines | Cerebrospinal fluid levels of cytokines (pg/mL) | | | | Statistically significant (p) |
|---|---|---|---|---|---|
| | Concussion (1st group) | Mild TBI (2nd group) | Moderate TBI (3rd group) | Severe TBI (4th group) | |
| | n = 25 | n = 30 | n = 31 | n = 24 | |
| IL-6 | 2.5 (1.1-3.7) | 19.59 (8.4-46.5) | 103.6 (27.4-138.7) | 2225.0 (872.3-3739.0) | $p_{1-2} = 0.011$ $p_{1-3} < 0.0001$ $p_{1-4} < 0.0001$ |

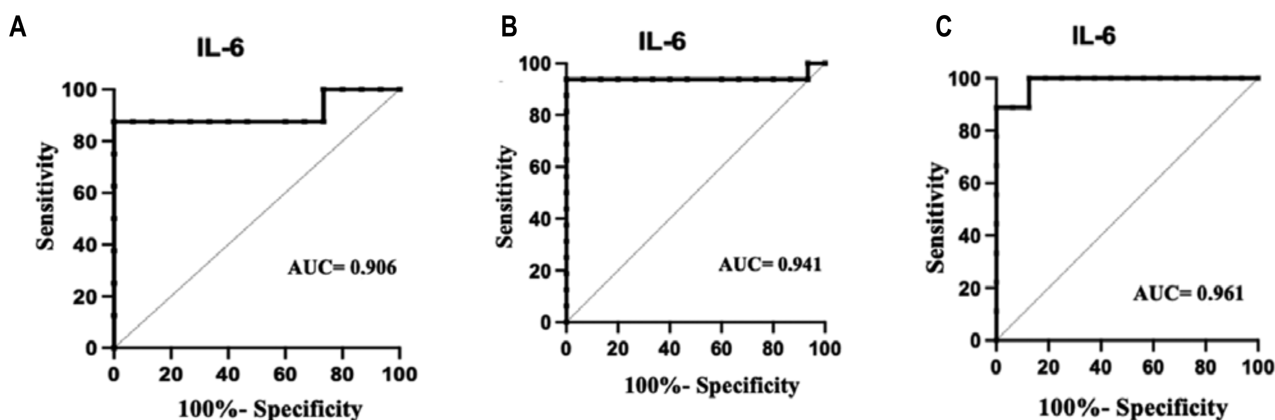


**Figure 1. ROC curves and AUC of CSF IL-6: mild TBI (A), moderate TBI (B) and severe TBI (C) compared to control**

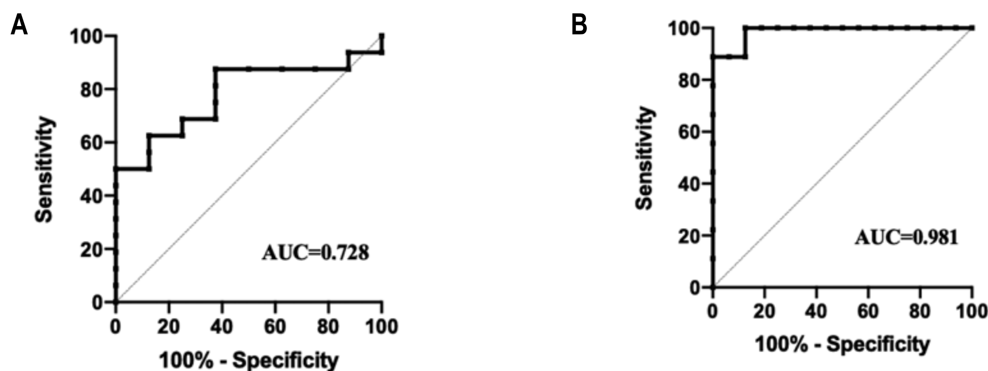


**Figure 2. ROC curve and AUC of CSF IL-6: mild TBI (A) and moderate TBI (B) compared to severe TBI**

The use of ROC analysis data allows for differential diagnosis of brain contusion based on the assessment CSF IL-6 showed that IL-6 ≥ 11.4 pg/mL – Mild TBI, CSF IL-6 ≥ 46.14 pg/mL – Moderate TBI, CSF IL-6 ≥ 240.9 pg/mL – Severe TBI (Figure 4).

Also, based on the fact that IL-6 is a pro-inflammatory cytokine and can influence the degree and risk of demyelination, a correlation analysis was performed between IL-6 and MBP level. Thus, MBP is a specific structural component of neuronal membranes, which is necessary for myelination of oligodendrocytes and maintenance of myelin structure. The detection of MBP in the CSF is a sensitive marker of myelin degradation. In healthy people, this indicator is not determined. Myelin degradation in addition to TBI is possible with neuroinfectious and demyelinating diseases. Additionally, the level of IL-6 was positively correlated with the level of MBP ($r = 0.465$; $p = 0.017$).

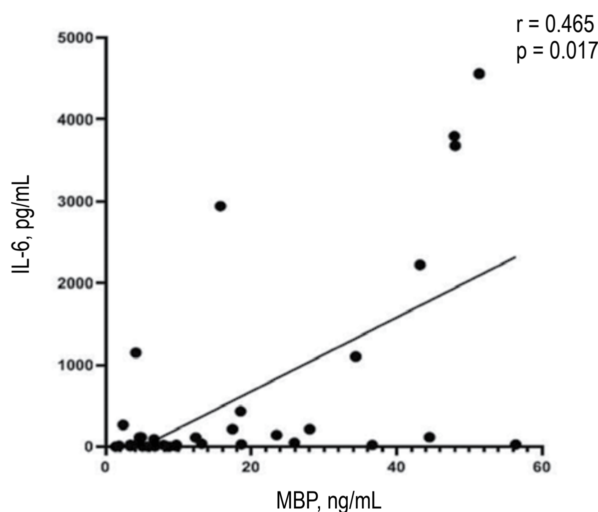


**Figure 3. Correlation between the concentration of myelin basic protein (ng/mL) and IL-6 (pg/mL)**

Note. r, Spearman's correlation coefficient; MBP, myelin basic protein.

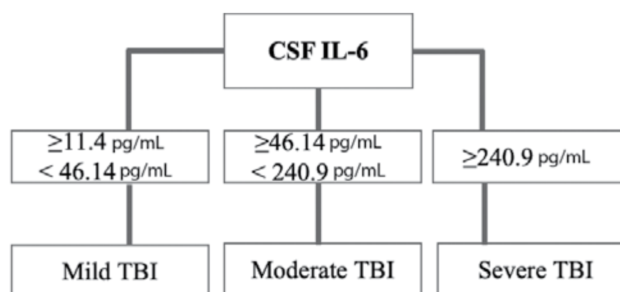


**Figure 4. Algorithm in differential diagnosis of brain injury**

## Conclusion

Thus, TBI is a reaction of the whole organism to CNS injury. It causes a violation of homeostasis and is accompanied by a complex of morphofunctional changes not only in the area of damage, but throughout the brain, and then in other organs and systems. The inflammatory process that occurs during traumatic damage to brain tissues, accompanied by hypersecretion of pro-inflammatory cytokines (IL-6), is essential during and after TBI.

All of these results suggest the important role of IL-6 in TBI immunopathogenesis, as a cytokine that supports neuroinflammation and which is involved in white matter damage.

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# ОЦЕНКА ВЗАИМОСВЯЗИ ПОЛИМОРФИЗМА IL17A G-197A С ИММУНОЛОГИЧЕСКИМИ НАРУШЕНИЯМИ И СТРУКТУРНЫМИ ИЗМЕНЕНИЯМИ МОЗГА ПРИ ШИЗОФРЕНИИ

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**Резюме.** Шизофрения – хроническое психическое заболевание, которое вызывается сложной палитрой генетических, эпигенетических факторов и повреждающих воздействий окружающей среды. Шизофрения сопровождается структурными изменениями головного мозга, ассоциированными с клинической симптоматикой. К значимым патогенетическим компонентам шизофрении относятся иммунологические нарушения и системное воспаление, приводящие к нейровоспалению, которое является важным фактором в развитии структурных изменений мозга. Ранее нами показано, что повышение уровня интерлейкина-17A связано с морфометрическими изменениями мозга, активацией системного воспаления и Th2-звена адаптивного иммунитета при шизофрении. Генетический полиморфизм IL17A G-197A (rs2275913) может влиять на уровень секреции интерлейкина-17A. Целью данной работы было изучение ассоциаций между полиморфизмом IL17A G-197A, изменениями

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иммунитета и морфометрическими показателями мозга у больных для получения новых данных об иммунопатогенезе шизофрении. В основную группу вошли 60 человек с диагнозом «шизофрения» в возрасте от 18 до 42 лет. Контрольную группу составили 85 человек без когнитивных нарушений, сопоставимых с больными по полу и возрасту. Для определения концентрации цитокинов и хемокинов в сыворотке крови использовался мультиплексный анализ. Полиморфизм гена IL17A G-197A определяли методом полимеразной цепной реакции с электрофоретической детекцией продуктов амплификации. В результате проведенного исследования впервые был выявлен ряд взаимосвязей между генетическим полиморфизмом IL17A G-197A и показателями иммунитета у больных шизофренией. У носителей аллеля G наблюдалось значительное повышение IFNγ – ключевого цитокина Th1-звена адаптивного иммунитета, и IL-8 – хемокина, являющегося медиатором воспаления. Также у больных – носителей аллели G было отмечено повышение уровня хемокина CXCL16, который может стимулировать секрецию других провоспалительных хемокинов и участвует в активации Th1-звена адаптивного иммунитета. Кроме того, в данном исследовании была впервые обнаружена ассоциация гетерозиготного генотипа GA полиморфизма IL17A G-197A со снижением толщины коры головного мозга в ряде областей фронтальной коры при шизофрении. Изменения толщины коры в некоторых из этих областей, включая среднюю лобную извилину и орбитофронтальную кору, связано с патогенезом негативной симптоматики у больных. Полученные результаты свидетельствуют о важности иммуногенетических факторов в патогенезе шизофрении и показывают перспективность дальнейшего изучения полиморфизма IL17A G-197A на расширенных выборках больных как потенциального биомаркера иммунной дисрегуляции и морфометрических изменений мозга при шизофрении.

*Ключевые слова: хемокины, цитокины, IL-17A, магнитная резонансная томография, однонуклеотидный полиморфизм, средняя толщина коры, шизофрения*

# ASSOCIATIONS OF IL17A G-197A SINGLE NUCLEOTIDE POLYMORPHISM WITH IMMUNOLOGICAL PARAMETERS AND STRUCTURAL CHANGES OF THE BRAIN IN SCHIZOPHRENIA

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**Abstract.** Schizophrenia is a chronic mental disorder that is caused by a complex palette of genetic, epigenetic and environmental factors. Some of the important components of its pathogenesis are systemic inflammation and the dysfunction of immunity, which lead to neuroinflammation, contributing to development of structural brain changes. Earlier we have shown that increase in interleukin-17A levels is associated with morphometric changes and immune dysregulation in schizophrenia. IL17A G-197A (rs2275913) genetic polymorphism is involved in determining interleukin-17A secretion. The goal of this work was to investigate the associations between rs2275913 polymorphism, immune disorders and structural neurovisualization findings in schizophrenia to provide new insights into the immunopathogenesis of this disease. 60 patients aged 18 to 42 years diagnosed with schizophrenia were enrolled. 85 healthy volunteers were included into the control group. Multiplex assay was used to determine cytokine and chemokine serum levels. Rs2275913 polymorphism was assessed by polymerase chain reaction with electrophoretic detection of amplification products. A number of relationships between rs2275913 polymorphism and the immune parameters in schizophrenia were revealed. Carriers of G allele showed significant increase in IFNγ, a key cytokine of Th1-link of adaptive immunity, and

IL-8, an inflammatory chemokine. Also, increased levels of CXCL16 were observed in patients carrying the G allele. CXCL16 activates secretion of other proinflammatory chemokines and is involved in activation of Th1 adaptive immunity. Associations of heterozygous GA genotype with reduced cortical thickness in a number of areas of the frontal cortex in schizophrenia were found. Changes in cortical thickness in some of these areas, including middle frontal gyrus and orbitofrontal cortex, can be relevant to the pathogenesis of schizophrenia. The results highlight the importance of immunogenetic factors in the pathogenesis of schizophrenia and indicate that the rs2275913 polymorphism requires further studies as a potential biomarker of immune dysregulation and morphometric brain changes in schizophrenia.

*Keywords: chemokines, cytokines, IL-17A, magnetic resonance imaging, mean cortical thickness, schizophrenia, single nucleotide polymorphism*

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## Introduction

Schizophrenia is a chronic mental disorder that is caused by a complex palette of genetic, epigenetic and environmental factors [5]. Symptoms of schizophrenia include disturbances in thinking, cognitive function, affects, emotional and psychomotor disorders. Schizophrenia is also characterized by structural brain changes that are associated with the severity and character of clinical symptoms, as well as with the duration of the disease [13].

The modern genetic, immunological, neuroimaging and other research methods allow to accumulate a large amount of data on the multiple factors of schizophrenia pathogenesis, which have not been fully deciphered yet. One of the most important components of the pathogenesis of schizophrenia is associated with dysfunction of immunity and systemic inflammation, which lead to the development of neuroinflammation and dysregulation of the immune response in the CNS [7]. Neuroinflammation is an important factor in the development of structural changes of the brain in schizophrenia. Therefore, it is important to study the role of immunogenetic factors that can influence systemic inflammation, immune dysregulation and inflammation in the development of morphometric changes of the CNS in schizophrenia.

In our previous studies, elevated levels of the cytokine IL-17A were found to have significant associations with morphometric changes of the brain in schizophrenia patients, and were also associated with systemic inflammation and activation of the Th2 type of the adaptive immune response [3].

The cytokine IL-17A plays a key role in protection against extracellular bacterial and fungal infections. However, overproduction of this protein is associated with immunoinflammatory and autoimmune diseases. The main source of IL-17A are Th17 cells, which induce local inflammatory processes in response to extracellular pathogens and autoimmune responses [1]. The pathogenetic role of IL-17A in neurodegenerative diseases and inflammatory CNS diseases is widely studied and is due to the fact that it can stimulate proinflammatory cytokine synthesis by microglial cells of the central nervous system (CNS), increase the permeability of the blood-brain barrier (BBB), permeate the BBB, support neuroinflammation and promote excessive activation of the glutamatergic neurotransmitter system. This leads to excitotoxic neuronal damage and has a depressing effect on neurogenesis in the hippocampus [2].

Therefore, it is relevant to study the factors influencing IL-17A levels in schizophrenia and their possible contribution to the development of morphometric changes of the brain in patients. The IL17A G-197A (rs2275913) genetic polymorphism is involved in determining IL-17A secretion. This SNP is located in the promoter region of the gene, near the nuclear factor of activated T cells (NFAT) binding motif. It has been suggested that substitution of nucleotide G for A in this position can lead to changes in IL-17A cytokine production [8]. Therefore, it is relevant to study the relationship of IL17A G-197A SNP with immunological disorders and morphometric parameters of the CNS in patients with schizophrenia [10].

The aim of this work was to investigate the associations between the IL17A G-197A polymorphism, immune disorders and the findings of structural brain MRI in schizophrenia patients to provide new insights into the immunopathogenesis of schizophrenia.

## Materials and methods

The clinical sample consisted of 60 patients aged 18 to 42 years diagnosed with schizophrenia (F20.0) who were undergoing treatment at N.A. Alekseev Psychiatric Clinical Hospital No.1. 85 persons without cognitive impairment (31 men, 54 women), comparable with patients with schizophrenia by sex and age, were included into the control group. All

participants signed an informed voluntary consent form. The conduct of the study was approved by the local ethical committee of the National Research Center "Kurchatov Institute" (No. 5 of 05.04.2017).

The content and functional activity of lymphocyte subpopulations were analyzed by flow cytoflowmetry. Monoclonal antibodies for immunophenotyping manufactured by Becton Dickinson (USA) were used for cell staining.

A multiplex assay (Merck Millipore, Germany) was used to determine cytokine and chemokine concentrations in blood serum.

IL17A G-197A gene polymorphism was determined by PCR with electrophoretic detection of amplification products. Two parallel amplification reactions with two pairs of allele-specific primers were performed. PCR amplification products were separated in a 3% agarose gel. Ethidium bromide solution was used as a dye to visualize PCR products in the gel.

MRI brain scans were performed on a Siemens Magnetom Verio 3T magnetic resonance imager (Siemens GmbH, Germany). A 32-channel brain coil was used to acquire data. High-resolution anatomical data based on T1-weighted sequences (TR = 1900 ms, TE = 2.21 ms, 176 slices, voxel size $1 \times 1 \times 1$ mm$^3$) were obtained for gyrification, grey and white matter morphometry, and cerebrospinal volume for each subject. The obtained structural images were analyzed in Freesurfer program designed for processing and analysis of human brain MRI. This program allowed a complete morphometry of the brain. Based on the analyzed data, the index of local cerebral gyrification was calculated.

Excel (Microsoft, 2010) and STATISTICA 10 (Stat Soft, 2010) programs were used for statistical processing. The Shapiro-Wilk test was used to assess the normality of the distribution. Results were presented as means with 95% confidence intervals; when comparing two groups, the significance of differences was assessed using Student's test. In the case of discrete variables, Fisher's exact test was used to assess the significance of differences. Differences between variables were considered statistically significant at $p < 0.05$.

**TABLE 1. FREQUENCY OF IL17A G-197A SNP IN PATIENTS WITH SCHIZOPHRENIA AND IN HEALTHY CONTROLS**

| Polymorphism | Group | Total number of patients | Homozygotes for the 1st allele | | Heterozygotes | | Homozygotes for the 2nd allele | |
|---|---|---|---|---|---|---|---|---|
| | | | n | % | n | % | n | % |
| IL17A G-197A (rs2275913) | Schizophrenia | 85 | 29* | 34.1 | 39* | 45.9 | 17* | 20.0 |
| | Controls | 100 | 80 | 80.0 | 19 | 19.0 | 1 | 1.0 |

Note. *, significant differences with the control group (p < 0.05).

