Levels of circulating cytokines in children with multiple sclerosis with different effectiveness of interferon therapy

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Tatiana V. Radygina, Svetlana V. Petrichuk, Olga V. Kurbatova, Daria G. Kuptsova, Andrei P. Fisenko, Elena L. Semikina, Ekaterina V. Freydlin, Luizat M. Abdullaeva, Bella I. Bursagova

FSAI "National Medical Research Center of Children's Health" under the Ministry of Health of Russia, 2/1, Lomonosovskij prospect, 119296, Moscow, Russia

Tatiana Vyacheslavovna Radygina — PhD (Medicine), Senior Research Associate of Laboratory of Experimental Immunology and Virology of FSAI "National Medical Research Center of Children's Health" under the Ministry of Health of Russia, ORCID ID: 0000-0003-4704-6885, e-mail: radigina.tv@nczd.ru.

Svetlana Valentinovna Petrichuk — PhD, MD (Biology), Professor, Chief Scientist of Laboratory of Experimental Immunology and Virology of FSAI "National Medical Research Center of Children's Health" under the Ministry of Health of Russia, ORCID ID: 0000-0003-0896-6996, e-mail: cito@list.ru.

Olga Vladimirovna Kurbatova — PhD (Medicine), Senior Research Associate, Head of Laboratory of Experimental Immunology and Virology of FSAI "National Medical Research Center of Children's Health" under the Ministry of Health of Russia, ORCID ID: 0000-0002-9213-5281, e-mail: putintseva@mail.ru.

Daria Gennadievna Kuptsova — Junior Researcher of Laboratory of Experimental Immunology and Virology of FSAI "National Medical Research Center of Children's Health" under Ministry of Health of Russia, ORCID ID: 0000-0001-7771-3314, e-mail: dg.kuptsova@gmail.com.

Andrei Petrovich Fisenko — PhD, MD (Medicine), Professor, Head of FSAI "National Medical Research Center of Children's Health" under the Ministry of Health of Russia, ORCID ID: 0000-0001-8586-7946, e-mail: fisenko@nczd.ru.

Elena Leonidovna Semikina — PhD, MD (Medicine), Chief Scientist of Laboratory of Experimental Immunology and Virology, Head of Laboratory Department of FSAI "National Medical Research Center of Children's Health" under the Ministry of Health of Russia, ORCID ID: 0000-0001-8923-4652, e-mail: semikinaelena@yandex.ru.

Ekaterina Valeryevna Freydlin — Research assistant of Laboratory of Experimental Immunology and Virology of FSAI "National Medical Research Center of Children's Health" under the Ministry of Health of Russia, ORCID ID: 0000-0002-5645-5181, e-mail: frdl-cito@mail.ru.

Luizat Muslimovna Abdullaeva — Junior Research Assistant of Laboratory of Rare Hereditary Diseases in Children of the Medical Genetic Center, Neurologist of Department of the Psychoneurology and Neurorehabilitation of Pediatric Center of Psychoneurology of FSAI "National Medical Research Center of Children's Health" under the Ministry of Health of Russia, ORCID ID: 0000-0003-1574-2050, e-mail: abdullaeva.lm@nczd.ru.

Bella Ibragimovna Bursagova — PhD (Medicine), Senior Research Associate of Laboratory of Rare Hereditary Diseases in Children of the Medical Genetic Center, Deputy for Medical Work of Department of the Psychoneurology and Neurorehabilitation of Pediatric Center of Psychoneurology of FSAI "National Medical Research Center of Children's Health" under the Ministry of Health of Russia, ORCID ID: 0000-0001-8506-2064, e-mail: bursagova@nczd.ru.

Resume. Multiple sclerosis (MS) is a chronic, demyelinating disease that leads to disability. Understanding the etiology of MS contributes to the development of pathogenetic methods of treatment, and the search for informative biomarkers of the effectiveness of treatment will allow the patient to adjust therapy in time. The aim of this work was to determine informative cytokines and cytokine profiles to predict the effectiveness of IFN-β1a therapy in children with MS.
Materials and methods. 66 children with MS aged 16 [14.2–17.5] years who are on INFß-1a therapy were examined: group 1 — patients with exacerbation of MS (with active foci of demyelination by MRI), n = 34; group 2 — patients in remission of MS (without active foci), n = 32. The content of cytokines in the blood serum of patients was assessed using the multiplex panel Human Th17 Magnetic Bead Panel.
Results: There was a significant increase in cytokine concentrations in patients with exacerbation of MS compared with children in remission: IL5, IL6, IL9, IL12p70, IL17E/IL25, IL21, IL28A, GM-CSF, TNFß. Threshold values for IL9 (AUC = 0,785), IL6 (AUC = 0,750), TNFß

For correspondence:
Tatiana Vyacheslavovna Radygina, Senior Research Associate of Laboratory of Experimental Immunology and Virology of FSAI "National Medical Research Center of Children's" under Ministry of Health of Russia.

Address: Address: 2/1 Lomonosovskij prospect, 119296, Moscow, Russia.

E-mail: radigina.tv@nczd.ru.

Для корреспонденции:
Радыгина Татьяна Вячеславовна, старший научный сотрудник лаборатории экспериментальной иммунологии ФГАУ «НМИЦ здоровья детей» Минздрава России.

Адрес: 119296, Россия, г. Москва, Ломоносовский пр., 2/1.

E-mail: radigina.tv@nczd.ru.

(0,740), IL28A (AUC=0,744) were obtained above which it is possible to predict an exacerbation of MS in patients: IL9 — 3.9 pg/ml (Sn — 70.6, Sp — 71.9), IL6 — 4.0 pg/ml (Sn — 70.6, Sp — 68.8), TNFß — 6.6 pg/ml (Sn — 70.6, Sp — 71.9), IL28A — 243.0 pg/ml (Sn — 70.6, Sp — 71.9). Cytokine profiles associated with T-lymphocytes and their functions were evaluated using z-score.
Conclusions. For the first time, an increase in cytokine levels was demonstrated in children with active foci of demyelination compared to patients in remission of MS. An increase in proinflammatory cytokines and cytokine profiles associated with Th1 and Th17, as well as with Th2 and Th22 has been shown. The use of threshold values for IL9, IL6, TNFβ, IL28A will help predict the development of exacerbation in patients with MS.
Keywords: cytokines, multiple sclerosis, children, Th1-cytokines, Th2-cytokines, Th17-cytokines, ИНФ-β1a.

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Уровни циркулирующих цитокинов у детей с рассеянным склерозом при разной эффективности интерфероновой терапии

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Радыгина Т. В., Петричук С. В., Курбатова О. В., Купцова Д. Г., Фисенко А. П., Семикина Е. Л., Фрейдлин Е. В., Абдуллаева Л. М., Бурсагова Б. И.

ФГАУ «НМИЦ здоровья детей» Минздрава России, 119296, г. Москва, Ломоносовский проспект, д. 2, стр. 1, Россия

Радыгина Татьяна Вячеславовна — к. м. н., старший научный сотрудник лаборатории экспериментальной иммунологии и вирусологии ФГАУ «НМИЦ здоровья детей» Минздрава России, ORCID ID: 0000-0003-4704-6885, e-mail: radigina.tv@nczd.ru.

Петричук Светлана Валентиновна — д. б. н., профессор, главный научный сотрудник лаборатории экспериментальной иммунологии и вирусологии ФГАУ «НМИЦ здоровья детей» Минздрава России, ORCID ID: 0000-0003-0896-6996, e-mail: cito@list.ru.

Курбатова Ольга Владимировна — к. м. н., старший научный сотрудник, и. о. заведующего лаборатории экспериментальной иммунологии и вирусологии ФГАУ «НМИЦ здоровья детей» Минздрава России, ORCID ID: 0000-0002-9213-5281, e-mail: putintseva@mail.ru.

Купцова Дарья Геннадьевна — младший научный сотрудник лаборатории экспериментальной иммунологии и вирусологии ФГАУ «НМИЦ здоровья детей» Минздрава России, ORCID ID: 0000-0001-7771-3314, e-mail: dg.kuptsova@gmail.com.

Фисенко Андрей Петрович — д. м. н., профессор, директор ФГАУ «НМИЦ здоровья детей» Минздрава России, ORCID ID: 0000-0001-8586-7946, e-mail: fisenko@nczd.ru.

Семикина Елена Леонидовна — д. м. н., главный научный сотрудник лаборатории экспериментальной иммунологии и вирусологии, заведующий лабораторным отделом ФГАУ «НМИЦ здоровья детей» Минздрава России, ORCID ID: 0000-0001-8923-4652, e-mail: semikinaelena@yandex.ru.

Фрейдлин Екатерина Валерьевна — лаборант-исследователь лаборатории экспериментальной иммунологии и вирусологии ФГАУ «НМИЦ здоровья детей» Минздрава России, ORCID ID: 0000-0002-5645-5181, e-mail: frdl-cito@mail.ru.