**TABLE 2. ASSOCIATIONS BETWEEN IL17A G-197A SNP, CYTOKINE, CHEMOKINE LEVELS AND CELL IMMUNITY PARAMETERS IN PATIENTS WITH SCHIZOPHRENIA**

| Parameter | GG | GA | AA | Controls |
|---|---|---|---|---|
| IFNγ, pg/mL | 106.6±54.0 * p = 0.036 | 169.3±109.8 * p = 0.038 | 85.8±61.5 | 48.1±26.9 |
| IL-8, pg/mL | 71.5±32.7 * p = 0.028 | 89.2±36.9 * p = 0.005 | 56.6±50.9 | 26.3±14.6 |
| CCL3, pg/mL | 1194.8±2441.1 | 47.1±25.3 * p = 0.026 | 27.2±22.6 | 25.4±9.0 |
| CXCL16, pg/mL | 774.4±147.78 | 706.8±271.0 * p = 0.048 | 690.0±56.4 | 639.9±36.7 |
| $CD3^-CD16^+CD56^+$ (NK cells), % | 12.3±2.7 | 10.0±2.3 | 16.1±4.1 * p = 0.04 | 11.9±1.9 |
| $CD3^-CD8^+CD16^+CD56^+$ ($CD8^+$ expressing NK cells), % | 4.2±1.5 | 3.3±1.9 | 6.6±3.2 * p = 0.04 | 3.7±0.6 |

Note. *, significant differences with the control group (p < 0.05).

## Results and discussion

The study to identify the IL17A G-197A (rs2275913) polymorphism of the IL17A gene revealed statistically significant differences in the frequency of this SNP between the schizophrenia patient group and the control group (Table 1).

As a result of this study, a number of relationships between the studied genetic polymorphism IL17A G-197A (rs2275913) and the immune parameters in schizophrenia patients were revealed for the first time (Table 2).

Thus, carriers of G allele of the investigated SNP showed significant increase in IFNγ, a key cytokine of Th1-link of adaptive immunity, and IL-8, a chemokine mediating inflammation. In AA homozygotes, these indices did not differ from the norm. However, they had an increased content of $CD3^-CD16^+CD56^+$NK cells and of $CD8^+$ expressing $CD3^-CD8^+CD16^+CD56^+$NK cells. According to the literature, this subpopulation of NK cells has an immunoregulatory function and may contribute to neuroprotection in brain diseases [6]. The association between IL17A G-197A SNP and $CD3^-CD8^+CD16^+CD56^+$NK cells content has not been previously studied and requires further research.

In addition, carriage of the G allele of SNP IL17A G-197A was found to be associated with a tendency for elevated levels of the chemokine CCL3. This proinflammatory chemokine has a marked chemotactic effect on neutrophils, monocytes and macrophages. Previous studies involving this chemokine in patients with schizophrenia did not show significant differences with controls, which may have been due to insufficient statistical power of sampling [11].

Also, increased levels of CXCL16 were observed in patients carrying the G allele of SNP IL17A G-197A. This chemokine is expressed by mononuclear phagocytes after stimulation by the proinflammatory cytokines IFNγ and TNFα. It can activate the secretion of other proinflammatory chemokines and is involved in activation of the Th1 type of adaptive immune response [14].

According to our previous studies, elevated IL-17A levels are associated with marked immune abnormalities and with cortical morphometric changes in schizophrenia [4]. In this study, the G allele of SNP IL17A G-197A was shown to be associated with activation of systemic inflammation and signs of Th1 activation of the adaptive immune system in patients. It is possible that the effect of this SNP on the state

**TABLE 3. MEAN CORTICAL THICKNESS IN PATIENTS WITH SCHIZOPHRENIA DEPENDING ON THE SNP ALLELE IL-17A G-197A**

| | AA | | AG | | GG | | Controls | |
|---|---|---|---|---|---|---|---|---|
| **Indicator** | **m** | **σ** | **m** | **σ** | **m** | **σ** | **m** | **σ** |
| **Caudal part of the middle frontal gyrus (right)** | 2.56 | 0.13 | 2.45* | 0.12 | 2.58 | 0.14 | 2.66 | 0.14 |
| **Precentral gyrus (left)** | 2.49 | 0.12 | 2.46* | 0.10 | 2.65 | 0.10 | 2.62 | 0.14 |
| **Rostral part of the middle frontal gyrus (left)** | 2.41 | 0.13 | 2.40* | 0.09 | 2.46 | 0.11 | 2.54 | 0.13 |
| **Lateral orbitofrontal cortex (right)** | 2.72 | 0.14 | 2.66* | 0.11 | 2.72 | 0.11 | 2.81 | 0.11 |
| **Rostral part of the middle frontal gyrus (right)** | 2.36 | 0.13 | 2.37* | 0.10 | 2.39 | 0.11 | 2.53 | 0.16 |
| **Precentral gyrus (right)** | 2.45 | 0.19 | 2.40* | 0.12 | 2.59 | 0.14 | 2.56 | 0.16 |
| **Pars orbitalis of the inferior frontal gyrus (IFG) (left)** | 2.79 | 0.20 | 2.75* | 0.20 | 2.82 | 0.12 | 2.96 | 0.17 |
| **Pars triangularis of the inferior frontal gyrus (IFG) (left)** | 2.42 | 0.18 | 2.46* | 0.14 | 2.49 | 0.07 | 2.60 | 0.14 |

Note. *, significance of differences $p < 0.005$ compared to the control group.

of immunity is due to changes in the local IL-17A secretion, although this requires further research.

Considering the results obtained in this study indicating the effect of SNP IL17A G-197A on the severity of immune disorders in patients, the association of SNP IL17A G-197A (rs2275913) with the average thickness of the large hemispheric cortex in schizophrenia was assessed (Table 3).

The data presented in Table 3 suggest an association of the heterozygous GA genotype of the SNP IL17A G-197A with reduced cortical thickness in a number of areas of the frontal cortex in schizophrenia. Changes in cortical thickness in some of these areas can be relevant to the pathogenesis of the disease, particularly to the development of negative symptoms in patients, which include apathy and abulia, as well as disturbances in memory, attention, thinking and speech. One such area is the middle frontal gyrus, which is divided into upper and lower parts by the middle frontal sulcus. Reduced middle frontal gyrus volume has been found to be associated with impaired episodic memory [9]. It is also known that the middle frontal gyrus is associated with reading, writing and numerical literacy skills, while the left middle frontal gyrus is also active in tasks requiring the actualization of verbal memory and word articulation, and supports the feedback system during verbal activity [15].

The orbitofrontal cortex is an area of the brain that plays an important role in the implementation of volitional functions and the regulation of behavior, as well as in emotional reinforcement in learning. According to the literature, structural changes in the orbitofrontal cortex and disruptions in its functional connections with surrounding structures can lead to cognitive and behavioral disorders associated with impaired decision-making and emotion regulation. Decreased cortical thickness in these areas has been confirmed by other studies of brain morphometrics in schizophrenic patients, but the association of these changes with the SNP IL17A G-197A has been shown for the first time [12]. The findings indicate that SNP IL17A G-197A requires further research as a potential biomarker of adaptive immunity dysregulation and morphometric abnormalities in the brain in schizophrenia.

## Conclusion

In conclusion, the results of this study demonstrate the association of increased chemokine levels and changed content of NK-cell subpopulations in schizophrenia patients with carriage of SNP IL17A G-197A (rs2275913) and also demonstrate the association of carriage of this polymorphism with morphometric changes in the cerebral cortex. The results indicate that SNP IL17A G-197A requires further studies on expanded samples of patients as a potential biomarker of immune dysregulation and morphometric abnormalities in the brain in schizophrenia. The findings can be translated into practice for use in predicting the course of the disease, the development of structural brain abnormalities, and the selection of therapy in schizophrenia.

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# УРОВЕНЬ ХЕМОКИНОВ И ДРУГИХ МЕДИАТОРОВ ВОСПАЛЕНИЯ У ПАЦИЕНТОВ С МЯГКИМ КОГНИТИВНЫМ СНИЖЕНИЕМ, ПРОХОДЯЩИХ РЕАБИЛИТАЦИЮ

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**Резюме.** Болезнь Альцгеймера – наиболее распространенное нейродегенеративное заболевание в пожилом возрасте. В части случаев ее развитию предшествует додементная стадия – мягкое когнитивное снижение. Важным компонентом патогенеза нейродегенерации является хроническое нейровоспаление (воспалительная активация микроглии и астроцитов в мозге), развитию и поддержанию которого может способствовать системный воспалительный ответ вследствие нарушения иммунной регуляции. Изучение уровня хемокинов у пациентов с MCI и его взаимосвязи с клиническими проявлениями – актуальное направление исследований, так как показано участие ряда из них в патогенезе нейродегенерации. Целью данного исследования было изучение уровня хемокинов и других медиаторов воспаления в динамике у пациентов с мягким когнитивным снижением на фоне реабилитации, а также исследование его связи с выраженностью когнитивных нарушений. В основную группу исследования вошли 48 пациентов, проходящих курс реабилитации в Клинике памяти Психиатрической клинической больницы № 1. Продолжительность курса составляла 6 недель, программа включала когнитивные тренировки, психотерапию и самостоятельное выполнение заданий. Пациенты прошли иммунологические исследования и клиническую оценку в динамике. Повторное обследование проводилось через 6 месяцев после начальной точки. В контрольную группу вошли 46 здоровых добровольцев, сопоставимые с пациентами по возрасту и полу. Для определения концентрации цитокинов и хемокинов в сыворотке крови использовали мультиплексный анализ. Для оценки достоверности различий использовали критерий Стьюдента. Оценка когнитивных функций проводилась с исполь-

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зованием международных нейропсихологических шкал, включая Монреальскую когнитивную шкалу и Краткую шкалу оценки психического статуса. Обнаружено повышение у пациентов уровня ряда цитокинов и хемокинов (TNFα, CXCL10/IP10, CCL22/MDC), регулирующих системное воспаление, клеточные и гуморальные механизмы адаптивного иммунитета. Выявлена взаимосвязь уровня хемокина CCL7 с параметрами нейропсихологического обследования пациентов: обнаружено, что снижение его содержания ассоциировано с более высокой тяжестью когнитивных расстройств. На фоне проведенной реабилитации отмечалось увеличение числа баллов по шкале MMSE, снижение уровня провоспалительного цитокина TNFα, а также хемокинов CXCL10, CCL22 более чем у 50% пациентов. Полученные данные вносят вклад в понимание роли хемокинов в патогенезе мягкого когнитивного снижения и указывают, что их уровень может являться потенциальным биомаркером тяжести когнитивных нарушений. Для последующей трансляции полученных данных в клиническую практику необходима их валидация в более крупных исследованиях, а также оценка взаимосвязи уровней хемокинов с выраженностью когнитивных нарушений при MCI в динамике долгосрочного наблюдения.

*Ключевые слова: болезнь Альцгеймера, воспаление, Монреальская когнитивная шкала, мягкое когнитивное снижение, реабилитация, хемокины*

# LEVELS OF CHEMOKINES AND OTHER INFLAMMATORY MEDIATORS IN PATIENTS WITH MILD COGNITIVE IMPAIRMENT UNDERGOING REHABILITATION

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**Abstract.** Alzheimer’s disease is the most common neurodegenerative disease in old age. In some cases, it is preceded by mild cognitive impairment (MCI). One of the important components in the pathogenesis of neurodegeneration is chronic neuroinflammation (inflammatory activation of microglia and astrocytes in the brain). Systemic inflammatory response and immune dysregulation may contribute to neuroinflammation. The purpose of this study was to investigate the level of chemokines and other inflammatory mediators in patients with MCI who underwent medical rehabilitation, and to study its associations with the severity of cognitive impairment. The study group included 48 patients with MCI undergoing rehabilitation. Rehabilitation included cognitive therapy, psychotherapy and tasks for unaided performance. Repeated examination was conducted 6 months after the completion of rehabilitation. The control group included 46 healthy volunteers. Multiplex assay was used to determine serum cytokine and chemokine concentrations. Student’s t-test was used to assess the significance of differences. Assessment of cognitive functions was performed using international neuropsychological scales. In patients with MCI, we have found an increase in the levels of several cytokines and chemokines (TNFα, CXCL10/IP10, MDC) that regulate systemic inflammation, cellular and humoral mechanisms of adaptive immunity. After the rehabilitation course their levels returned to normal. It was also found that decrease in CCL7 level in the patients before the rehabilitation course is associated with the severity of cognitive impairment. The findings contribute to understanding the role of chemokines in the pathogenesis of MCI, and indicate that their levels can be potential biomarkers of the severity of cognitive impairment. For translation of the findings into clinical practice, their validation in larger studies is needed, as well as assessing the associations between chemokine levels and the severity of cognitive impairment in MCI over long-term follow-up.

*Keywords: Alzheimer’s disease, inflammation, mild cognitive impairment, Montreal Cognitive Assessment, rehabilitation, chemokines*

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## Introduction

Alzheimer's disease (AD) is the most prevalent neurodegenerative disease in people older than 65 years. The typical morphological attributes of AD include amyloid plaques containing deposits of amyloid-β peptide, neurofibrillary bundles composed of tau protein, and chronic neuroinflammation [4]. An important component of the pathogenesis of neurodegeneration in AD is prolonged activation of innate immune response mechanisms in the central nervous system (CNS) (neuroinflammation), the development and maintenance of which can be facilitated by systemic inflammatory response and immune regulation disorders. In neuroinflammatory conditions, activated microglial cells secrete high levels of free oxygen species, cytokines, chemokines and other inflammatory mediators, which causes neuronal damage, disruption of trophic functions of astrocytes and deposition of neurotoxic amyloid-β oligomers [8].

In a number of cases, the development of AD-type dementia is preceded by mild cognitive impairment (MCI), a clinical condition that is characterized by deficits of memory and other cognitive functions that don't reach the severity of dementia. Patients with MCI have an increased risk of dementia, but a number of patients remain cognitively stable. Neurovisualization and morphological methods detect neurodegeneration in the patients with MCI, and finding new potential prognostic markers in MCI, including immunological markers, is important for clinical practice.

Chemokines are a superfamily of small structurally related cytokines that form a complex network. They normally regulate the movement of white blood cells, but also perform a very wide variety of immune and non-immune functions. Studying the level of chemokines in the MCI patients and its relationship with the clinical manifestations of the disease is a promising area of research, since animal models show that a number of chemokines are involved in the pathogenesis of MCI and AD by stimulating and maintaining neuroinflammation, activation of amyloid-β oligomer deposition and tau protein hyperphosphorylation [7]. Chemokines can be produced not only by innate and adaptive immunity cells, but also by other cells of the body, including CNS cells: microglia, astrocytes, oligodendrocytes, endotheliocytes and neurons [12]. They can contribute to the maintenance of chronic inflammation by attracting T cells to the inflammation focus and activation of mononuclear phagocytes.

The basis of the work was our earlier findings on the associations between systemic inflammation, deficiency of the humoral immune response and progression of cognitive disorders in MCI [10]. The goal of this study was to assess the serum concentrations of chemokines and other inflammatory mediators in patients with mild cognitive impairment undergoing rehabilitation, to study their associations with the severity of cognitive impairment in patients.

## Materials and methods

The main study group consisted of 48 patients with mild cognitive decline undergoing medical rehabilitation at the Memory Clinic of Psychiatric Clinical Hospital № 1 (4 men, 44 women, mean age 73.16±2.06 years).

Patients came in with complaints of subjective cognitive impairment, including forgetfulness, attention and concentration deficits, occasional difficulty finding their way home, difficulty expressing thoughts, decreased professional and social productivity, impaired motor skills, and difficulty performing everyday household activities (paying bills, shopping). The rehabilitation course was carried out in face-to-face and half-distance mode under conditions of established restrictions because of COVID-19: face-to-face – once a week (cognitive training and psychotherapy session) and remotely – independent daily performance of tasks by the project participants directed on maintenance and self-rehabilitation of cognitive functions, including use of the developed program of the "Memory Clinic" portal.

Repeated examination of the patients was conducted 6 months after the completion of the rehabilitation course. The duration of the rehabilitation course was 6 weeks, and the total duration of sessions was 96 hours. Rehabilitation was conducted in a group format. The neurocognitive training program was aimed at restoration of visual-spatial recognition, memory, kinesthetic, tactile and somatognostic functions, attention, goal setting, and control functions. Psychotherapy included methods of psychological aid adapted to the rehabilitation program [13].

Patients underwent immunologic studies, clinical evaluation and neuropsychological testing at the starting point of the study and at follow-up. Re-examination was performed 6 months after the initial point.

The control group included 46 healthy volunteers without a diagnosis of MCI, AD or other neurological and psychiatric diseases, and without acute infectious

diseases or systemic diseases in the decompensation phase, comparable with the patients in age and gender.

The study was approved by the local ethics committee of the Kurchatov Institute Research Center. All participants were acquainted with the details of the study and signed a voluntary informed consent sheet, a questionnaire, and a consent to process personal data.

We used a multiplex assay panel (Merck Millipore, Germany) to determine the concentration of cytokines and chemokines in blood serum.