Абдуллаева Луизат Муслимовна — младший научный сотрудник лаборатории редких наследственных болезней у детей Медико-генетического центра, врач-невролог отделения психоневрологии и нейрореабилитации Центра детской психоневрологии ФГАУ «НМИЦ здоровья детей» Минздрава России, ORCID ID: 0000-0003-1574-2050, e-mail: abdullaeva.lm@nczd.ru.

Бурсагова Бэлла Ибрагимовна — к. м. н., старший научный сотрудник лаборатории редких наследственных болезней у детей Медико-генетического центра, заместитель по лечебной работе отделения психоневрологии и нейрореабилитации Центра детской психоневрологии ФГАУ «НМИЦ здоровья детей» Минздрава России, ORCID ID: 0000-0001-8506-2064, e-mail: bursagova@nczd.ru

Аннотация. Рассеянный склероз (РС) — это хроническое, демиелинизирующее заболевание, приводящее к инвалидизации. Понимание этиологии РС способствует разработке патогенетических методов лечения, а поиск информативных биомаркеров эффективности лечения позволит вовремя скорректировать терапию. Целью данной работы было выявить информативные цитокины и цитокиновые профили для прогноза эффективности терапии ИНФ-β1а у детей с РС.
Материалы и методы. Обследовано 66 детей с РС в возрасте 16 [14,2–17,5] лет, находящихся на терапии ИНФ-β1а: 1-я группа — пациенты в обострении РС (с активными очагами демиелинизации, по данным МРТ), n=34; 2-я группа — пациенты в ремиссии РС (без активных очагов), n=32. Содержание цитокинов в сыворотке крови пациентов измеряли с помощью мультиплексной панели Human Th17 Magnetic Bead Panel.
Результаты: у пациентов в обострении РС выявлено достоверное увеличение концентрации цитокинов IL5, IL6, IL9, IL12p70, IL17E/IL25, IL21, IL28A, GM-CSF, TNFβ по сравнению с детьми в ремиссии. Пороговые значения cut-off соста-

вили для IL9 (AUC = 0,785), IL6 (AUC = 0,750), TNFβ (0,740), IL28A (AUC = 0,744), выше которых можно прогнозировать у пациентов обострение РС: IL9 — 3,9 пг/мл (Sn — 70,6, Sp — 71,9), IL6 — 4,0 пг/мл (Sn — 70,6, Sp — 68,8), TNFβ — 6,6 пг/мл (Sn — 70,6, Sp — 71,9), IL28A — 243,0 пг/мл (Sn — 70,6, Sp — 71,9). Проведена оценка уровней цитокиновых профилей, ассоциированных с Т-лимфоцитами, а также с их функциями с использованием метода z-score.

Выводы. Впервые продемонстрировано увеличение уровней цитокинов у детей с активными очагами демиелинизации по сравнению с пациентами, находящимися в ремиссии РС. Обнаружено увеличение провоспалительных цитокинов, ассоциированных с Th1- и Th17-, а также с Th2- и Th22-лимфоцитами. Применение пороговых значений cut-off для IL9, IL6, TNFβ, IL28A позволяет прогнозировать развитие обострения у пациентов с РС.

Ключевые слова: цитокины, рассеянный склероз, дети, Th1-цитокины, Th2-цитокины, Th17-цитокины, ИНФ-β1а.

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INTRODUCTION

Multiple sclerosis (MS) is a chronic, demyelinating disease that occurs in a genetically predisposed individual under the influence of various adverse environmental factors and leads to damage to the central nervous system with subsequent disability. About 2,8 million people suffer from MS worldwide [1]. The prevalence of MS in Russia ranges in different regions from 36 to 79 cases per 100,000 population, while children constitute some 4–5% of all patients [2]. The disease has a remitting course in most children (97–99%), and the average age of the nosology onset is 14,2 ± 1,3 years [2]. Modern drugs allow to reduce the number of exacerbations and the progression of the disease [3]. Patients are prescribed long-term therapy with drugs, changing the course of MS (PITRS). There are 2 approaches of treating MS — escalatory and inductive. Escalatory approach implies prescription of I line PITRS, in case of their inefficiency, drugs of II line are used. Inductive approach uses drugs of II line at the initial stage of therapy that are considered more efficient, but have a large number of adverse reactions [4]. IFN-β1a — I-line drug (allowed from 12 years) and fingolimod (II-line drug, allowed from 10 years) are referred to drugs allowed for MS treatment in children in Russia [5]. The mechanism of IFN-β1a action is to reduce T-cell activation and adhesiveness, the inhibition of matrix metalloproteinases and the loss of the ability for lymphocytes to pass through the blood-brain barrier. The effect of fingolimod is aimed at suppressing lymphocyte release from the lymph nodes [4].

The main mechanism of MS pathogenesis is immune system dysfunction [6]: particularly, the prevalence of T-helpers type 1 (Th1) over T-helpers type 2 (Th2) during an exacerbation and the production of increased levels of such pro-inflammatory cytokines as IFNγ and IL-12 [7]. There is also the critical role of T-helpers 17 (Th17) in the pathogenesis of MS, producing IL-17 and IL-23 [8]. Adults with MS showed an increase in the cytokine concentration of both Th1- (IL-1β, IL-2 и TNFα) and Th17-lymphocites (IL-17A, IL-21 and IL-22) that confirms both populations of T-lymphocytes to be involved in the pathogenesis of MS [9]. The inflammatory response and impaired interaction of immune cells with MS, mediated by cytokines, constitute an attractive target for MS immunotherapy [10]. Over the last decade there has been an increase in the number of studies, dedicated to the search for predictive biomarkers of disease progression and response to drug treatment [11, 12]. It has been shown in adult patients with MS that an increase in concentration of cytokines IL-1α, IL-4, IL-18, CCL7 CCL27, INFγ, LIF, M-CSF, SCF and TNFα allows to differentiate the phase of MS with a high degree of accuracy [13]. There are very few works on studies of the levels of cytokines with MS in children. An increase in the number of cytokines (IL-10, IL-21, IL-23, IL-27) was shown in children with MS both in an exacerbation state and in remission, compared to the group of healthy children [14]. Understanding of the pathogenesis of MS will contribute to the development of treatments, modifying the course of the disease, and the search for predictors of inefficiency of drugs used will allow to adjust therapy on time.

The purpose of this work was to identify the main cytokines and cytokine profiles to prognose the efficacy of INF-β1a therapy in children with multiple sclerosis.

MATERIALS AND METHODS

66 children with MS, aged 14,2-17,5 years (average age — 16 years) were examined, undergoing ING-β1α therapy. The patients were divided into groups based on clinical anamnestic and the presence of active foci of demyelination by the results of magnetic resonance analysis (MRA): group 1 contained patients with exacerbation of MS (with active foci) (n = 34); group 2 — patients in remission of MS (without active foci) (n = 32). The groups were compared by duration of the disease: exacerbation — [Me 1,2 (0,6–2,2)], remission — [Me 1,5 (0,7–3,4)], p = 0,545 — and by the duration of INF-β1a therapy: exacerbation — [Me 67,4 (14,8–95,7)], remission — [Me 41,4 (4,9–138,0)], p = 0,498.

All children were examined according to regulatory documents of the Russian Federation after receiving approval from the local ethics committee FSAI "NMRC for Children's Health", the Health Ministry of the Russian Federation (protocol № 6 of 11 June, 2019) and informed consent of parents and children over 14 in accordance with the Declaration of Helsinki.

Venous blood samples for the study were obtained from the cubital vein on an empty stomach in BD Vacutainer® clot activator test tubes. The test tubes with blood were centrifuged at 1500 rpm for 10 min, the resulting serum was stored at −80 °C until analysis.

The measurement of cytokine concentration in samples was performed using multiplex analysis (xMAP-technology) with MILLIPLEXMAP Human Th17 Magnetic Bead Panel (EMD Millipore Corporation, USA). The analysis made was according to the manufacturer's instruction, followed by analysis on Bio-Plex™ 200 Assay System (Bio-Rad, США) flow fluorometer using xPONENT 4.2 and Milliplex Analyst 5.1 software. The panel of the studied cytokines included: IL-17F, GM-CSF, IFN-γ, IL10, CCL20/MIP3α, IL12p70, IL13, IL15, IL17A, IL22, IL9, IL1β, IL33, IL2, IL21, IL4, IL23, IL5, IL6, IL17E/IL25, IL27, IL31, TNFa, TNFβ, IL28A. The results of cytokines were obtained in pg/ml. Differences between the groups of patients in exacerbation and remission of MS were analyzed on separate cytokines as well as on the complex of cytokines, associated with different cells and their functions: macrophage M (IL-1+IL-6+TNF-α), regulatory Reg (IL4+IL5+IL10+IL13+IL33), associated with particular cells (c), cell functions (f): Th1-assoiciated (cTh1) — (IFN-γ+IL12p70+TNF-β+IL2); Th2-associated (cTh2) — (IL4+IL5+IL10+IL13+IL17E/IL25+IL33), Th17-associated (cTh17) — (IL1β+IL6+IL17A+IL17F+IL21+IL22+IL23), fTh2+mast-associated fTh2+mast) — (IL4+IL5+IL31+IL13); family IL-12 (fIL12) — (IL12+IL23+IL27); fTh17 — (IL17A+IL17F); fTh2 (IL4+IL5+IL13); fTh22 (IL13+IL22) [14, 15, 16, 17].