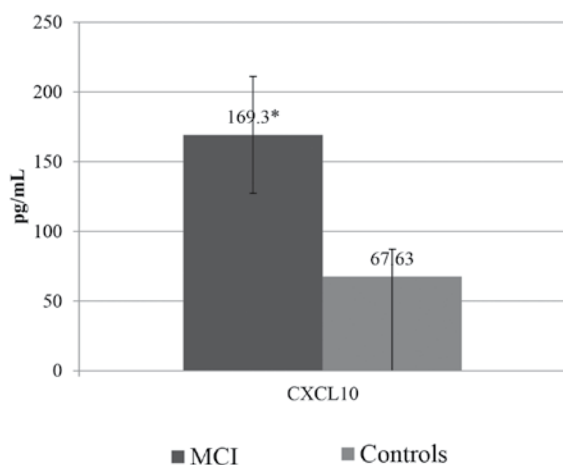


**Figure 1. Levels of CXCL10 chemokine in MCI patients and in the control group**

Note. *, significant differences with the control group ($p < 0.05$).

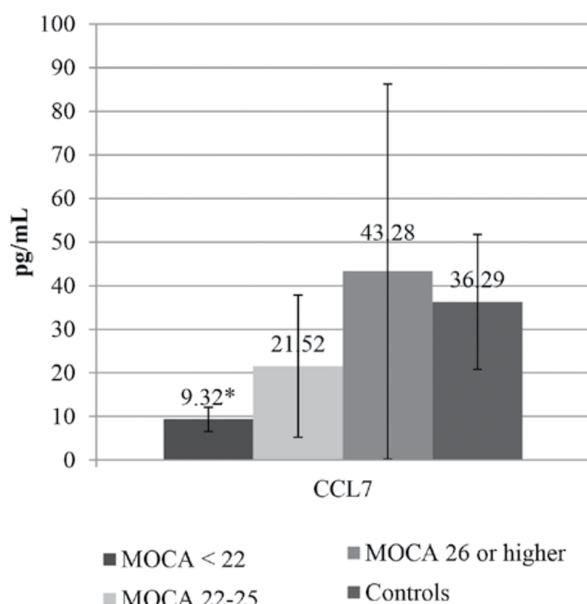


**Figure 2. Levels of CCL7 chemokine in MCI patients with different severity of cognitive impairment according to the MOCA scale and in the control group**

Note. *, significant differences with the control group ($p < 0.05$).

Cognitive functions were assessed at the Memory Clinic of PKB No. 1 using international neuropsychological scales, including the Montreal Cognitive Scale (MOCA), the Mini Mental Examination Scale (MMSE), the Hospital Anxiety and Depression Scale (HADS), and the clock drawing test.

Excel (Microsoft, 2010) and STATISTICA 10 (StatSoft, 2010) software were used for statistical processing. Normality of distribution was assessed using Shapiro-Wilks criterion. Group results were presented as averages with 95% confidence intervals. Student's t-test was used to assess the significance of differences.

## Results and discussion

It was found that levels of the proinflammatory cytokine TNFα (tumor necrosis factor-α), CXCL10/IP10 (interferon-γ inducible protein 10) and MDC (monocyte-derived chemokine, CCL22), which is involved in Th2-link activation of adaptive immunity, were significantly increased in MCI patients (Figure 1, Table 1).

CXCL10/IP10 is a proinflammatory chemokine involved in the effects of cytokine interferon-γ (IFNγ). As reported [2], the content of this chemokine is elevated in the cerebrospinal fluid in Alzheimer's disease in the early stages of dementia and in MCI, and in Alzheimer's disease there is a significant positive correlation between CXCL10 levels and the MMSE score, while in MCI no such correlation was found. Serum CXCL10/IL10 levels in MCI have not previously been studied.

TNFα is one of the major proinflammatory cytokines, secreted mainly by macrophages, NK cells, and lymphocytes. TNFα has 2 receptors, TNFR1 and TNFR2. The TNFR2 receptor is associated with homeostatic functions, whereas activation of the TNFR1 receptor triggers systemic inflammation cascades and causes a wide range of biological effects, including activation of innate immune cells, endothelial activation, acute phase protein synthesis, pyrogenesis, dendritic cell migration to lymph nodes and activation of the adaptive immune response. Depending on the state of the target cell and the microenvironment, TNFα can cause stimulation of survival, proliferative activity of target cells, their necrosis or apoptosis [5]. Increased TNFα levels indicate activation of the systemic inflammatory response in MCI patients, confirming our earlier findings [10]. Data obtained by other researchers also indicate an increase in this cytokine in the blood serum in MCI [15].

MDC is a chemokine expressed by dendritic cells, NK-cells and some T cell subpopulations. The main functions of MDC include the induction

TABLE 1. LEVELS OF CHEMOKINES AND INFLAMMATORY MEDIATORS IN MCI PATIENTS AT BASELINE AND 6 MONTHS AFTER THE REHABILITATION COURSE

| Indicator | Baseline | 6 months point | Controls |
|---|---|---|---|
| **CXCL10, pg/mL** | 169.3±41.9*, ** | 75.1±16.6 | 67.63±19.59 |
| **CCL7/MCP-3, pg/mL** | 21.49±11.51 | 21.72±10.01 | 38.81±21.99 |
| **TNFα, pg/mL** | 8.17±1.36** | 7.61±2.97 | 5.57±1.45 |
| **MDC, pg/mL** | 1230.3±323.8*, ** | 483.1±90.4 | 597.75±84.74 |

Note. *, the significance of differences between baseline and the 6 months point, $p < 0.05$; **, the significance of differences with the control group, $p < 0.05$.

and maintenance of activation of the Th2-link of adaptive immunity. This chemokine participates in the pathogenesis of allergic diseases and malignant tumors [11]. According to the literature, increased MDC levels in the cerebrospinal fluid is one of the markers of increased permeability of the blood-brain barrier in AD, which may play an important role in the pathogenesis of AD, contributing to the development and maintenance of chronic neuroinflammation due to violation of the CNS's immuno-privileged status [1].

It was also detected that the content of the interferon-inducible chemokine CCL7/MCP-3 (monocyte chemotactic protein 3) was significantly reduced in MCI patients with a significant degree of cognitive impairment (less than 22 points on the MOCA scale) (n = 13) (Figure 2). The chemokine CCL7 is one of the mediators that promote extravasation of monocytes and neutrophils to the site of the inflammatory reaction. This chemokine is important for the initiation of adaptive immune response to viruses and other intracellular pathogens (*Listeria monocytogenes*, etc.) [14]. Its deficiency leads to impaired monocyte functions, including antigen presentation. The low level of CCL7 in MCI patients with severe cognitive decline may contribute to the deficiency of mechanisms of antigen-dependent cytotoxicity in these patients.

Assessment of the cognitive functions of MCI patients before and after rehabilitation showed that before rehabilitation, the mean MMSE score was 26.08±0.54 and MOCA score was 22.61±0.91. After rehabilitation, the mean MMSE score was 28.18±0.74 ($p = 3.75 \times 10^{-5}$) and MOCA score was 24.00±1.33 ($p > 0.05$). MOCA scores higher than 26 are considered normal and scores from 18 to 25 are characteristic for MCI. For MMSE, scores higher than 26 are considered normal and scores from 18 to 25 are characteristic for MCI. Thus, the rehabilitation course had a favorable effect on the cognitive functions of MCI patients in the short-term dynamics, which was expressed in a significant increase in the MMSE score.

We studied the effect of the rehabilitation course on the level of chemokines and inflammatory mediators in MCI patients. The main results are shown in the Table 1.

It was revealed that at repeated examination after the rehabilitation course in MCI patients the content of proinflammatory cytokine TNFα in blood serum decreased to normal levels, as well as the content of MDC chemokine. MDC, a chemokine that regulates Th2 type of the adaptive immune response and the permeability of BBB. The levels of MDC have been found to increase in the cortex APP/PS1 mice that are susceptible to AD [6]. The decrease of MDC levels in systemic circulation can be beneficial for the patients with MCI, reducing the BBB permeability and neuroinflammation.

It was also found that the content of chemokine CXCL-10/IP-10 (interferon-gamma inducible protein 10) decreased to normal levels after the rehabilitation course. CXCL-10/IP-10 is an important component of the antiviral response, stimulating migration to the site of infection and adhesion of activated type 1 T helpers. CXCL10 is expressed by neurons, glial and stromal cells in various CNS diseases and can play both protective and damaging roles [3]. The chemokine CXCL10 binds to the CXCR3 receptor, which is mainly expressed on activated T cells and natural killer cells. CXCL10 mediates leukocyte influx in various inflammatory diseases of the central nervous system and may be involved in the development of neuroinflammation, contributing to the pathogenesis of Alzheimer's disease [9]. Given the association of the level of this chemokine with the severity of cognitive impairment in MCI, it is of interest to further study it as a marker of the effectiveness of MCI therapy.

Therefore, in this work, we found increase in the levels of several major chemokines that regulate systemic inflammation, cell and humoral mechanisms of adaptive immunity in MCI patients. This increase may reflect activation of inflammation, participate in the development of neuroinflammation, and contribute to the progression of Alzheimer's disease. In particular, we revealed an important fact about the

relationship between the CCL7 chemokine level and the parameters of neuropsychological examination of patients with MCI: decrease in CCL7 content was found to be associated with the severity of cognitive impairment according to the MOCA scale. The findings indicate that decrease in the content of the CCL7 chemokine is a potential marker of the severity of cognitive impairment in MCI.

According to this study, the medical rehabilitation course had a beneficial effect on the cognitive functions of the patients, and was associated with a decrease of the levels of TNFα, CXCL10 and MDC to normal values. These fascinating results show the importance of further research of the influence of medical rehabilitation in MCI on immunological parameters. Some of the factors that can possibly explain these results are a decrease in anxiety, better emotional stability, better organized schedule and higher physical activity in the patients who underwent the rehabilitation course. These factors could have an effect on neuroimmune interactions in the patients, reducing systemic inflammation and the activation of adaptive immunity.

The elevated serum levels of TNFα, CXCL10 and MDC in patients with MCI at the initial examination and their decrease after the rehabilitation course indicate the importance of further study of these proteins as markers of the effectiveness of rehabilitation measures in mild cognitive impairment.

## Conclusion

The findings of this study make a contribution to understanding the role of chemokines in the pathogenesis of mild cognitive impairment, and indicate that their levels may be a biomarker of the severity of cognitive decline in patients. It is necessary to validate the obtained data in larger studies, as well as to evaluate the relationship between chemokine levels and the severity of cognitive impairment in MCI in the dynamics of long-term follow-up. Translation of the obtained data and methods into practice is promising for predicting the course of the disease and selection of therapy.

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# ОСОБЕННОСТИ ПОСТВИРУСНОГО СИНДРОМА ХРОНИЧЕСКОЙ УСТАЛОСТИ, АССОЦИИРОВАННОГО С МЯГКИМ КОГНИТИВНЫМ СНИЖЕНИЕМ, У ПАЦИЕНТОВ С АТИПИЧНЫМИ ХРОНИЧЕСКИМИ АКТИВНЫМИ ГЕРПЕСВИРУСНЫМИ ИНФЕКЦИЯМИ

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**Резюме.** Согласно современным представлениям, дисфункциональные изменения в работе иммунной системы оказывают влияние на иммунные процессы в ЦНС, способствуя развитию нейроиммуновоспаления, и тем самым опосредованно влияют на скорость прогрессирования нейродегенеративных процессов. Целью нашего исследования явилось изучение распространенности поствирусного синдрома хронической усталости и когнитивных нарушений (aMCI) среди пациентов, страдающих атипичной, хронической активной герпесвирусной инфекцией (АХА-ГВИ).

Под нашим наблюдением находились 126 пациентов обоих полов в возрасте от 18 до 60 лет, страдающих АХА-ГВИ.

Установлено, что моно-ВЭБ инфекцией страдают 27,7%, микст-ВЭБ инфекция наблюдается у 72,3% пациентов. При оценке когнитивного функционирования с использованием шкал CGI, MMSE выявлена частота встречаемости aMCI – 68,3%: при микст-ГВИ она составила – 87,4%, при моно ГВИ – 38,8%. В процессе исследования были выявлены существенные ограничения в применении использованных стандартных шкал в связи с невозможностью проведения комплексной оценки параметров клинического статуса и когнитивных дисфункций, а также корреляции этих параметров и оценки динамики на фоне проводимой иммунотерапии. Для реализации этой цели на дальнейших этапах исследования была использована разработанная нами Шкала оценки критериальных клинических признаков/симптомов пациентов, страдающих АХА-ГВИ с СХУ. Показано, что при микст-ГВИ выраженность симптомов достоверно превышала выраженность симптомов пациентов с моно-ГВИ и составляла 52,7 (43,1-62,2) и 38,0 (31,9-42,8) баллов соответственно ($p \geq 0,05$). Таким образом было

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установлено, что пациенты, страдающие микст-ГВИ, имеют более выраженные, тяжелые проявления СХУ и aMCI, которые в 1,5 раза превышают аналогичные проявления у пациентов с моно-ГВИ, снижая качество жизни этих пациентов, ухудшая их социальную адаптацию, создавая риск развития психогенной депрессии.

Длительная персистенция герпес-вирусов в организме иммуннокомпрометированных людей создает условия для постоянной антигенной стимуляции и иммунного дисбаланс с дебютом вторичного иммунодефицита или клинической манифестацией имеющихся первичных нарушений в иммунной системе, что создает предпосылки для развития нейроиммувопоспалительных изменений в ЦНС и ПНС с последующим формированием клинических проявлений миелоэнцефалита и синдрома хронической усталости с различными когнитивными нарушениями, которые могут быть классифицированы как мягкое когнитивное снижение.

*Ключевые слова: герпесвирусные инфекции, иммунная дисфункция, синдром хронической усталости, когнитивные расстройства, интерферонопатии, иммунотерапия*

# PECULIARITIES OF POST-VIRAL CHRONIC FATIGUE SYNDROME ASSOCIATED WITH MILD COGNITIVE DECLINE IN PATIENTS WITH ATYPICAL CHRONIC ACTIVE HERPESVIRUS INFECTIONS

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**Abstract.** According to modern ideas, changes in the functioning of the immune system affect the immune processes in the nervous system, contributing to the development of neuro-immuno-inflammation and thereby indirectly affect the rate of progression of neurodegenerative processes. The aim of our study was to investigate the prevalence of post-viral chronic fatigue syndrome and cognitive impairment (aMCI) among patients with atypical, chronic active herpesvirus infections (ACA-HVI).

Under our supervision were 126 patients of both sexes aged 18 to 60 years with ACA-HVI.

It was established that mono-EBV infection affects 27.7%; mixed EBV infection is observed in 72.3% of patients. When assessing cognitive functioning using CGI, MMSE scales, the incidence of aMCI was found to be 68.3%: with mixed HVI – 87.4%, with mono HVI – 38.8%. During the study, significant limitations were identified in the use of standard scales due to the impossibility of conducting a comprehensive assessment of clinical status parameters and cognitive dysfunctions, as well as correlation of these parameters and assessment of dynamics of the immunocorrection. To achieve this goal the Scale of assessment of the criterion clinical symptoms of patients with ACA-HVI with CFS was used. It was shown that in mixed-HVI, the severity of symptoms exceeded the severity of symptoms of patients with mono-HVI and was 52.7 (43.1-62.2) and 38.0 (31.9-42.8) points, respectively ($p \geq 0.05$). Thus, it was found that patients suffering from mixed HVI have more pronounced, severe manifestations of CFS and aMCI, which are 1.5 times higher than similar manifestations in patients with mono-HVI, significantly reducing the quality of life of these patients, worsening their social adaptation.

Prolonged persistence of herpes viruses in immune-compromised people creates conditions for constant antigenic stimulation and immune imbalance with the onset of secondary immunodeficiency or clinical manifestation of existing primary disorders in the immune system, which creates the prerequisites for the development of neuro-immuno-inflammatory changes in nervous system, followed by the formation of clinical manifestations of ME/CFS with different cognitive impairments that may be classified as aMCI.

*Keywords: herpesvirus infections, immune dysfunction, chronic fatigue syndrome, cognitive disorders, interferonopathies, immunotherapy*

## Introduction

In recent years, the prevalence of viruses belonging to the Herpesviridae family has become pandemic [1, 3]. In this regard, the Herpesviridae family study is of great interest in the etio-and immunopathogenesis of some infectious and non-infectious diseases, tending to a chronic progressive course and characterized by torpidity to ongoing standard therapy, the clinical picture of which is often atypical, polysymptomatic and polysyndromic. Possessing pronounced neuro- and immunotropism, herpes family viruses are able to persist for a long time in neuroglial cells, neurons, cells of the immune system, causing the development of chronic systemic and neuroimmune inflammation, clinically accompanied by the meningoencephalitis (ME) symptoms, post-viral chronic fatigue syndrome and immune dysfunction, including signs of amnestic mild cognitive impairment (aMCI) [2, 7, 11, 12, 14, 15]. It is known that one of the aMCI causes is neuroinflammation induced by a viral process of a predominantly integrative type [5, 6, 8, 10]. The leading clinical manifestations of aMCI are: progressive memory impairment, visual-spatial functions, fatigue, rapid exhaustion with little physical and mental stress, emotional lability, and personality changes in general [8].

According to modern concepts, dysfunctional changes in the immune system affect the immune processes in the CNS, contributing to the development of neuro-immuno-inflammation and thus indirectly affect the rate of neurodegenerative progression. Along with this, the results of studies evaluating various parameters of the cellular and humoral parts of the immune system, cytokine profile, interferon system in aMCI are few and often contradictory.

**The aim of our study** was to study the prevalence of aMCI among patients suffering from atypical, active chronic mono and mixed herpesvirus infections (ACA-HVI), as well as to clarify immuno-pathogenetically significant disorders in the mechanisms of immune antiviral defense and the interferon system dysfunction.

## Materials and methods

Study group (SG) included 126 patients of both sexes aged 18 to 60 years, suffering from atypical ACA-HVI. The comparison group (CG) consisted of 30 apparently healthy individuals matched by sex and age with GI patients. The study was approved by the Ethics Commission, and informed consent was obtained from all patients to participate in the study and to process personal data in accordance with the World Medical Association's Declaration of Helsinki (WMA Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects, 2013).