To analyze and normalize data for cytokine profiles, we have implemented the approached, described in Cataldi C. work [18]. Z-standardization (z-score) was conducted for cytokines, using the following formula: $z = (x_i - \mu)/\sigma$, where z — standardized score, x_i — initial sampling unit, μ — arithmetic mean, σ — standard deviation. Z of separate cytokines was summed up to define z-score of cytokine complexes.

Statistical processing of the data obtained was carried out using Statistica 10.0 (StatSoft, USA) programs. Descriptive statistics of quantitative trait are presented in the format: median (lower and upper quartiles) — Me ($Q_{0,25}$–$Q_{0,75}$). Significance of differences between groups in the condition of exacerbation and remission was evaluated using nonparametric the Mann-Whitney U-test. Differences were considered statistically significant at $p < 0,05$. ROC-analysis was used to identify thresholds of cytokines in exacerbation and remission, the area under the curve, sensitivity and specificity were determined (SPSS, version 25, USA). Spearman correlation analysis was carried out to evaluate the effect of disease and therapy duration on the level of cytokines in patients with MS.

RESULTS AND DISCUSSION

The content of cytokines in the blood serum in patients with MS in groups with and without active foci of demyelination is presented in the table 1. In particular, significant differences for 9 pro-inflammatory cytokines out of 25 studied ones (IL5, IL6, IL9, IL12p70, IL17E/IL25, IL21, IL28A, GM-CSF, TNFβ) were found when comparing two groups. Concentrations of all the above cytokines were higher as well in the group of patients with active foci, compared to the group of patients without active foci (table 1).

Table 1. **Cytokine content in groups of MS patients with active foci and without active foci**
Таблица 1. **Содержание цитокинов в группах пациентов с РС с активными очагами и без активных очагов**

Cytokine name	Group 1 Me ($Q_{0,25}$–$Q_{0,75}$), pg/ml N = 34	Group 2 Me ($Q_{0,25}$–$Q_{0,75}$), pg/ml N = 32	p_{12}
IL1β	1,7 (0,3–2,0)	1,7 (0,5–2,0)	0,084
IL2	6,3 (4,3–10,8)	5,2 (2,5–10,8)	0,237
IL4	95,2 (27,6–130,4)	38,6 (4–124,2)	0,165
IL5	5,3 (3,7–6,5)	1,9 (0,1–4,1)	**0,013**
IL6	11,7 (0,9–30,2)	1,5 (0,3–5,4)	**<0,001**
IL9	8,5 (1,8–25,5)	0,2 (0,2–4,9)	**<0,001**
IL10	2,6 (0,1–5,1)	2,4 (0,1–5,1)	0,814
IL12p70	2,2 (1,9–2,6)	1,7 (0,2–2,0)	**0,021**
IL13	198,2 (101,7–313,1)	133,9 (57,4–235,7)	0,092
IL15	5,5 (3,9–9,2)	5,5 (1,9–10,8)	0,784
IL17A	0,5 (0,3–0,8)	0,3 (0,2–1,2)	0,0985
IL17E/IL25	25,6 (10,5–33,4)	7,7 (2,0–29,9)	**0,049**
IL17F	45,6 (9,7–55,9)	16,8 (2,2–39,6)	0,062
IL21	7,5 (5,3–8,8)	5,3 (0,9–7,7)	**0,031**
IL22	709,3 (36,0–1444,0)	36,0 (36,0–797,8)	0,146
IL23	928,9 (8,0–1343,0)	83,6 (8,0–1025,0)	0,185
IL27	518,7 (394,5–745,5)	558,6 (334,5–860,9)	0,924
IL28A	369,9 (45,0–808,9)	45,0 (45,0–255,1)	<0,001
IL31	35,1 (18,2–66,3)	4,2 (0,7–50,5)	0,098
IL33	20,9 (6,2–28,2)	2,4 (0,1–22,1)	0,105
CCL20	21,5 (16,9–25,6)	20,1 (8,5–32,0)	0,579
GM-CSF	212,4 (148,1–255,0)	12,4 (2,0–217,2)	0,019
IFNɣ	3,8 (3,0–9,0)	3,3 (0,9–8,3)	0,495
TNFa	15,0 (9,7–16,8)	11,1 (7,4–30,7)	0,479
TNFβ	71,9 (3,0–149,0)	3,0 (3,0–7,9)	**<0,001**

Note: group 1 — with active foci, group 2 — without active foci.

Примечание: группа 1 — с активными очагами, группа 2 — без активных очагов.

A weak inverse correlation is revealed between disease duration in children with MS and the level of cytokines: IL33 (R = −0,26); IL23 (R = −0,27); IL6 (R = −0,32); IL31 (R = −0,27); IL28A (R = −0,25). A similar correlation is found between therapy duration in children with MS and the level of cytokines: IL17F (R = −0,38); IL10 (R = −0,48); IL12p70 (R = −0,49); IL2 (R = −0,37); IL4 (R = −0,40); IL5 (R = −0,34); IL6 (R = −0,34); IL17E/IL25 (R = −0,33); TNFβ (R = −0,33); IL28A (R = −0,37). Thus, there was reduced level of cytokines in the blood serum in children with MS with an increase in duration of the disease and INF-β1a therapy. ROC-analysis showed a good quality of the separation model for exacerbation/remission conditions for the following cytokines: IL9 (AUC = 0,785), IL6 (AUC = 0,750), TNFβ (0,740), IL28A (AUC = 0,744) (fig. 1).

Threshold cut-off values are obtained, above which it is possible to prognose exacerbations of MS in patients (table 2.)

There was evaluation of cytokine complexes on z-score in groups of patients with MS. Analysis of the results showed that cytokine profiles M and with Th1 on z-score were significantly different in patients with active foci, compared to patients in remission, whereas cytokine profiles, associated with Th2 and Th17 cells, между группами did not differ significantly between groups (fig. 2A). Meanwhile, difference between groups of patients with MS for