**Anamnesis and physical examination**

All Herpes virus (HI) patients were surveyed using questionnaires specially developed by us in order to identify the features of the epidemiological and infectious anamnesis, and identify the main clinical/criteria signs and symptoms of ACA-HVI [4].

Cognitive functioning was assessed by the CGI (Clinical Global Impression) and MMSE (Mini-Mental State Examination) scales [13].

**Laboratory diagnostics**

The study included complex analysis consisting of the traditional methods (history taking, physical examination methods, CBC, etc.), serodiagnostic methods (IgM VCA EBV, IgG VCA EBV, IgM CMV, IgG CMV IgM HSV1 / 2, IgG HSV1 / 2) using ELISA test systems, NPO Diagnostic Systems (Russia), and the PCR method of the AmpliSense test system (Russia) to detect the virus genome in biomaterials (blood, saliva, urine, scrapings from the tonsils and posterior pharyngeal wall). To assess the functioning of antiviral immunity (immunogram, INF-status, etc.) features, flow cytometry and ELISA methods were used. Statistical analysis was performed using the Microsoft Excel 2019, Statistica 2.0 software package.

## Results and discussion

When analyzing the etiological structure of morbidity in the observed groups, we established the frequency of occurrence of mono- and mixed-HVI. It is noteworthy that the Epstein-Barr virus (EBV) was the dominant virus in both groups of patients. According to the data obtained, 27.7% of patients suffered from mono-EBV infection, and 72.3% – from mixed-EBV infection (Figure 1).

In the structure of these infections, combinations of EBV + CMV + HHV type 6 are in the lead (14.7%); EBV+CMV+HSV type 1 (12.4%); EBV + CMV + HSV type 1 + HHV 6 type (12.4%), as well as EBV + HHV 6 type and EBV + HSV type 1 – 8.8% each. Further, the distribution of mixed infections according to the occurrence of combinations is as follows: EBV+CMV (8.8%); EBV+CMV+HSV2, EBV+CMV+ HHV type 6 (14.7%) and rarer combinations (less than 27.3%) (Figure 2).

In the patients suffering from mono- ACA-HVI clinical picture, the leading clinical manifestations of HSV1/HSV2 were vesicular rashes on the skin and mucous membranes of various prevalence and localization. Among the predominant symptoms, subfebrile condition, regional lymphadenopathy, the appearance of chills, headache, hyper- and paresthesias in certain dermatomes, prodromes preceding and accompanying HVI recurrence, as well as the presence of cognitive impairment, sleep disorders, insomnia, and a pronounced decrease in working capacity were identified.

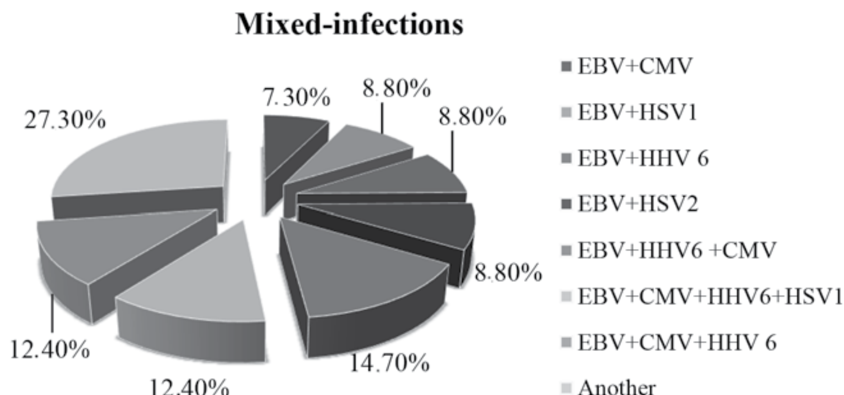


**Figure 1. Etiological structure of mixed EBV infections**

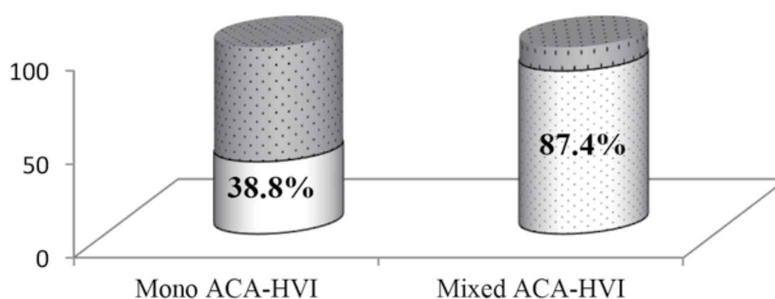


**Figure 2. Frequency of aMCI occurrence in patient groups with mono- and mixed herpesvirus infections (%)**

In patients suffering from mixed ACA-HVI there were more pronounced complaints and clinical manifestations of dysfunction in the CNS, ANS and PNS, that included a prolonged feeling of severe weakness, chronic fatigue, and poor tolerance to adequate physical activity. In addition, patients were worried about excessive sweating, intermittent pain in the throat, in muscles and joints, headaches, subfebrile temperature, lymphadenopathy, sleep disturbance, decreased memory, attention, intelligence (cognitive dysfunction), less often – psychogenic depression.

When assessing cognitive functioning using the CGI (Clinical Global Impression) scale and the Mini-Mental State Examination (MMSE), it was found that in patients with ACA-HVI, the incidence of aMCI was 68.3%. At the same time, in patients with mixed HVI, the incidence of aMCI was higher (87.4%), than in patients with mono HVI (38.8%) (Figure 2).

The SG (patients diagnosed with aMCI) included patients with ACA-HVI who had an MMSE score of ≥ 27 and met the diagnostic criteria for aMCI syndrome at the stage of mild cognitive decline.

It is known that MCI can be an initial stage of many neurodegenerative diseases, established on the results of neuropsychological testing, complaints of cognitive impairment by the patient or his relatives, the absence of significant disturbances in the patient's daily activities, and the exclusion of other causes of cognitive decline. Indicators of neuropsychological scales for aMCI are intermediate between the corresponding age norm and dementia values characteristic.

In our study, the use of these scales was of great diagnostic value in the period of screening assessing amnestic and cognitive impairment in order to identify cognitive dysfunctions that meet the aMCI criteria in CFS. However, these scales had some limitations as they did not allow to assess comprehensive parameters of the clinical status and cognitive dysfunctions, as well as the dynamics and correlation of these parameters against the background of the ongoing integration program for correcting the immune system of immunocompromised patients suffering from ACA-HVI. To achieve this goal, at further stages of the study, we developed the Scale for assessing the criterial clinical signs/symptoms of patients suffering from ACA-HVI with CFS [4].

A comparative assessment of the criterial CFS signs/symptoms severity in patients suffering from

**TABLE 1. COMPARATIVE ASSESSMENT OF THE CRITERIAL CFS SIGNS/SYMPTOMS SEVERITY IN MONO AND MIXED HVI, Me ($Q_{0.25}$-$Q_{0.75}$)**

| Symptom | Mixed HVI | Mono HVI |
|---|---|---|
| **Severe fatigue** | 5.0 (5.0-5.0) | 5.0 (5.0-5.0) |
| **Poor tolerance to adequate exercise** | 5.0 (5.0-5.0) | 5.0 (5.0-5.0) |
| **Prolonged subfebrile condition** | 4.0 (3.5-4.5) | 2.5 (2.0-3.0)* |
| **Pain and discomfort in the throat** | 4.0 (3.5-4.5) | 3.0 (2.5-3.5)* |
| **Excessive sweating, chilliness, sensitivity to cold** | 3.5 (2.5-4.5) | 2.5 (2.0-3.0) |
| **Headache, migraine** | 4.0 (3.5-4.5) | 2.5 (2.0-3.0)* |
| **Regional lymphadenopathy** | 4.5 (4.0-5.0) | 3.5 (3.0-4.0)* |
| **Increased fatigue, decreased productivity** | 5.0 (4.5-5.0) | 3.5 (3.0-4.0)* |
| **Neurological disorders (paresthesia, synesthesia, sensory disorders, low muscle tone, etc.)** | 3.5 (2.5-4.0) | 2.0 (1.4-2.5) |
| **Decreased memory, concentration** | 2.4 (1.5-4.0) | 2.0 (1.0-2.3) |
| **Cephalgia, arthralgia, myalgia** | 3.3 (2.1-4.2) | 2.0 (1.5-2.5) |
| **Sleep disorders (insomnia or increased sleepiness)** | 4.0 (2.5-5.0) | 2.5 (2.0-2.7) |
| **Panic attacks, mood disorders, emotional lability, psychogenic depression, etc.** | 4.5 (3.0-5.0) | 2.0 (1.5-2.3)* |
| **Sum of points** | 52.7 (43.1-62.2) | 38.0 (31.9-42.8)* |

Note. *, significant differences between the parameters of the mono-HVI and mixed-HVI , $p < 0.05$.

mono and mixed herpes virus infections was made (Table 1).

It was shown that in patients suffering from mixed HVI, the severity of symptoms significantly exceeded the severity of symptoms in patients with mono-HVI: 52.7 (43.1-62.2) and 38.0 (31.9-42.8) points, respectively ($p \geq 0.05$). Thus, it was found that patients suffering from mixed HVI have more pronounced, severe manifestations of CFS and aMCI, that were 1.5 times higher than similar manifestations in patients with mono- HVI, significantly reducing patient quality of life, worsening their social adaptation, placing them at risk for the psychogenic depression development.

It is obvious that the presence of an adequate, simple and convenient tool for identifying and assessing the criterial signs/symptoms of CFS helping the doctor assess the existing disorders of the clinical and cognitive status, determine their severity, will allow timely diagnosis of existing disorders, and dynamic assessment of the therapy effectiveness, as well as in a timely manner to include the patient in the increased risk of developing severe cognitive impairment and psychogenic disorders.

According to modern concepts, the progression of neurodegeneration in aMCI is facilitated by a long-term latent activation of the innate immune response mechanisms in the central nervous system (neuroinflammation), one of the causes of which may be the atypical active chronic course of herpes virus infections, mostly with neutrotropism. In the brain with aMCI, pathological activation of microglial cells is noted, their secretion of excessive levels of pro-inflammatory cytokines, free radicals, and the neurotransmitter glutamate, increasing neuronal damage. The relationship between markers of systemic inflammation, individual indicators of immunity and neuroinflammation in aMCI is being studied. It has been shown that the presence of chronic systemic inflammation increases the risk of developing aMCI by 1.5-1.8 times.

## Conclusion

Long-term persistence of viral and bacterial agents in the body of immunocompromised people creates conditions for constant antigenic stimulation. As a result, there is a breakdown of adaptation mechanisms with depletion of immune homeostasis reserves and immune imbalance with the onset of secondary immunodeficiency or clinical manifestation of existing primary disorders in the immune system (congenital immunity errors). All together, it leads to the chronic pathology, allergization of the body, changes in the autoimmune profile of patients, and creates the prerequisites for the neuro-immuno-inflammatory changes in the CNS and PNS, followed by the clinical manifestations of encephalomyelitis and chronic fatigue syndrome (ME/CFS), and various cognitive impairments that can be classified as mild cognitive impairment (aMCI).

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# РОЛЬ ПРОВОСПАЛИТЕЛЬНЫХ ЦИТОКИНОВ ПРИ ТИРЕОИДИТЕ ХАШИМОТО, АССОЦИИРОВАННОМ С ПСИХИЧЕСКИМИ РАССТРОЙСТВАМИ

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**Резюме.** Психические нарушения часто сопровождают аутоиммунные заболевания, например, с 1949 года известно о «микседематозном безумии» – это психоз, причиной которого является гипотиреоз. Самая частая причина гипотиреоза – аутоиммунный тиреоидит Хашимото. Известно также и о другом психоневрологическом расстройстве, ассоциированном с аутоиммунным тиреоидитом – это энцефалопатия Хашимото. Энцефалопатия Хашимото – это тяжелое нарушение функций центральной нервной системы, патогенез которого не связан с гормональными нарушениями. Известно, что цитокины являются регуляторами и участниками воспаления, в том числе и аутоиммунного. Разумеется, когда речь идет о высоких концентрациях провоспалительных цитокинов, мы можем говорить о системном воспалении. Однако минимальные или незначительные колебания цитокинов в пределах диапазонов, характерных для здоровых или для нормергического острофазового ответа при болезни, не могут быть интерпретированы с точки зрения бинарной эндокринологической логики. Известно, что в центральной нервной системе цитокины способны влиять на нейроэндокринный контроль системно регулируемых функций. Нельзя забывать и о том, что глиальные клетки (астроглия, микроглия) способны к продукции ряда цитокинов и могут оказывать таким путем влияние на нейроны и развитие поведенческих изменений. Кроме того, доказана способность ряда цитокинов вне самой ЦНС действовать на вагальные афференты и через них доносить информацию в ЦНС, влияя на ее состояние и функции. Разумно предположить, что минимальные колебания уровней провоспалительных цитокинов могут оказывать влияние на состояние и функции ЦНС. Целью исследования было изучить уровни провоспалительных цитокинов у пациентов с аутоиммунным тиреоидитом; у пациентов с аутоиммунным тиреоидитом, ассоциированным с психическими нарушениями; у группы здоровых лиц; и оценить влияние уровней цитокинов на клинические проявления. В группе пациентов с тиреоидитом и психическими расстройствами уровни CCL20/MIP3α, IL-13, IL-2, IL-27, IL-5 были

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достоверно выше, чем в других группах. При этом между клиническими проявлениями психических расстройств и уровнями цитокинов положительной корреляции установлено не было. Но была выявлена положительная корреляция между уровнями некоторых цитокинов и свободным трийодтиронином, а также уровнем антитиреоидных антител. Психические расстройств, ассоциированные с аутоиммунным тиреоидитом могут быть связаны с изменениями в цитокиновом профиле и являться результатом нейровоспаления.

*Ключевые слова: цитокины, тиреоидит, нейровоспаление, психические расстройства, энцефалопатия Хашимото, шизофрения*

# ROLE OF PROINFLAMMATORY CYTOKINES IN HASHIMOTO'S THYROIDITIS ASSOCIATED WITH PSYCHIATRIC DISORDERS

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**Abstract.** Mental disorders often accompany autoimmune diseases, for example, since 1949 it has been known about "myxedematous madness", a psychosis caused by hypothyroidism. The most common cause of hypothyroidism is Hashimoto's autoimmune thyroiditis. It is also known about another neuropsychiatric disorder associated with autoimmune thyroiditis, Hashimoto's encephalopathy. It is a severe dysfunction of the central nervous system, the pathogenesis of which is not associated with hormonal disorders. Cytokines are regulators and participants of inflammation, including autoimmune. Certainly, when we are talking about high concentrations cytokines, we mean systemic inflammation. The minimal or mediocre fluctuations in cytokines within the ranges that are characteristic of healthy status or normergic acute phase response in disease cannot be interpreted from the point of view of binary endocrinological logic. In the CNS, cytokines are able to influence on the neuroendocrine control of systemically regulated functions. It is also important that glial cells (astroglia, microglia) are capable of producing a number of cytokines and can affect neurons and develop behavioral changes. In addition, the ability of a number of cytokines outside the CNS itself to act on vagal afferents and through them to convey information to the CNS, affecting its state and functions, has been proven. It is reasonable to assume that minimal fluctuations in cytokine levels may also affect the state and function of the CNS. The aim of the study was to investigate the levels of cytokines in patients with thyroiditis; in patients with thyroiditis associated with mental disorders; in a group of healthy individuals; and evaluate the effect of cytokine levels on clinical manifestations. In the group of patients with thyroiditis and mental disorders, the levels of CCL20/MIP3α, IL-13, IL-2, IL-27, IL-5 were significantly higher than in other groups. At the same time, no positive correlation was found between the clinical manifestations of mental disorders and the levels of cytokines. A positive correlation was found between the levels of some cytokines and free triiodothyronine, as well as the level of antithyroid antibodies. Mental disorders associated with autoimmune thyroiditis may be associated with changes in the cytokine profile and result from neuroinflammation.

*Keywords: cytokines, thyroiditis, neuroinflammation, psychiatric manifestations, Hashimoto's encephalopathy, schizophrenia*

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## Introduction

Mental disorders often accompany autoimmune diseases. In 1949, "myxedematous madness" was described, a psychosis associated with hypothyroidism. The main cause of hypothyroidism in areas without iodine deficiency is Hashimoto's autoimmune thyroiditis (AIT), affecting up to 15% of the female and 1-5% of the male population in some regions [1]. The focus of modern thyroidology is Hashimoto's encephalopathy (HE), a severe dysfunction of the central nervous system (CNS) against the background of AIT, manifested by various psychoneurological and behavioral disorders, the pathogenesis of which is not associated with hormonal disorders, since HE is observed in euthyrosis [15].

When evaluating data regarding the systemic concentrations of cytokines in mental disorders, it is worth to notice that cytokines are not hormones, but chemical bioregulators of short-distance focal, contact and zonal action (paracrine, juxtacrine, and autocrine modes of signaling). The minimal or mediocre fluctuations in concentrations within the ranges that are characteristic of healthy status or normergic acute phase response in disease cannot be interpreted from the point of view of binary endocrinological logic. The direction of a particular cytokine effect on various cells differs in paracrine versus systemic modes of action, depending on the contextual permissive background of other bioregulators acting locally or systemically at the moment [8, 14].

However, the central nervous system (CNS), in particular, its hypothalamic area, undoubtedly is accessible to the effects of cytokines through the systemic circulation and from local glial/neuronal interactions. Both may alter the neuroendocrine control of systemically regulated functions [11]. The elements of the intra-barrier and extra-barrier immune systems of the brain, in particular, glial cells are capable of producing a number of cytokines and can thus influence neurons and induce behavioral changes [9, 11]. In addition, it has been proven that cytokines outside the CNS itself are able to affect vagal afferents thus conveying information to the brain [6].