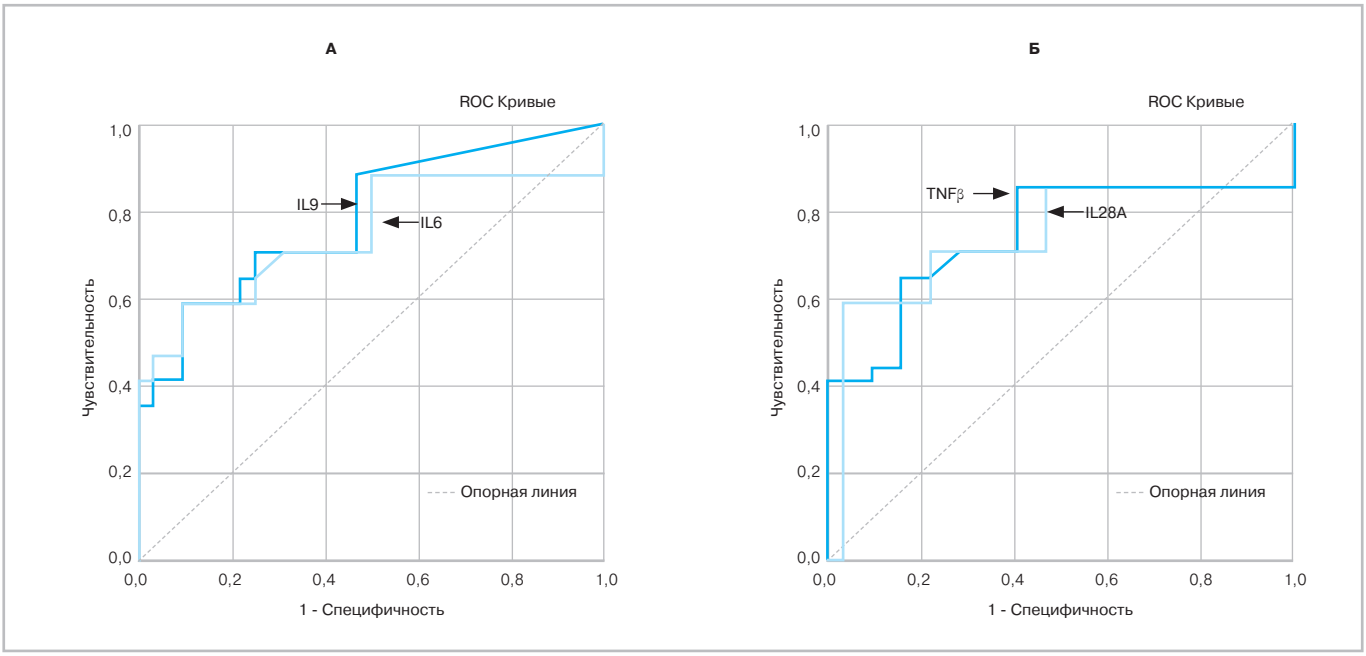

Fig. 1. **ROC curves for serum cytokines in children with MS. A — IL9, IL6; B — TNFβ, IL28A**
Рис. 1. **ROC-кривые для цитокинов сыворотки крови у детей с РС. А — IL9, IL6; Б — TNFβ, IL28A**

Reg, fTh22 and fTh2 cytokine profiles were reliable (fig. 2Б).

Thus, patients with MS exacerbation showed a significant increase in pro-inflammatory cytokines, involved in the differentiation and activation of different types of Th-cells, compared to patients in remission of the disease. It has been also found that patients in MS exacerbation experience a significant increase in cytokine production, necessary for differentiation from naive $CD4^+$-cells in Th1-cells and a significant increase in cytokine profile, characteristic of M1-macrophages as well as levels of cytokines, associated with the activation and function of Th2-cells: IL5, IL17E/IL25, IL13, IL33. It is interesting to note that patients in exacerbation showed a significant increase in cytokines of the regulatory profile (IL4, IL6, IL10, IL11 и IL13, IL33). It is known that, on the one hand, these cytokines play a neuroprotective role, and on the over hand, may be involved in the damage to the brain tissues, activating Th2-cells with subsequent stimulation of B-cells and complement activation [19]. We have shown previously that patients with active foci of demyelination have a significantly lower number of T-regulatory lymphocytes, compared to the group in MS remission, which might be explained by the compensatory response of T-cells [20]. Recently there have been studies on Th22 role in autoimmune diseases that produce IL22 and IL13 [21, 22].

It is known that, depending on the microenvironment, Th22 may differentiate into Th1- and Th2-cells. Besides, IL22 can play synergistic role with IL17, damaging the integrity of blood-brain barrier [21]. As our study has shown, patients in exacerbation of MS have a significantly increased profile of cytokines, synthesized by Th22-cells.

Table 2. **Cytokine thresholds for exacerbation/remission conditions in children with MS**
Таблица 2. **Пороговые значения цитокинов для состояний обострение/ремиссия у детей с РС**

Cytokine	Sensitivity (Sn), %	Specificity (Sp), %	cut-off, pg/ml
IL9	70,6	71,9	3,9
IL6	70,6	68,8	4,0
TNFβ	70,6	71,9	6,6
IL28A	70,6	71,9	243,0