**Aim:** to study the cytokine profile in Hashimoto's autoimmune thyroiditis (AIT), both in mentally intact patients and in those having psychiatric disorders (PD), and to evaluate the relationships of cytokine levels with clinical manifestations

## Materials and methods

We have studied three groups of patients: Three groups of patients were involved: 1) AIT+PD, 27 patients (mean age 53.7±16.0 years), having various psychiatric diagnoses verified at specialized hospital (among them: schizophrenia (n = 15), obsessive compulsive disorder (n = 1), Alzheimer disease (n = 1), dementia (n = 4), bipolar affective disorder (n = 4), organic delusional disorder (n = 1), depression (n = 1)). 2) AIT, 30 mentally healthy patients with Hashimoto's thyroiditis (mean age 48.1±12.0 years) 3) HC – 30 mentally and somatically healthy individuals (mean age 40.6±12.4 years). An informed consent was obtained from all participants prior to the study. The study was approved by the Ethics Committee of St Petersburg State University (protocols No. 76 dated 06/30/2017, No. 84 dated 06/20/2018 and No. 10/19 dated 10/17/2019).

The cytokine concentrations in peripheral venous blood serum were measured by multiparametric fluorescent analysis on a Luminex device, MagPix model (Luminex Inc., USA) with a commercial Human Th17 Magnetic Bead Panel kit (Merck, Germany), the study was carried out according to the manufacturer's instructions.

Laboratory studies of autoantibodies and hormones in peripheral venous blood serum were carried out on a BioMark xMark plate spectrophotometer (Bio-Rad, USA). Quantitative assessments of serum concentrations of anti-thyroperoxidase antibodies (anti-TPO), anti-thyroglobulin antibodies (anti-TG), free thyroxine (FT4), free triiodothyronine (FT3), thyroid stimulating hormone (TSH), and prolactin were carried out using commercial kits of reagents from Hema-Medica (Russia). The level of autoantibodies to alpha-enolase were measured using reagent kits from Cusabio Biotech Co., Ltd (PRC). Each study was performed according to the kit manufacturer's instructions.

Common methods of variation statistics were applied. Absolute values and fractions of the whole – n (%) were used to describe categorical variables. Continuous, discrete, and rank variables were described by the median and quartiles: Me ($Q_{0.25}$-$Q_{0.75}$) [12]. Intergroup analysis was performed using the Mann–Whitney test (U-statistics). Spearman's test (r-statistics) was used to perform correlation analysis between quantitative, countable and ordinal characteristics [12]. To assess the association of categorical variables with quantitative ones, a logistic model with an ordered choice was used, where the coefficient of the model (the logarithm of the odds ratio (log (odds)) served as a measure of association [4]. Improvement for multiple testing of hypotheses was carried out by the Benjamini–Hochberg correction [3]. The results were considered statistically significant if the probability of error of the first kind (p) was < 0.05 [13]. The calculations were performed in the programming language R v. 4.1.0.

## Results and discussion

The serum concentrations of 12 cytokines in 3 groups of patients were analyzed. The levels of CCL20/MIP-3α (from the English "Macrophage Inflammatory Protein-3") and IL-13 were statistically significantly different in all three groups of patients, the highest levels of these cytokines were in patients from the AIT + PD group, and the lowest in healthy individuals. And the levels of IL-2 and IL-27 were statistically significantly higher in patients from the AIT + PD group than in healthy individuals, while the level of IL-27 was statistically significantly higher in patients with psychiatric disorders, compared with patients only with Hashimoto's. IL-21 and IL-5 levels were statistically significantly higher in the AIT group and in the AIT+PD group compared to the HC group. And the level of IL-15, on the contrary, was statistically significantly higher in the group of heal-

TABLE 1. RESULTS OF ANALYSIS OF THE LEVELS OF VARIOUS CYTOKINES IN THE STUDIED GROUPS, Me ($Q_{0.25}$-$Q_{0.75}$)

| Cytokine | Control group of healthy individuals- HC | Group of mentally healthy patients with Hashimoto's thyroiditis – AIT | Group of patients with Hashimoto's thyroiditis, associated with psychiatric disorders – AIT+PD | Group comparison options | Statistical significance (p) |
|---|---|---|---|---|---|
| **CCL20/MIP3α** | 7.36 (4.25-12.76) | 15.12 (10.06-25.20) | 26.04 (13.00-52.75) | HC - AIT | p = 0.006* |
| | | | | HC - AIT + PD | p < .001* |
| | | | | AIT - AIT + PD | p = 0.013* |
| **IFNγ** | 0.82 (0.00-5.02) | 1.68 (0.21-6.25) | 1.68 (0.41-3.78) | HC - AIT | p > 0.999 |
| | | | | HC - AIT + PD | p > 0.999 |
| | | | | AIT - AIT + PD | p > 0.999 |
| **IL-1β** | 1.08 (0.26-2.00) | 1.49 (0.73-3.71) | 1.29 (1.08-2.10) | HC - AIT | p = 0.220 |
| | | | | HC - AIT + PD | p = 0.280 |
| | | | | AIT - AIT + PD | p = 0.665 |
| **IL-12P70** | 0.38 (0.00-1.59) | 0.99 (0.00-5.32) | 2.07 (0.00-5.00) | HC - AIT | p = 0.738 |
| | | | | HC - AIT + PD. | p = 0.306 |
| | | | | AIT - AIT + PD | p = 0.738 |
| **IL-13** | 0.61 (0.00-2.07) | 50.84 (17.15-91.30) | 102.9 (51.78-172.60) | HC - AIT | p < 0.001* |
| | | | | HC - AIT + PD | p < 0.001* |
| | | | | AIT - AIT + PD | p = 0.038* |
| **IL-15** | 50.84 (32.87-70.60) | 0.56 (0.00-3.98) | 1.45 (0.15-3.08) | HC - AIT | p < 0.001* |
| | | | | HC - AIT + PD | p < 0.001* |
| | | | | AIT - AIT + PD | p = 0.289 |
| **IL-17A** | 0.0 (0.00-1.91) | 0.0 (0.00-1.87) | 1.44 (0.00-3.37) | HC - AIT | p = 0.625 |
| | | | | HC - AIT + PD | p = 0.138 |
| | | | | AIT - AIT + PD | p = 0.282 |
| **IL-2** | 0.39 (0.00-3.83) | 3.25 (0.24-4.50) | 4.59 (3.26-6.79) | HC - AIT | p = 0.125 |
| | | | | HC - AIT + PD | p = 0.008* |
| | | | | AIT - AIT + PD | p = 0.065 |
| **IL-21** | 0.0 (0.0-0.5) | 8.30 (0.00-18.77) | 7.43 (0.00-13.59) | HC - AIT | p < 0.001* |
| | | | | HC - AIT + PD | p < 0.001* |
| | | | | AIT - AIT + PD | p = 0.527 |
| **IL-27** | 0.48 (0.36-0.71) | 0.56 (0.47-0.75) | 0.78 (0.64-1.05) | HC - AIT | p = 0.310 |
| | | | | HC - AIT + PD | p = 0.003* |
| | | | | AIT - AIT + PD | p = 0.003* |
| **IL-5** | 0.0 (0.00-0.03) | 1.24 (0.01-4.15) | 1.06 (0.05-2.39) | HC - AIT | p < 0.001* |
| | | | | HC - AIT + PD | p < 0.001* |
| | | | | AIT - AIT + PD | p = 0.729 |
| **TNFα** | 15.86 (10.28-21.4) | 21.32 (15.47-25.75) | 19.5 (16.38-24.96) | HC - AIT | p = 0.192 |
| | | | | HC - AIT + PD | p = 0.192 |
| | | | | AIT - AIT + PD | p = 0.804 |

Note. *, are marked values $p < 0.05$ (according to the Mann–Whitney U test).

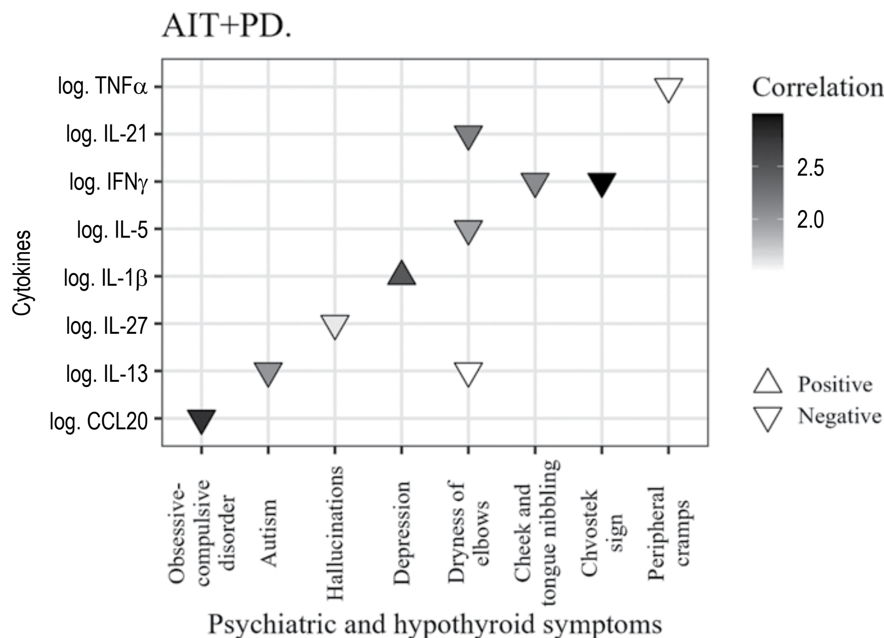


**Figure 1. Results of correlation analysis between cytokine levels and various psychiatric and hypothyroid symptoms in patients with Hashimoto's thyroiditis and psychiatric disorders**

thy individuals than in other groups. And the levels of IFNγ, IL-1β, IL-12P70, IL-17A and TNFα did not differ significantly in the study groups. The results are presented in Table 1.

Thus, both IL-13 and IL-5 were significantly higher in patients with mental disorders. Both of these cytokines belong to Th2-dependent, and their production is closely related, which probably explains the concordance of their changes in AIT. The increase in their production in AIT was also recorded by other authors. IL-13 is associated with the development of von Basedow-Graves disease, it is expressed not only in lymphocytes, but also in thyrocytes, and its production depends on TSH and its receptor [2].

To assess the mechanistic role of these changes, we evaluated the correlations between cytokines and hormones or autoantibodies. In AIT+ PD group, statistically significant direct correlations were found between the concentrations of FT3 and two cytokines: IL-1β ($r = 0.42$; $p = 0.031$) and IL-15 ($r = 0.51$; $p = 0.015$). This could result from the ability of these cytokines to alter hypothalamic-pituitary regulation of endocrine functions through vagal afferents that bear their receptors [8]. The finding is also quite consistent with the exclusive role of FT3 in the central nervous system: it is this particular hormone that has receptors on microglial cells and controls the processes of their neuroinflammatory activation and phagocytic behavior [10].

While analyzing the relationships between cytokines and various autoantibodies in AIT + PD group, we found significant direct correlations between the concentration of antibodies to TG and some cytokines: IL-15 ($r = 0.55$; $p = 0.008$) and IL-27 ($r = 0.4$; $p = 0.038$). IL-15 is known to be a cytokine stimulating memory T cells, $CD8^{+}$ lymphocytes and enhancing immune responses against intracellular parasites, but may also promote autoimmune inflammation in some autoimmunopathies. However, it also promotes T regulator differentiation under certain permissive conditions [5] which is in agreement with our results.

AIT patients without mental disorders had statistically significant inverse correlations between antibodies to TPO and IL-12P70 ($r = -0.54$; $p = 0.021$) and IL-17A ($r = -0.72$; $p = 0.003$) During the analysis of the relationship between cytokine levels and various clinical symptoms of mental disorders or hypothyroidism, almost all cytokines were negatively correlated with clinical manifestations, that is, the higher was the cytokine level, the less pronounced was the symptom (Figure 1). However, IL-1β levels were positively correlated with signs of depression (Figure 1). The data obtained in this study can be interpreted taking into account that cytokines can shift tryptophan metabolism in the CNS towards kynurenine pathway, which contributes to neurotransmitter disorders involved in the pathogenesis of schizophrenia [7].

## Conclusion

Mental disorders in AIT are related to characteristic changes in cytokine profile, which may result from neuroinflammation and associated with the level of anti-thyroid autoimmunity (anti-TG antibodies) as well as with the level of FT3.

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# ИММУНОМОДУЛИРУЮЩИЕ ЭФФЕКТЫ ПРОТИВООПУХОЛЕВЫХ ПРЕПАРАТОВ – ИНГИБИТОРОВ ТИРОЗИНКИНАЗЫ БРУТОНА – И ВОЗМОЖНОСТИ ИХ ИСПОЛЬЗОВАНИЯ ПРИ АЛЛЕРГИЧЕСКИХ И ИНФЕКЦИОННЫХ ЗАБОЛЕВАНИЯХ

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**Резюме.** Ингибиторы тирозинкиназы Брутона (BTK) представляют собой класс препаратов, которые показали свою эффективность и безопасность у больных хроническим лимфоцитарным лейкозом и неходжкинскими лимфомами, считавшихся невосприимчивыми к любому ранее применяемому типу терапии. BTK играет ключевую роль на всех стадиях развития В-лимфоцитов, однако в последние годы появились данные о том, что BTK также задействована и в активации миелоидных клеток.

Целью данного исследования является анализ и систематизация всех опубликованных материалов об иммуномодулирующих эффектах ингибиторов BTK (ибрутиниб, акалабрутиниб и др.).

Систематический обзор научной литературы был выполнен с использованием процесса пошагового поиска в электронных базах данных (PubMed, Web of Science, ScienceDirect и Scopus). При поиске в базе данных использовались следующие ключевые слова: “CLL”, “BTK”, “ibrutinib”, “COVID-19”, “allergy”, “inflamation”. Поиск исследований проводился с момента появления первого препарата ингибитора BTK (Ибрутиниб) в 2009 г. до декабря 2022 г.

Представлены имеющиеся на сегодняшний день результаты исследования влияния ингибиторов BTK на функциональное состояния В- и Т-лимфоцитов, нейтрофилов и моноцитов/макрофагов, описаны иммуномодулирующие эффекты ибрутиниба на клетки адаптивной и врожденной иммунной системы, включая $CD4^+$ и $CD8^+$Т-лимфоциты, NK-клетки. Поскольку ингибиторы BTK изменяют функциональную активность фагоцитарных клеток и соотношение популяций Т-клеток,

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появилось предположение о возможности использования этих препаратов для лечения ряда других нозологических форм, не только В-клеточных злокачественных новообразований, что на данный момент изучается в клинических исследованиях. Суммированы данные о применении ингибиторов БТК для борьбы со сверхострым воспалением, а также с целью подавления аллергических реакций, в том числе анафилаксии. Кроме того, обсуждается целесообразность кратковременного применения ингибиторов ВТК для снижения риска побочных эффектов при оральной иммунотерапии, а также для десенсибилизации к лекарственным средствам.

Приведенные данные свидетельствуют, что ингибиторы БТК является перспективными препаратами с иммуномодулирующим эффектом. Однако ингибиторам БТК следующего поколения предстоит повысить селективность для снижения нецелевого воздействии на другие киназы.

*Ключевые слова: тирозинкиназа Брутона, ингибиторы тирозинкиназы Брутона, ибрутиниб, акалабрутиниб, Т-лимфоциты, NK-клетки, нейтрофилы, моноциты/макрофаги*

# IMMUNOMODULATING EFFECTS OF ANTITUMOR DRUGS BRUTON TYROSINE KINASE INHIBITORS AND THE POSSIBILITY OF THEIR USE IN ALLERGIC AND INFECTIOUS DISEASES

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**Abstract.** Bruton's tyrosine kinase (BTK) inhibitors represent a class of drugs that have demonstrated their efficacy and safety in patients with chronic lymphocytic leukemia and non-Hodgkin's lymphomas who were considered refractory to any previously used type of therapy. BTK plays a key role in all stages of B lymphocyte development, but in recent years, there have been data indicating that BTK is also involved in the activation of myeloid cells.

The aim of this study is to analyze and systematize all published materials on the immunomodulatory effects of BTK inhibitors (ibrutinib, acalabrutinib, etc.).

A systematic review of the scientific literature was performed using a step-by-step search process in electronic databases (PubMed, Web of Science, ScienceDirect, and Scopus). The following keywords were used in the database search: "CLL", "BTK", "ibrutinib", "COVID-19", "allergy", "inflammation." The search for studies was conducted from the time of the first BTK inhibitor drug (ibrutinib) appearance in 2009 until December 2022.

The results of the study on the influence of BTK inhibitors on the functional state of B and T lymphocytes, neutrophils, and monocytes/macrophages are presented. The immunomodulatory effects of ibrutinib on adaptive and innate immune system cells, including $CD4^+$ and $CD8^+$T lymphocytes and NK cells, are described. Since BTK inhibitors alter the functional activity of phagocytic cells and the ratio of T cell populations, there is a suggestion about the possibility of using these drugs for the treatment of other nosological forms, not only B cell malignancies, which is currently being studied in clinical trials. Data on the use of BTK inhibitors to combat hyperacute inflammation and to suppress allergic reactions, including anaphylaxis, are summarized. In addition, the expediency of short-term use of BTK inhibitors to reduce the risk of side effects during oral immunotherapy and for desensitization to drugs is discussed.

The presented data indicate that BTK inhibitors are promising drugs with immunomodulatory effects. However, BTK inhibitors need to increase selectivity to reduce off-target effects on other kinases.

*Keywords: Bruton's tyrosine kinase, Bruton's tyrosine kinase inhibitors, ibrutinib, acalabrutinib, T lymphocytes, NK cells, neutrophils, monocytes/macrophages*

# Introduction

Bruton's tyrosine kinase (BTK) inhibitors are a new class of drugs for the treatment of chronic lymphocytic leukemia (CLL) and small lymphocytic lymphoma and mantle cell lymphoma. Since their FDA approval, ibrutinib and acalabrutinib have significantly changed the possibilities of CLL and mantle cell lymphoma therapy, increasing progression-free survival time, especially in patients with a high risk of unfavorable disease progression. However, during the use of these drugs, new data emerged that, in addition to their antiproliferative action on malignant B cells, BTK inhibitors also affect other immune system cells such as T lymphocytes, NK cells, granulocytes, monocytes, macrophages, demonstrating immunomodulatory and anti-inflammatory effects. The aim of this study is to analyze and systematize all published materials on the immunomodulatory effects of BTK inhibitors.

# Materials and methods

A systematic review of scientific literature was conducted using a step-by-step search process in electronic databases (PubMed, Web of Science, ScienceDirect, and Scopus). The following keywords were used in the database search: "CLL", "BTK", "ibrutinib", "COVID-19", "allergy", "inflammation". The search for studies was conducted from the time of the first BTK inhibitor drug (Ibrutinib) approval in 2009 until December 2022.

# Results and discussion

BTK is a cytoplasmic non-receptor tyrosine kinase that is essential for transmitting signals from the BCR and thus plays an important role in the development, survival, proliferation, differentiation, and activation of B cells at different stages of their development [1]. Upon BCR activation, BTK forms a signaling complex together with spleen tyrosine kinase (SYK), VAV protein, phosphoinositide 3-kinase (PI3K), adapter protein SLP65, and phospholipase Cγ2 (PLCγ2). BTK phosphorylates PLCγ2, transcriptional nuclear factor κB (NF-κB), nuclear factor of activated T cells (NF-AT), as well as extracellular signal-regulated kinase 1 and 2 (ERK1, ERK2), which in turn mediate subsequent functional responses [1]. In addition to BCR, BTK also regulates signaling pathways of chemokine receptors, including CXCR4 and CXCR5, which play a key role in chemotaxis and migration of B lymphocytes. Similarly, BTK plays an important role in regulating the survival, proliferation, and migration of malignant B lymphocytes, significantly affecting the development of malignant B cell neoplasms.

In recent years, BTK inhibitors are increasingly used instead of combined immunochemotherapy regimens (such as FCR, BR), especially in CLL and MCL patients with high risk of unfavorable prognosis. The use of ibrutinib and acalabrutinib has also been approved for the treatment of refractory and relapsed forms of lymphoplasmacytic lymphoma, marginal zone lymphoma, and Waldenstrom's macroglobulinemia.

Studies on the mechanisms of action of BTK inhibitors have shown that BTK is expressed not only in B lymphocytes but also in myeloid cell populations, including monocytes, macrophages, granulocytes, myeloid-derived suppressor cells (MDSC), dendritic cells (DC), osteoclasts, adipocytes, megakaryocytes, platelets, as well as NK cells and T lymphocytes [1].

**Immune Modulatory Effects of Ibrutinib and Acalabrutinib**

As BTK is involved in the activation of many immune cell populations, its inhibition by ibrutinib exerts a complex immune modulatory effect on both adaptive and innate immune system cells, including $CD4^+$ and $CD8^+$T lymphocytes, NK cells, and cells of most (all?) myeloid lineages [2, 3].

Impaired T lymphocyte function has been observed in patients with CLL, manifested by exhaustion of effector T cells, memory T cells, and immunosuppressive Treg. Most of the immune modulatory effects of BTK inhibitors on T cells (shift towards Th1 phenotype, decrease in Th2 cell numbers, alteration of Th17/Treg balance towards Treg dominance, reduction in cytokine production) are mediated by inhibiting ITK in TCR signaling pathways [3]. In response to CD3 and CD28 stimulation, BTK-/- T cells demonstrate reduced expression of activation marker CD69 and defective proliferation, as well as reduced cytokine production [4].

High levels of BTK expression are also characteristic of monocytes and macrophages [4]. In these cells, BTK critically regulates signal transduction from Toll-like receptors (TLRs), directly interacting with their cytoplasmic Toll/IL-1 (TIR) domains. Upon ligand binding, TLRs induce BTK phosphorylation, which promotes activation of transcription factor NF-κB and interferon regulatory factors (IRFs), necessary for upregulation of inflammatory cytokine, chemokine, and interferon gene expression [5, 6]. In monocytes, macrophages, and dendritic cells, ibrutinib and acalabrutinib inhibit signal transduction from other receptors that recruit BTK, including TREM-1 and Dectin-1, leading to reduced production of inflammatory cytokines and chemokines, as well as impaired phagocytosis of tumor cells and infectious pathogens. It has been shown that treatment with ibrutinib leads to decreased serum levels of various chemokines and inflammatory cytokines in patients with CLL. These changes may contribute to predisposition to infectious diseases and the possibility of cytokine imbalance.

In granulocytes, ibrutinib also inhibits activation and effector functions induced by BTK-dependent mechanisms upon activation of TLR, TREM-1, and NLRP3 inflammasome formation [7]. It has

been shown that ibrutinib suppresses inflammatory processes by blocking NLRP3 inflammasome assembly and subsequent caspase-1 activation, which prevents the maturation of IL-1β in neutrophils infiltrating the site of injury [7]. Neutrophils isolated from CLL patients treated with ibrutinib had reduced oxidative burst and bactericidal activity, as well as impaired ability to form extracellular traps (NETs) [8]. Treatment with BTK inhibitors in a mouse model of pneumococcal pneumonia led to a reduction in systemic neutrophil activation and their migration to the lungs [9]. In CLL patients in the early stages of treatment with ibrutinib, neutrophils produce less IL-8 (mediated by immune complexes through FcγR) and show reduced degranulation in response to opsonized *E. coli*, leading to a decrease in the release of neutrophil elastase, myeloperoxidase, and lactoferrin. In addition, neutrophils with BTK deficiency exhibit increased sensitivity to apoptosis, impaired maturation and differentiation, and decreased production of active oxygen species [8, 9].

Since ibrutinib and acalabrutinib alter the functional activity of phagocytic cells and the ratio of T cell populations, there is a hypothesis about the possibility of using these drugs to treat a number of other pathological conditions, including various malignancies of the hematopoietic and lymphoid systems, solid tumors, autoimmune diseases, atherothrombosis, and autoimmune diseases [8, 9]. The effectiveness of BTK inhibitors in the treatment of diseases in these groups is currently being studied in several clinical trials.

**Possible use of BTK inhibitors in allergies**

The main effector cells in allergic diseases, including food allergies, drug allergies, allergic rhinitis, asthma, and chronic spontaneous urticaria, are mast cells and basophils. When an allergen cross-links allergen-specific IgE bound to Fc RI on the surface of mast cells and basophils, a powerful activation signal is generated, which triggers rapid degranulation with the release of numerous allergic mediators, including histamine, prostaglandins, leukotrienes, and cytokines, that determine the development of clinical symptoms. Until recently, specific histamine receptor blockers, leukotriene receptor antagonists, and corticosteroids have been mainly used to treat allergic diseases to suppress pathological immune reactions and allergic inflammation. In recent years, target immunobiological agents have emerged to alleviate the symptoms of asthma and urticaria, aimed at reducing the level of circulating immunoglobulin E (IgE) (omalizumab), cytokines IL-4, IL-5 IL-13 or blocking their receptors. However, the use of these drugs does not allow achieving stable positive effects in a number of treated patients.

Recent studies have shown that BTK is involved in signal transduction through high-affinity Fc RI in human mast cells and basophils, and it is a critical signaling component for inducing histamine secretion, leukotriene C4, and IL-4. BTK inhibitors can prevent IgE-mediated degranulation and production of inflammatory cytokines by human mast cells and basophils [10]. Ibrutinib has inhibitory activity against a number of kinases involved in transmitting signals from Fc RI to ITK (proto-oncogenic tyrosine protein kinase FYN and tyrosine protein kinase LYN). Thus, BTK inhibitors can potentially be used to prevent allergic reactions, including anaphylaxis.

It is noteworthy that in human basophils, IgE-mediated reactions depend so much on the activity of kinases SRC, LYN, SYK, BTK, and PI3-kinase delta that selective inhibition of any of these kinases leads to complete inhibition of the release of all mediators. In 2017, a pilot study by Regan et al. demonstrated that ibrutinib completely eliminates skin prick test reactivity and IgE-mediated basophil activation test response to aeroallergens in CLL patients within 7 days after starting treatment. Data were obtained that only two doses of ibrutinib can reduce or eliminate skin prick test reactivity to food products and aeroallergens in individuals with allergies.

Currently, several new BTK inhibitors are undergoing clinical trials as potential drugs for the treatment of chronic spontaneous urticaria. The efficacy of fenebrutinib in chronic urticaria has been demonstrated during clinical trials, however, in a phase IIa study, a temporary increase in liver enzyme levels was recorded; this hepatotoxicity, not previously considered an effect characteristic of BTK inhibitors, may interfere with further trials of fenebrutinib [11].

In numerous studies, it has been shown that food oral immunotherapy (OIT) can lead to desensitization to food products in patients with food allergies [11]. However, OIT can be complicated by allergic reactions, ranging from minor (hives, upset stomach) to severe (systemic anaphylaxis requiring adrenaline treatment). Most adverse reactions during food OIT occur during the dose escalation phase. Currently, the feasibility of short-term use of BTK inhibitors to reduce the risk of side effects during OIT is being discussed. It is assumed that short courses of BTK inhibitors can reduce the frequency and/or severity of side effects during the escalation phase, allowing patients to safely reach the maintenance dose.

BTK inhibitors can also be used episodically in desensitization to drugs. It should be noted that ibrutinib is unlikely to prevent reactions to drugs that cause IgE-independent allergies, such as non-specific activation of mast cells due to iodine-containing contrast agents, as it is believed that BTK is not involved in the development of these hypersensitivity reactions. Thus, the use of BTK inhibitors is likely to be limited to preventing immediate-type hypersensitivity reactions (ITHRs), which have IgE-mediated mechanisms, particularly to beta-lactam antibiotics or platinum-based chemotherapeutic agents. BTK

inhibitors act quickly and have a short-term effect that wears off after discontinuation of the drug, potentially making episodic use of drugs to prevent ITHRs such as IgE-mediated anaphylaxis possible [11].

Despite the promising prospects for the use of BTK inhibitors to develop new treatment concepts for patients with IgE-dependent allergies, their introduction into allergology practice may be hindered by both their relatively high cost and the lack of clinical trial data on the safety and efficacy of these drugs – none of the BTK inhibitors currently used in clinical practice have been tested in children, and food OIT is typically indicated for children with food allergies.

**The use of BTK inhibitors in patients with COVID-19**

The presence of certain anti-inflammatory immunomodulatory effects of BTK inhibitors in patients with hematologic and oncologic diseases has led to the suggestion of using these drugs to control the hyperacute inflammation in patients with COVID-19. Positive expectations were supported by experimental studies that showed that the use of BTK inhibitors saved mice infected with influenza virus from lethal acute lung injury. Interesting data were also obtained when observing patients with hematologic malignancies who developed COVID-19 while taking the BTK inhibitor ibrutinib. For example, six patients with Waldenstrom's macroglobulinemia were reported to have only mild upper respiratory tract symptoms with COVID-19.

As of 2022, there are seven clinical trials registered to study the effectiveness of acalabrutinib (four trials) and ibrutinib (three trials) in COVID-19. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes acute respiratory distress syndrome, often leading to a fatal outcome. In some patients, a hyperactive inflammatory response of macrophages develops, manifested as a cytokine storm, which leads to respiratory failure. Pathological examination of the lungs in patients who died from coronavirus infection revealed extensive cellular infiltration with predominance of macrophages. It is assumed that the recruitment of macrophages in the development of infection may be associated with an increase in the expression of angiotensin-converting enzyme 2 (ACE2), regulated by increased concentrations of locally produced alpha-interferon. Macrophages can perceive the single-stranded RNA of the SARS-CoV-2 virus through TLR-7 with subsequent signal transmission, leading to NF-κβ activation. In the presence of the virus, NLRP3 inflammasome activates caspases, cleaving pro-IL-1β protein and releasing mature cytokine into the surrounding environment [7].

Ibrutinib has been shown to have potential anti-inflammatory effects in terms of lowering levels of inflammatory cytokines that are often elevated in severe COVID-19 [12]. Treatment of patients with severe COVID-19 with acalabrutinib also improved oxygenation and reduced IL-6 production by monocytes. In another clinical trial, acalabrutinib was administered to 19 patients hospitalized with severe COVID-19 (11 on non-invasive ventilation, 8 on mechanical ventilation). Patients were found to have improved oxygenation after 10-14 days of treatment with acalabrutinib [13]. These results confirm that BTK inhibition with ibrutinib or acalabrutinib may provide some degree of protection against the development of severe disease. Thus, BTK inhibition is one of the possibilities to reduce excessive inflammation in severe COVID-19.

## Conclusion

Bruton tyrosine kinase was discovered as a key factor in the development of B lymphocytes, which determined the possibility of using BTK inhibitors in B lymphoproliferative diseases. The emergence of a new class of therapeutic agents has led to a significant improvement in treatment outcomes in patients who were considered refractory to any previously used type of therapy. Initially, only B cells were considered to be targets of these medicinal products, but subsequently a significant role of BTK in the activation of myeloid cells became clear, since it enhances signals transduced not only from BCR, but also from other activating receptors. Due to the discovery of the mechanism of BTK activity in myeloid cells during the COVID-19 pandemic, new opportunities have opened for the use of BTK inhibitors in the suppression of hyperacute inflammation. In addition, the possibility of using this class of drugs for the control of allergic inflammation has been shown. However, it is important to consider that the currently marketed BTK inhibitors are not selective enough and have off-target effects also on several other TEC family kinases. Further research is needed to more accurately determine the possible areas of their use.

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# ПУПОВИННАЯ КРОВЬ КАК ПЕРСПЕКТИВНЫЙ ИСТОЧНИК NK-КЛЕТОК ДЛЯ ИММУНОТЕРАПИИ

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**Резюме.** В настоящее время большое количество исследований по генной модификации NK-клеток пуповинной крови (UCB-NK) проводится как на клиническом, так и доклиническом уровне. Иммунотерапия на основе UCB-NK-клеток обладает большим терапевтическим потенциалом для использования в противоопухолевой терапии. Однако, несмотря на известные преимущества перед PB-NK-клетками, такие как высокая концентрация в пуповинной крови, низкий процент передачи вируса от донора, а также возможность сохранения фенотипа после криоконсервации, UCB-NK-клетки преимущественно характеризуют в научной литературе как незрелые и низкофункциональные NK-клетки. В данной работе были изучены фенотипические характеристики UCB-NK-клеток и возможность стимуляционной компенсации сниженной функциональной активности UCB-NK-клеток. Проведенные исследования выявили, что фенотипически UCB-NK-клетки можно охарактеризовать как малодифференцированные и слабоактивированные клетки, экспрессирующие высокий уровень ингибирующего рецептора NKG2A, низкий уровень активирующего рецептора NKG2C и молекулы активации HLA-DR, что соответствовало литературным данным. Для стимуляции свежевыделенных UCB-NK-клеток было выбрано два вида стимулов: 1) 100 ед IL-2; 2) комбинация 100 ед IL-2 и фидерных клеток K-562, экспрессирующих мембраносвязанный IL-21 (K562-mbIL21). Было показано, что при стимуляции UCB-NK-клеток в течение 7 дней комбинацией IL-2 и K562-mbIL21 уровень дегра-

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нуляции (LAMP-1) и пролиферативная активность этих клеток была выше, чем у параллельно культивируемых в тех же условиях *Ex vivo* PB-NK-клеток, при этом стимул в виде IL-2 и K562-mbIL21 оказался более перспективным способом получения большого количества пролиферативно активных UCB-NK-клеток, по сравнению со стимуляцией только IL-2. Поскольку генетическая модификация NK-клеток является перспективным направлением улучшения противоопухолевых свойств NK-клеток, для дальнейшего изучения полученных UCB-NK-клеток была проведена процедура ретровирусной трансдукции. UCB-NK-клетки, стимулированные комбинацией IL-2 и K562-mbIL21, трансдуцировались на 8-й день культивирования. В данной работе применялась направленная оверэкспрессия адапторной молекулы DAP12, участвующей в сигналинге активирующих NK-клеточных рецепторов. PB-NK-клетки и UCB-NK-клетки трансдуцировали параллельно, в одинаковых экспериментальных условиях при равном объеме вирусных частиц. В результате было выявлено, что эффективность трансдукции вирусными частицами, несущими ген адапторной молекулы DAP12, в более чем 4 раза выше для UCB-NK-клеток по сравнению с PB-NK-клетками. Таким образом UCB-NK-клетки представляются перспективным инструментом для дальнейших исследований в области иммунотерапии рака.