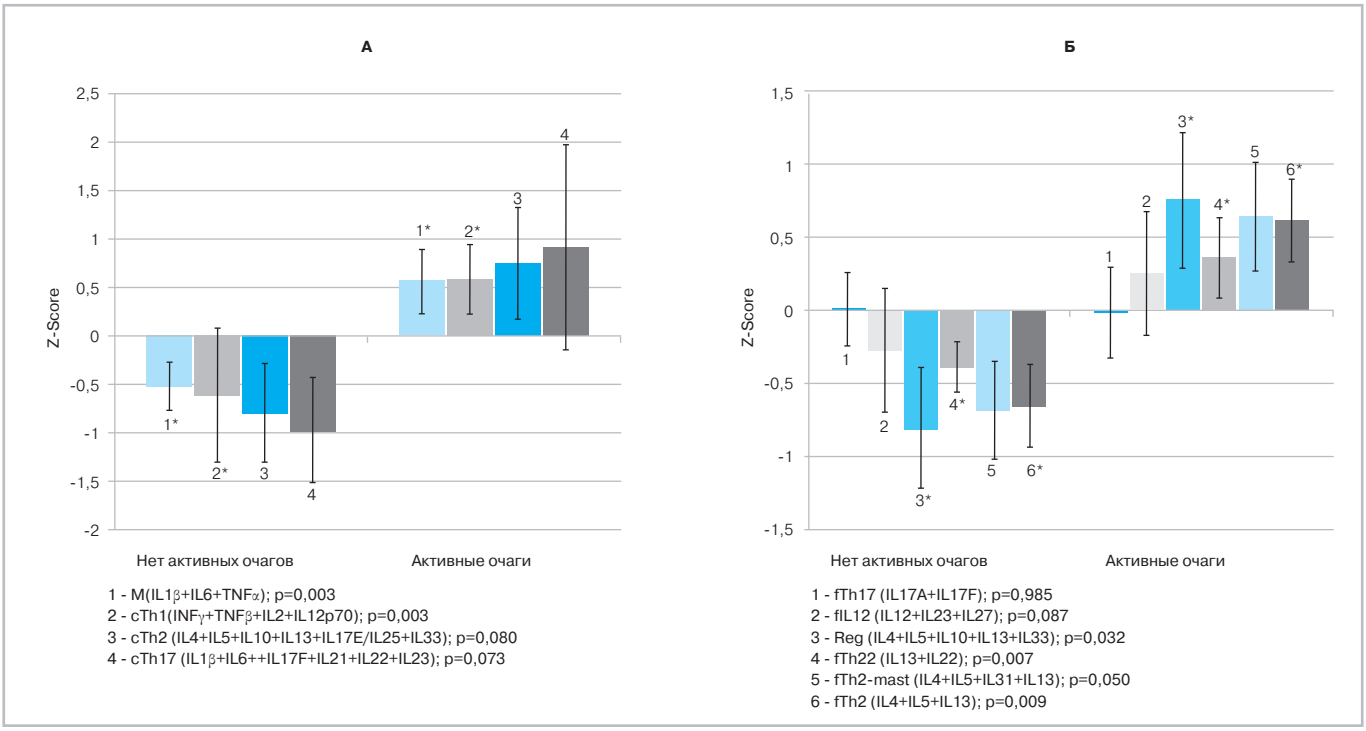

Fig. 2. **Z-score of cytokine profiles. A — M, cTh1, cTh2, cTh17. B — cytokine profiles associated with the function: fTh17, fIl12, Reg, fTh22, fTh2-mast, fTh2**

Рис. 2. **Z-score профилей цитокинов у пациентов с активными очагами и без активных очагов. А — M, cTh1, cTh2, cTh17. Б — профили цитокинов, ассоциированные с функцией: fTh17, fIl12, Reg, fTh22, fTh2-mast, fTh2**

Примечание: * отмечены достоверные результаты (p < 0,05); по оси OY — отложены средние значения в z-трансформации с указанием стандартной ошибки.

Note: * — reliable results are marked (p < 0,05); on the OY axis — the average values in the z-transformation are postponed, indicating the standard deviation.

CONCLUSION

For the first time, we obtained the data that demonstrate an increase in cytokine levels in patients with active foci of demyelination compared to patients in remission of MS. There is an increase in proinflammatory cytokines, necessary for differentiation of naïve $CD4^+$ T-cells into effector cells and activation of not only Th1 and Th17, but also Th2 and Th22-helper populations. Most significant differences were noted for cytokines, associated with Th1-cells. Levels of pro-inflammatory IL10 and IL13 cytokines did not differ between patient group. However, the profile of regulator cytokines was higher in patients in exacerbation, which may indicate the compensatory cell response to inflammation. Threshold cut-off values, obtained for IL9, IL6, TNFβ, IL28A, will help predict the development of exacerbation in patients with MS.

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THE AUTHORS' CONTRIBUTION TO THE WORK

Tatiana V. Radygina — collection of material, conduct of study, text writing, statistical analysis, text editing.
Svetlana V. Petrichuk — text writing, statistical analysis, text editing.
Olga V. Kurbatova, Daria G. Kuptsova — text writing, text editing.
Andrei P. Fisenko, Elena L. Semikina, Luizat M. Abdullaeva, Bella I. Bursagova — text editing.
Ekaterina V. Freydlin — processing and preparation of biological material for research.

ВКЛАД АВТОРОВ В РАБОТУ

Радыгина Т. В. — сбор материала, проведение исследования, написание текста, статистическая обработка, редактирование текста.
Петричук С. В. — написание текста, статистическая обработка, редактирование текста.
Курбатова О. В., Купцова Д. Г. — написание текста, редактирование текста.
Фисенко А. П., Семикина Е. Л., Абдуллаева Л. М., Бурсагова Б. И. — редактирование текста.
Фрейдлин Е. В. — обработка и подготовка биологического материала к исследованию.