*Ключевые слова: UCB-NK-клетки, фенотип, стимуляция NK-клеток, функциональная активность, трансдукция, DAP12*

# UMBILICAL CORD BLOOD AS A PROMISING SOURCE OF NK CELLS FOR IMMUNOTHERAPY

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**Abstract.** Currently, a large number of studies on genetic modification of cord blood NK cells (UCB-NK) are carried out at both clinical and preclinical levels. Immunotherapy based on UCB-NK cells has great potential for antitumor therapy. However, despite having known several advantages over peripheral blood NK cells (PB-NK), including a high concentration in cord blood and low virulence rate, UCB-NK cells are predominantly characterized in the scientific literature as immature and low-functioning NK cells. In this work, we studied the phenotypic characteristics of UCB-NK cells and the possibility of stimulatory compensation of the decreased functional activity of UCB-NK cells. Our studies revealed UCB-NK cells can be characterized as poorly differentiated and weakly activated cells with high level of inhibitory receptor NKG2A and low level of activating receptor NKG2C and HLA-DR, accordingly with the literature data. Two types of stimuli were chosen to stimulate freshly isolated UCB-NK cells: 1) 100 units of IL-2; 2) combinations of 100 units IL-2 and K-562 feeder cells expressing membrane-bound IL-21 (K562-mbIL21). It was shown the degranulation (LAMP-1) and proliferative activity was higher than for parallel cultured *ex vivo* PB-NK cells under the same conditions for UCB-NK cells stimulated for 7 days with IL-2 + K562-mbIL21. Moreover, stimulation in the way of IL-2 + K562-mbIL21 seemed to be a more perspective way to obtain a large number of proliferatively active UCB-NK cells compared to stimulation with IL-2 only. Since genetic modification of NK cells is a promising way to improve the antitumor properties of NK cells, retroviral transduction procedure was performed to study of the stimulated UCB-NK cells. UCB-NK cells stimulated with IL-2 + K562-mbIL21 were transduced on day 8 of cultivation. In this study, we used targeted overexpression of the adaptor molecule DAP12, which is involved in

the signaling of activating NK cell receptors. PB-NK cells and UCB-NK cells were transduced under the equal experimental conditions in same volume of viral particles. As a result, the transduction efficiency was found to be more than 4-fold higher for UCB-NK cells compared to PB-NK cells. Thus, UCB-NK cells appear to be a promising tool for further research in cancer immunotherapy.

*Keywords: UCB-NK cells, phenotype, NK cell stimulation, functional activity, transduction, DAP12*

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## Introduction

NK cells are lymphocytes of the innate immune system with great cytotoxic potential against tumor cells as well as cells infected by viruses [14]. Unlike T cells, NK cells do not require prior sensitization by antigen or presentation of antigen by HLA-I molecules to recognize “targets” [7], making them an attractive tool for tumor immunotherapy. Therapeutically useful NK cells can be derived from various sources, but currently the greatest number of clinical studies are focused on NK cells from peripheral blood (PB-NK) [9] and NK cells from cord blood (UCB-NK) NK cells [13]. Thus, UCB-NK cells have several advantages: 1) low risk of virus transmission from donor to recipient; 2) high concentration and, as a consequence, high availability of NK-cells and rapid receipt of the “ready” product; 3) possibility to preserve phenotypic characteristics and functional activity after cryopreservation [12]. Nevertheless, the use of cord blood as a source of NK cells for immunotherapy has its limitations: 1) difficult blood sampling; 2) UCB contains low numbers (between 10-100-fold fewer) nucleated cells blood [2] 3) UCB have specific phenotypic characteristics compared to PB-NK. It has been shown that UCB-NK cells have an immature phenotype [8], which is comparable to data reporting about decreased expression of the activating receptor NKG2C and increased expression of the inhibitory receptor NKG2A compared to PB-NK cells [15], although some groups of researchers have found that UCB-NK cells are sufficiently mature and functional in their phenotypic characteristics [11]. Currently, a large number of studies on UCB-NK gene modification are being conducted at both clinical and preclinical levels. One potentially promising area is genetic modification to improve the anti-tumor properties of NK cells. A variety of strategies aimed to enhance the cytotoxicity, survival and migration activity of NK cells have been developed for clinical application. One the approach is targeted overexpression of the adaptor molecule DAP12, which is involved in the signaling of activating NK cell receptors such as NKG2C, NKp44 and activating receptors of the KIR family [3].

## Materials and methods

Peripheral blood samples were collected from healthy volunteers agreed to participate in the study. The collection of umbilical cord blood samples of volunteer healthy adults was carried out based on the Federal Research and Clinical Center for Specialized Types of Medical Care and Medical Technologies (FMBA).

To isolate the umbilical cord mononuclear cells were collected in EDTA-containing test tubes and centrifuged in a Ficoll gradient with a density of 1.077 g/cm$^3$. NK cells were isolated from by negative magnetic separation from mononuclear cells using the NK cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s protocol. Freshly isolated NK cells were grown in NK MACS Medium (Miltenyi Biotech, Bergisch Gladbach, Germany) supplemented with 10% FCS (HyClone, USA), 2 mM L-glutamine, 2 mM sodium pyruvate (PanEco, Moscow, Russia), 2 mM antibiotic-antimycotic (Sigma-Aldrich, St. Louis, MO, USA), 1% of NK supplement (Miltenyi Biotech, Bergisch Gladbach, Germany).

To determine the most effective method of activation, peripheral blood or cord blood NK cells were cultured with stimuli in two variants: 1) 100 U/mL IL-2 (Hoffmann La-Roche, Basel, Switzerland) 2) in combination of 100 U/mL IL-2 (Hoffmann La-Roche, Basel, Switzerland) and irradiated K562-mbIL21 feeder cells with 1:2 ratio. The cells were cultured at 37 °C with 5% of $CO_2$. Cultivated NK cells were counted on eighth and tenth days by cell counter TC20 (Bio-Rad Laboratories, Hercules, CA, USA).

The proliferative activity of NK cells was evaluated using the internalizable fluorescent label CFSE passively penetrating the cells. The cells were incubated in a solution supplemented with 5 μmol/mL CFSE for 15 minutes in heat. The cells were washed three times with FBS serum medium to inactivate CFSE and cultured in complete medium with the addition of stimuli. The level of proliferation was measured at 7 and 10 days after staining using flow cytometry. Actively proliferating cells were detected by decreased CFSE levels.

NK cell degranulation was determined by the level of expression on the cell surface of the lysosomal marker LAMP-1 in the presence of brefeldin A and K562 target cells. For this purpose, NK cells were mixed with K562 in a 1:1 ratio and incubated for 2.5

hours in complete medium with CD107a antibodies and brefeldin. Cell surface staining and flow cytometry measurements were then performed.

UCB-NK were analyzed using a MACSQuant 10 flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany) equipped with lasers $\lambda$ = 405 nm, $\lambda$ = 488 nm, $\lambda$ = 635 nm; threshold was set to cut-off events with low CD45 staining. The following mouse anti-human fluorescent-labeled antibodies were used for surface cell staining: NKG2C-FITC (clone REA205, Miltenyi Biotec, Bergisch Gladbach, Germany), HLA-DR-PE (clone L243, Sony Biotechnology, San Jose, CA, USA), CD56-APC-Vio770 (clone REA196, Miltenyi Biotec, Bergisch Gladbach, Germany), KIR2DL2/L3-APC (clone DX27, Sony Biotechnology, San Jose, CA, USA), CD57-APC (clone HNK-1, Sony Biotechnology, San Jose, CA, USA).

The data was analyzed using FlowJo, GraphPad Prism X 10.0.7r2. Statistical analysis of differences in the data was conducted by Mann–Whitney one-tailed U test. $P < 0.05$ were considered significant.

Transduction of PB-NK and UCB-NK was performed in 24-well plates pre-treated with retronectin solution (Takara, USA) and concentrated viral particles containing the gene for the adaptor molecule DAP12 (TYROBP). To determine the infectious titer of the obtained retroviral particles, we preliminarily transduced the Raji cell line. Next, the number of transducing units (TU) was calculated using the formula (N cells * % transduction)/Vvir.particles) to predict the parameters for efficient PB-NK and UCB-NK transduction. Cytometric analysis of GFP luminescence was used to evaluate the efficiency of transduction, the measure was performed on day 3 after staging the experiment.

## Results and discussion

To study the phenotypic features of NK cells, the portion of the UCB mononuclear cell fraction obtained during isolation was taken for analysis. Expression of major NK cell surface markers, including the activation marker HLA-DR, inhibitory receptors KIR2DL2/3, NKG2A, activating receptor NKG2C, and differentiation marker CD57 were measured by flow cytometry. Phenotypic data characterize UCB-NK as poorly differentiated and weakly activated cells (Figure 1, see 3$^{rd}$ page of cover). However, it is worth noting that in comparison with the *ex vivo* PB-NK phenotype data we obtained earlier, a significant difference in the analyzed phenotypes of peripheral and cord blood NK cells was observed specifically in the expression of NKG2 family receptors; apparently, UCB-NK express higher levels of NKG2A and lower levels of NKG2C (Figure 1A, see 3$^{rd}$ page of cover), which corresponds to literature data [8, 15]. In addition, it has been shown decreased HLA-DR expression observed in UCB-NK cells (Figure 1, see 3$^{rd}$ page of cover) compared with PB-NK cells [5] suggests a priority stage of activation of peripheral blood NK cells over cord blood NK cells. However, also comparing with our earlier data, it has not been revealed significant differences in the percentage of CD56$^{bright}$, CD57$^{+}$ and KIR$^{+}$ subpopulations (Figure 1B, see 3$^{rd}$ page of cover) between PB-NK and UCB-NK cells were observed in the analysis [6].

The immaturity of UCB-NK cells makes it difficult to obtain clinically relevant antitumor therapy agents because such NK cells have reduced cytotoxicity compared to PB-NK cells [10]. However, it has been shown that various cytokine activation methods used, such as IL-2 or IL-15, or the combination of IL-15 with IL-2 or IL-18 can increase the functional activity of UCB-NK cells to the level observed for PB-NK cells [1]. Low number per unit of donor blood and immaturity of UCB-NK cells are the main obstacle to obtain a sufficient number of NK cells to create effective genetically modified antitumor therapy tools, so optimization of effective activation and genetic engineering processes is the basis for obtaining clinically meaningful results

To measure the level of proliferative activity, isolated PB-NK and UCB-NK cells were cultured under two types of stimulation: 1) with 100 units of IL-2; 2) in combination of IL-2 and K562-mbIL21 feeder cells. Cell counts were performed on days 8 and 14. The data obtained indicate that a steadily increasing induction of UCB-NK proliferation occurred in response to all stimuli added, and differences in proliferative activity were also revealed depending on the stimulation for PB-NK and UCB-NK stimulation (Figure 2, see 3$^{rd}$ page of cover). It was shown that only UCB-NK actively divided under conditions of stimulation with IL-2, whereas PB-NK had weak proliferative activity during the first 8 days of cultivation (Figure 2A, see 3$^{rd}$ page of cover), then the dynamics of the cell division process slowed and reached a plateau by day 21, in contrast to UCB-NK, which continued to proliferate actively (data not shown). In the stimulation way with K562-mbIL21 feeder cells, UCB-NK was found to outperform its own proliferation dynamics compared with the IL-2 only, whereas for PB-NK a high level of proliferation was observed only during the first 8 days after followed by a sharp decline in proliferative activity (Figure 2A, see 3$^{rd}$ page of cover). According to CFSE levels in cells on day 7 after IL-2 + K562-mbIL21 stimulation, the highest proportion of UCB-NK was involved in division (26.7%) compared with IL-2 and stimulation (10.6%) (Figure 2B, C, see 3$^{rd}$ page of cover). At day 10 after stimulation, there was a significant increase in proliferating UCB-NK stimulated by IL-2 + K562-mbIL21 (95.3%), whereas for IL-2, the proportion of divided cells was 62.2% (Figure 2B, C, see 3$^{rd}$ page of cover). In addition to the

analysis of NK proliferative activity, the cytotoxicity of UCB-NKs activated with combination of IL-2 and K562mbIL-21 was evaluated by measuring of the level of degranulation. Degranulation was determined by the level of expression of lysosomal marker LAMP-1 (CD107a) on the surface of UCB-NK. The baseline degranulation of NK cells without targets was taken as a negative control. It was shown that UCB-NK stimulated for 7 days with a combination of IL-2 and K562-mbIL21 had increased and even exceeded level of degranulation compared of PB-NK (Figure 2D, E, see 3rd page of cover).

Thus, UCB-NK cells proliferate in response to stimulation by both IL-2 and a combination of IL-2 and K562-mbIL21. Moreover, UCB-NK cells showed a more pronounced positive proliferative potential in response to cytokine stimulation, which is supported by literature data [1, 4]. Cultivation of *ex vivo* UCB-NK cells with IL-2 and K562-mbIL21 stimulation is a promising way to obtain a large number of proliferatively active UCB-NK cells with stable cytotoxicity.

After several transfection procedures of Phoenix Ampho cell line, sufficient volume of viral particles was accumulated to perform transduction of PB-NK and UCB-NK stimulated by a combination of IL-2 and K-562mbIL-21 for 7 days. Transduction of the Raji cell line (Figure 3A, see 3rd page of cover) was preliminarily performed and the infecting concentration of viral particles was determined using the formula TU = (Ncells*%transduction)/Vvir. particles) at a rate of 2TU for PB-NK and UCB-NK. All cells were transduced in parallel and with strictly the same volume of viral particles to compare the infection efficiency of PB-NK and UCB-NK. As a result, it was found that the transduction efficiency of viral particles carrying the DAP12 gene was more than 4-fold higher for UCB-NK compared to PB-NK (Figure 3B, C, see 3rd page of cover). Experimental data were confirmed in three independent repeats using a single accumulated stock of viral particles containing the DAP12 gene.

## Conclusion

The obtained data indicate that depending on the selection of cultivation conditions, stimulation can contribute to an increase in the efficiency of gene modification for UCB-NK cells compared to PB-NK cells. And despite the limitations [12] in use, UCB-NK cells remain a promising tool for application in antitumor therapy. In addition, the choice of a target gene for transduction did not fall by chance, since the adaptor molecule DAP12 is involved in signaling from a number of activating NK-cell receptors, such as NKG2C, NKp44, activating receptors of the KIR family [3]. In the future, we plan to evaluate the effect of DAP12 overexpression on the ratio of NKG2A and NKG2C receptors on the surface of UCB-NK cells, as well as on the way for “switching” the phenotype of NK cells from NKG2A+ to NKG2C+, thereby increasing the phenotypic and functional characteristics of genetically modified UCB-NK cells with further possibility of their use in clinical practice.

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# ИЗУЧЕНИЕ СОДЕРЖАНИЯ ИММУНОГЛОБУЛИНОВ И ОКИСЛЕННЫХ БЕЛКОВ В ЭЯКУЛЯТЕ ПРИ БЕСПЛОДИИ

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**Резюме.** Среди причин мужского бесплодия достаточно внимания уделяется окислительному стрессу, который в свою очередь является патогенетическим звеном воспалительного процесса. Однако практически нет информации о содержании окисленно модифицированных протеинов в спермальной жидкости, что затрудняет изучение патогенеза заболеваний репродуктивной системы мужчин. Отчасти окисление протеинов может быть обусловлено продукцией активных форм кислорода микроорганизмами как напрямую, так и опосредованно через активацию клеток иммунной системы. Цель исследования – изучить уровень окислено модифицированных белков и изменения концентрации иммуноглобулинов в спермальной жидкости при бактериоспермии. Проведено исследование эякулята 48 мужчин, обратившихся в клинику по поводу бесплодия в браке. Группу сравнения составили 32 практически здоровых мужчины, у которых отсутствовал рост микроорганизмов в образцах эякулята. При проведении бактериологического анализа исследуемые образцы разводили в 10 раз и использовали общепринятую методику. В спермальной жидкости определяли концентрацию альбумина, иммуноглобулинов A, M, G, E. Окислительную модификацию белков оценивали в реакции с 2,4-динитрофенилгидразином. Концентрацию окисленных белков выражали в нмоль/мг общего белка исследуемой биологической жидкости. Для определения концентрации белка использовали биуретовый метод. Статистический анализ результатов проводили с использованием методов описательной статистики и t-критерия Стьюдента для парных данных. Концентрация белка в семенной жидкости среди изучаемых групп существенно не отличалась. Концентрация альбумина (16,96±1,28 мг/мл) была статистически значимо ниже при отсутствии роста микроорганизмов, чем при бактериоспермии. При бактериоспермии отмечено снижение концентрации IgM и IgA и повышение уровня IgG. Степень окисления белков максимальна при выделении из семенной жидкости энтеробактерий. Таким образом, в ходе исследований было установлено, что, несмотря на отсутствие клиники, при бессимптомной бактериоспермии наблюдается секреция иммуноглобулинов G в спермальную жидкость. Показано накопление окисленных белков в семенной жидкости при бактериоспермии.

*Ключевые слова: окисленно модифицированные белки, спермальная жидкость, динитрофенилгидразин, альдегидные производные, кетоновые производные, иммуноглобулины*

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# STUDY OF THE IMMUNOGLOBULIN AND OXIDIZED PROTEIN CONTENT OF SEMEN UNDER INFERTILITY

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**Abstract.** Among the causes of male infertility, enough attention is paid to oxidative stress, which in turn is a pathogenetic link in the inflammatory process. However, there is practically no information on the content of oxidized modified proteins in the semen, which makes it difficult to study the pathogenesis of diseases of the male reproductive system. In part, protein oxidation may be due to the production of reactive oxygen species by microorganisms, both directly and indirectly through the activation of immune system cells. The aim of the research was to study the level of oxidized modified proteins and changes in immunoglobulin concentrations in the semen under bacteriospermia. A study was made of the ejaculate of 48 men who applied to the clinic for infertility in marriage. The comparison group consisted of 32 practically healthy men who had no growth of microorganisms in the ejaculate samples. When conducting bacteriological analysis, the studied samples were diluted 10 times and used the generally accepted method. The concentration of albumin, immunoglobulins A, M, G, E was determined in the spermatic fluid. The oxidative modification of proteins was evaluated in the reaction with 2,4-dinitrophenylhydrazine. The concentration of oxidized proteins was expressed in nmol/mg of the total protein of the studied biological fluid. The biuret method was used to determine the protein concentration. Statistical analysis of the results was performed using descriptive statistics and Student's t-test for paired data. The concentration of protein in the seminal fluid did not differ significantly among the studied groups. The albumin concentration (16.96±1.28 mg/mL) was statistically significantly lower in the absence of microorganism growth than in bacteriospermia. With bacteriospermia, a decrease in the concentration of IgM and IgA and an increase in the level of IgG were noted. The degree of protein oxidation is maximum when enterobacteria are isolated from seminal fluid. Thus, during the studies it was found that, despite the absence of a clinic, with asymptomatic bacteriospermia, the secretion of immunoglobulins G into the semen is observed. The accumulation of oxidized proteins in the seminal fluid in bacteriospermia has been shown.

*Keywords: oxidized proteins, semen, dinitrophenylhydrazine, aldehyde derivatives, ketone derivatives, immunoglobulins*

## Introduction

Numerous research report a decrease in the quality of semen and other markers of male reproductive health [6, 7]. There is a global crisis of male reproductive health [3], which is confirmed by a global decrease in the number of spermatozoa and an increase in anomalies of the male reproductive system, such as cryptorchidism, tumors of the urogenital tract [2]. The male factor of infertility occurs in about 40% of couples suffering from infertility [1, 5]. The data demonstrate a link between male infertility and general health [2].

It has been shown that asymptomatic bacteriospermia is often found in men who are in infertile marriages. It describes both the direct negative effect of microorganisms on the quality of ejaculate, and indirectly through the reactions of the macroorganism [4]. At the same time, humoral factors of acquired immunity are one of the links of protection against infection. The inflammatory response also implements mechanisms of auto-injury, in particular by hyperproduction of activated forms of oxygen and nitrogen, which trigger carbonyl stress and accumulation of oxidized modified proteins (OMP), which lose their function. The determination of the OMP-level in ejaculate can be a sensitive integral marker of a decrease in male fertility, as well as the development of urogenital pathology of both infectious and non-infectious nature.

**The aim of investigation** was to evaluate the content of immunoglobulins M, G, A, E and the intensity of the accumulation of OMP in the sperm fluid of men with asymptomatic bacteriospermia.

## Materials and methods

Investigation of the ejaculate of 48 men who applied to the clinic for infertile marriage was conducted. The comparison group consisted of 32 practically healthy men who had no growth of microorganisms in the ejaculate samples. The collection of the material and its study were carried out according to standardized methods proposed by WHO experts [9].

For bacteriological analysis, the studied samples were diluted tenfold. Bacteriological examination was performed according to the generally accepted method [4]. The total microbial number was expressed in lg of colony-forming units per 1 mL (lg CFU/mL). Albumin concentration was measured using reagent

kits with bromcresol green (Russia). To determine the concentration of immunoglobulins M (IgM), G (IgG), A (IgA) and E (IgE), an enzyme immunoassay was used (Russia). The concentration of Ig and albumin was expressed in mg/g of total protein.

The OMP was evaluated by reaction with 2,4-Dinitrophenylhydrazine [9], with preliminary incubation of the sperm fluid with a 10% solution of Streptomycin Sulfate. The resulting 2,4-dinitrophinylhydrazones (DNP) were recorded at the following wavelengths: at 365 nm for ketone-DNP of a neutral nature (kDNPn), at 432 nm – ketone-DNP of a basic nature (kDNFb) and at 530 nm – aldehyde-DNP of a neutral nature (aDNFn). When determining the carbonyl content, a molar absorption coefficient (e) equal to 22,000 $M^{-1}cm^{-1}$ was used. The molar extinction coefficient of DNP was used to calculate the concentration of carbonyl derivatives of OMP. Optical density was measured using a flatbed spectrophotometer (USA) with a quartz 96-well microplate.

The concentration of oxidized proteins was expressed in μmol/mg of total protein of the studied biological fluid [8]. To determine the protein concentration, a biuretic method was used (Russia). The protein concentration was measured in each sample after complete dissolution of the protein precipitate in urea solution. The total concentration of oxidized proteins was calculated by summing all oxidized derivatives.

Statistical analysis of the results was carried out using descriptive statistics and Student's t-test methods for paired data, correlation analysis was carried out using Spearman's rank correlation coefficient.

## Results and discussion

It was found that in men with bacteriospermia, 67% had gram-positive cocci (group 1; 4.51±0.26 lg CFU/mL), in (33%) – gram-negative bacilli (group 2; 5.38±0.29 lg CFU/mL, $p < 0.05$ to group 1), the 3rd group consisted of a comparison group without bacterial growth.

The protein concentration in the sperm fluid in the 3rd group was 54.5±2.6 mg/mL, which is not statistically significant from concentration in the 1st (59.0±2.7 mg/mL; $p > 0.05$) and 2nd groups (57.3±3.9 mg/mL; $p > 0.05$).

The albumin concentration of 16.96±1.28 mg/mL and its proportion to the total protein of 30.8±1.5% in group 3 is statistically significantly lower ($p < 0.05$) than in group 1 (21.12±1.41 mg/mL and 35.3±1.2%, respectively) and in group 2 (22.56±2.29 mg/mL and 38.6±2.2%, respectively). However, groups 1 and 2 did not differ statistically significantly from each other.

The highest levels of IgM and IgA were discovered in the comparison group. However, in contrast, the minimum level of IgG was recorded in this group (Table 1). Concentrations of different classes of immunoglobulins did not differ statistically significantly between group 1 and 2. Immunoglobulins E were not detected in any sample.

The total concentration of OMP in group 1 was 1252±22 μmol/mg of total protein, and the ratio of oxidized serum proteins aDNPn : kDNPb : kDNPn was expressed as: 1:1.7:3.2; in group 2, the sum of OMP was 1674±64 μmol/mg of total protein, the ratio of OMP fractions: 1:1,8:3,3. In group 3, the amount of OMP was 598±20 μmol/mg of total protein, and the fractions of OMP were correlated as: 1:1.4:2.2.

**TABLE 1. CONTENT OF IMMUNOGLOBULINS IN THE SPERM OF INFERTILE MEN**

| Group | Content of immunoglobulins, mg/g of total protein | | |
|---|---|---|---|
| | IgG | IgM | IgA |
| 1 | 231.8±11.4* | 4.6±0.9* | 34.6±3.5* |
| 2 | 237.6±23.0* | 5.0±0.8* | 30.8±3.8* |
| 3 | 167.2±12.7 | 12.4±2.9 | 96.2±10.0 |

Note. *, $p < 0.05$ when compared with the data of the 3rd group.

**TABLE 2. CONCENTRATION OF DIFFERENT OMP FRACTIONS IN SEMEN**

| Groups | Concentration of OMP, μmol/mg of total protein | | |
|---|---|---|---|
| | Ketone-DNP of a neutral nature | Ketone-DNP of a basic nature | Aldehyde-DNP of a neutral nature |
| 1 | 675±38#, * | 365±20#,* | 212±9#,* |
| 2 | 909±117* | 493±56* | 272±20* |
| 3 | 282±30 | 187±18 | 129±12 |

Note. *, $p < 0.05$ in comparison with the data of the 3rd group; #, $p < 0.05$ between the indicators in groups 1 and 2.

The concentrations of every DNP derivatives are presented in Table 2. It is worth noting that in the group with germination of microorganisms of the Enterobacteriaceae family, the concentrations of all OMP derivatives ($p < 0.05$) significantly prevail compared to the other groups.

Data analysis shows a moderate direct correlation between the concentration of IgG and OMP fractions in the comparison group ($r = 0.41$) and in the 1st group ($r = 0.44$). In group 2, inverse correlation was found between the concentration of IgA and OMP fractions ($r = -0.54$).

The increase of albumin concentrations in groups with bacteriospermia can be explained by the effect of microorganisms on the microcirculation, resulting in increased transudation of such "heavy" molecules, but this mechanism need to study. The elevation IgG levels in the groups from patients with bacteriospermia are explained by chronic bacterial infection in the genitourinary tract, but the decrease of IgA and IgM levels in these groups can be associated with the suppression of the pool of secretory immunoglobulins of both class A and M, which also need to study. In chronic bacterial infection, the spectrum of OMP shifts towards ketone derivatives of DNP, which according to some data indicates the irreversible nature of protein oxidation.

Probably due to a stronger of the opsonizing ability of IgG in comparison with immunoglobulins of other classes, the activation of the cellular link of innate immunity occurs, which leads to “oxidative stress" and the accumulation of OMP. The persistence of gram-negative flora leads to a more active accumulation of OMP, possibly due to a less secretory immunoglobulins level, whose less pronounced opsonizing activity (compared to IgG) does not lead to such active intensification of “oxidative stress".

## Conclusion

Thus, our investigation has shown that, despite the absence of a clinic, with asymptomatic bacteriospermia, the secretion of immunoglobulins G into the sperm is observed, and the level of immunoglobulin A and M is significantly reduced. The accumulation of oxidized proteins in seminal fluid from patients with bacteriospermia is also shown, and OMP accumulates more intensively during gram-negative bacilli contamination.

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**ИЛЛЮСТРАЦИИ К СТАТЬЕ «ПУПОВИННАЯ КРОВЬ КАК ПЕРСПЕКТИВНЫЙ ИСТОЧНИК NK-КЛЕТОК ДЛЯ ИММУНОТЕРАПИИ» (АВТОРЫ: ВЕЛИЧИНСКИЙ Р.А., ВАВИЛОВА Ю.Д., БОЙКО А.А., ШУСТОВА О.А., ПАЛАМАРЧУК А.И., ЮСУБАЛИЕВА Г.М., КУЧЕРОВА О.Н., СТРЕЛЬЦОВА М.А., КОВАЛЕНКО Е.И. [с. 1259-1264])**

ILLUSTRATIONS FOR THE ARTICLE "UMBILICAL CORD BLOOD AS A PROMISING SOURCE OF NK CELLS FOR IMMUNOTHERAPY" (AUTHORS: VELICHINSKII R.A., VAVILOVA J.D., BOYKO A.A., SHUSTOVA O.A., PALAMARCHUK A.I., YUSUBALIEVA G.M., KUCHEROVA O.N., STRELTSOVA M.A., KOVALENKO E.I. [pp. 1259-1264])

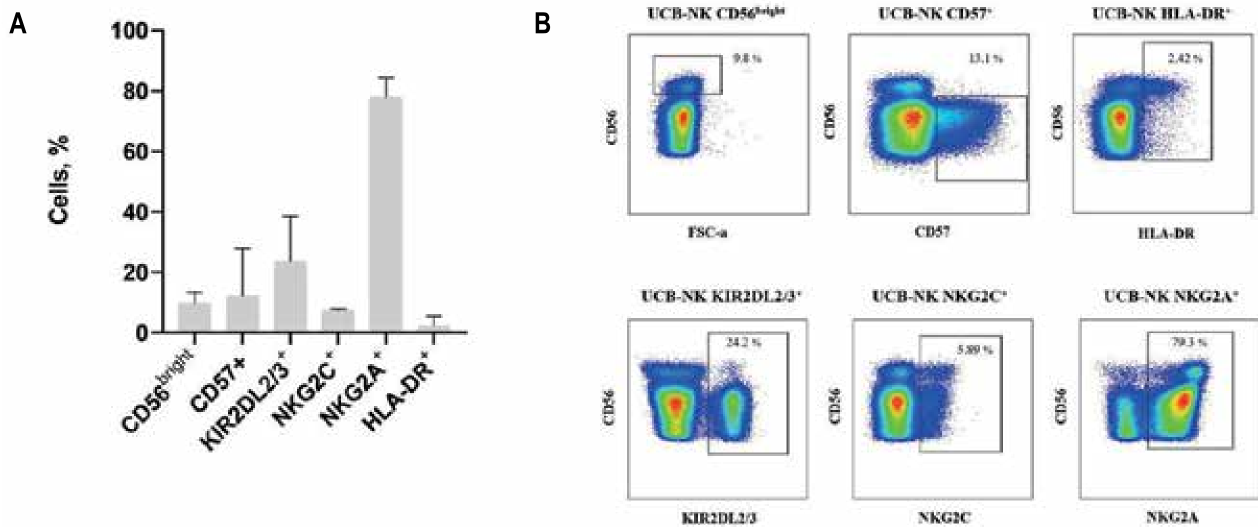


**Figure 1. Phenotypic analysis of *ex vivo* UCB-NK cells**

Note. (A) Percentages of NK cells expressing CD57, KIR2DL2/3, NKG2C, NKG2A, and HLA-DR. Data are presented as mean ± standard deviation. (B) Representative staining with CD57, HLA-DR, KIR2DL2/3, NKG2C, NKG2A distribution for NK cell subpopulations. NK cells in UCB samples were analyzed by flow cytometry after staining with fluorescent-labeled specific monoclonal antibodies. NK cells were defined as $CD3^-CD56^+$ cells in $CD45^{high}CD14^-$ cells in the FSC-SSC lymphocyte gate.

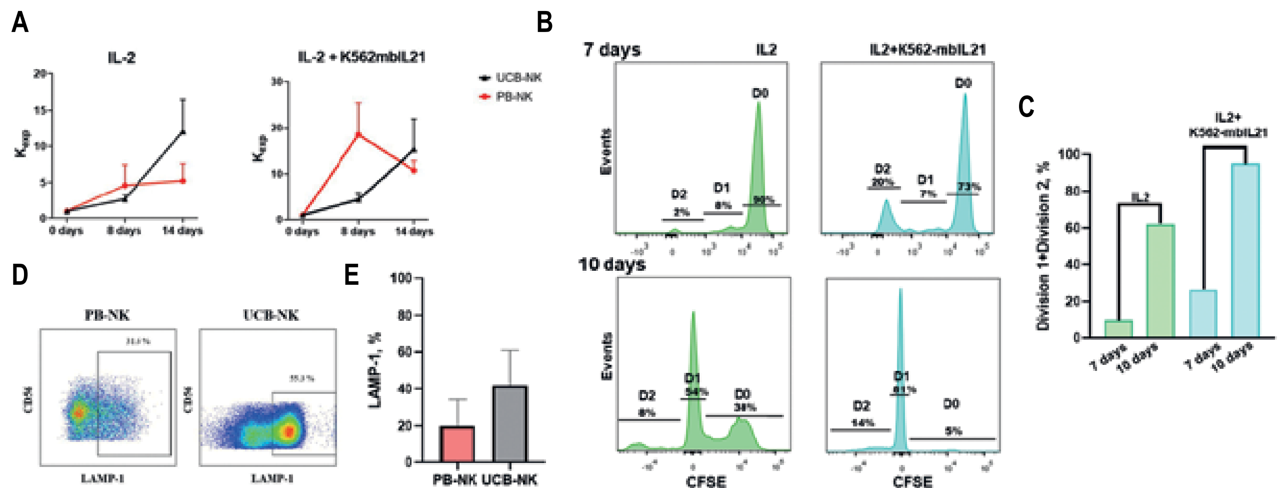


**Figure 2. Proliferative and functional activity of NK cells**

Note. (A) Comparison of PB-NK и UCB-NK expansion. Expansion coefficient (Kexp) = number of cells/initial number of cells (B) Representative CFSE profiles on day 7 and 10 after IL-2 and IL-2 + K562-mbIL21 stimulation. D1 and D2, percentage of CSFE-positive UCB-NK cells divided once and twice, respectively. (C) Proportion of CSFE-positive UCB-NK cells divided once (Division 1) + UCB-NK cells divided twice (Division 2) on days 7 and 10 after stimulation with two types of stimuli. (D) Representative dot plots of CD107a and expression in PB-NK and UCB-NK cells (E) Comparative analysis of $CD107a^+$ levels between PB-NK and UCB-NK stimulated with IL-2 in combination with K562-mbIL21 feeder cells for 8 days (target K562 cell line).

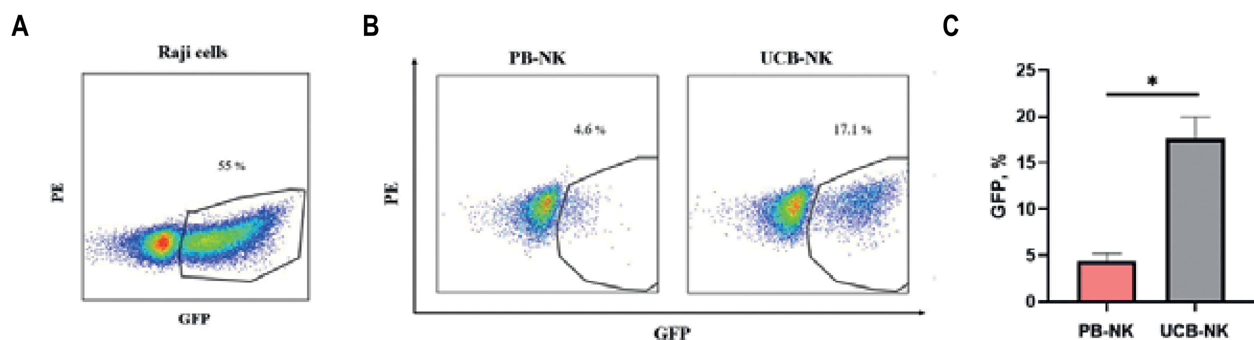


**Figure 3. Retroviral transduction of the DAP12 gene**

Note. (A) Estimation of the infectious titer for DAP12 viral particles on the Raji cell line. (B) Comparison of effectiveness for retroviral transduction of DAP12 gene on PB-NK and UCB-NK. Representative data from three independent repeats with use of one viral stock. (C) Comparison analysis of percentages of $GFP^+$ cells were conducted with nonparametric Mann–Whitney one-tailed U test.

**ПОДПИСНОЙ ИНДЕКС:**
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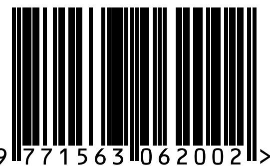
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