BULLETIN OF RUSSIAN STATE MEDICAL UNIVERSITY

BIOMEDICAL JOURNAL OF PIROGOV RUSSIAN NATIONAL RESEARCH MEDICAL UNIVERSITY

SUBMISSION http://vestnikrgmu.ru/login?lang=en
CORRESPONDENCE editor@vestnikrgmu.ru
COLLABORATION manager@vestnikrgmu.ru
ADDRESS ul. Ostrovityanova, d. 1, Moscow, Russia, 117997

Indexed in Scopus. CiteScore 2022: 0.6

Indexed in WoS. JCR 2021: 0.5

Five-year h-index is 8

SCImago Journal & Country Rank 2020: 0.14

Listed in HAC 31.01.2020 (№ 507)

Open access to archive

Issue DOI: 10.24075/brsmu.2024-03
The mass media registration certificate № 012769 issued on July 29, 1994
Founder and publisher is Pirogov Russian National Research Medical University (Moscow, Russia)

Approved for print 30.06.2024
Circulation: 100 copies. Printed by Print.Formula
www.print-formula.ru

ВЕСТНИК РОССИЙСКОГО ГОСУДАРСТВЕННОГО МЕДИЦИНСКОГО УНИВЕРСИТЕТА

НАУЧНЫЙ МЕДИЦИНСКИЙ ЖУРНАЛ РНИМУ ИМ. Н. И. ПИРОГОВА

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Журнал включен в Scopus. CiteScore 2022: 0,6

Журнал включен в WoS. JCR 2021: 0,5

Индекс Хирша (h^5) журнала по оценке Google Scholar: 8

SCImago Journal & Country Rank 2020: 0,14

Журнал включен в Перечень 31.01.2020 (№ 507)

Здесь находится открытый архив журнала

DOI выпуска: 10.24075/vrgmu.2024-03
Свидетельство о регистрации средства массовой информации № 012769 от 29 июля 1994 г.
Учредитель и издатель — Российский национальный исследовательский медицинский университет имени Н. И. Пирогова (Москва, Россия)

Подписано в печать 30.06.2024
Тираж 100 экз. Отпечатано в типографии Print.Formula
www.print-formula.ru

BULLETIN OF RSMU **3, 2024**
ВЕСТНИК РГМУ

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Nakonechnaya TO[1], Shagina IA[2,3], Myshkin MYu[2,3], Mutovina ZYu[4], Ryazantseva EV[4], Chudakov DM[1,2], Turchaninova MA[2,3], Britanova OV[2,3] ✉

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Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by inflammation of connective tissue and damage to various organs, including joints, skin, kidneys and heart. The disease has a significant gender predisposition and is more common in women. The pathogenesis of SLE is based on a violation of immunological tolerance, accompanied by activation of B lymphocytes and the production of autoantibodies. Recent advances in basic research have significantly deepened the understanding of the immunopathogenetic mechanisms of SLE, which justifies the use of new pharmacotherapeutic approaches. These approaches involve the use of biological drugs aimed at blocking the activity of type I interferon (IFN) or its receptors. The article discusses the molecular mechanisms of activation of the interferon response in SLE, modern methods for diagnosing the interferon signature, and new approaches to treatment aimed at blocking the interferon pathway. The possible role of the interferon signature in the stratification of SLE patients is also discussed. Such stratification will make it possible to more effective select treatment regimens taking into account the individual characteristics of the immune response of each patient. This may increase the effectiveness of treatment, reduce the likelihood of side effects and improve the prognosis for patients with SLE.

Keywords: systemic lupus erythematosus, interferon signature, anifrolumab

Financing: the study was supported by a grant from the Moscow Government (NIP No. 2412-63/22-1 dated May 13, 2022), sponsored by the Moscow Center for Innovative Technologies in Healthcare.

Author contribution: Myshkin MYu — literature analysis; Mutovina ZYu, Shagina IA — data collection in the field of rheumatology and medicine; Chudakov DM — concept, Turchaninova MA — analysis and interpretation of scientific data; Ryazantseva EV, Kazhdan MA — manuscript proofreading, Britanova OV, Nakonechnaya TO — literature analysis and manuscript preparation.

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Received: 24.06.2024 **Accepted:** 28.06.2024 **Published online:** 30.06.2024

DOI: 10.24075/brsmu.2024.027

АНАЛИЗ ЭКСПРЕССИИ ИФН-СТИМУЛИРОВАННЫХ ГЕНОВ КАК ИНСТРУМЕНТ ДЛЯ ОПТИМИЗАЦИИ ТЕРАПИИ СИСТЕМНОЙ КРАСНОЙ ВОЛЧАНКИ

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Системная красная волчанка (СКВ) представляет собой хроническое аутоиммунное заболевание, характеризующееся воспалением соединительной ткани и поражением различных органов, включая суставы, кожу, почки и сердце. Заболевание демонстрирует значительную гендерную предрасположенность, чаще встречается у женщин. В основе патогенеза СКВ лежит нарушение иммунологической толерантности, сопровождающееся активацией В-лимфоцитов и продукцией аутоантител. Достижения последних лет в фундаментальных исследованиях значительно углубили понимание иммунопатогенетических механизмов СКВ, что обосновывает применение новых фармакотерапевтических подходов, в том числе использование биологических препаратов, направленных на блокировку активности интерферона (ИФН) типа I или его рецепторов. В статье рассмотрены молекулярные механизмы активации интерферонового ответа при СКВ, современные методы диагностики интерфероновой сигнатуры и новые подходы к лечению, направленные на блокировку интерферонового пути. Обсуждается возможная роль интерфероновой сигнатуры для стратификации пациентов с СКВ. Стратификация позволит более точно подбирать терапевтические схемы, учитывая индивидуальные особенности иммунного ответа каждого пациента. Такой подход может повысить эффективность лечения, снизить вероятность развития побочных эффектов и улучшить прогноз для пациентов с СКВ.

Ключевые слова: системная красная волчанка, интерфероновая сигнатура, анифролумаб

Финансирование: исследование выполнено на средства гранта Правительства Москвы (НИР № 2412-63/22-1 НИР от 13.05.2022), спонсор — АНО «Московский центр инновационных технологий в здравоохранении».

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Статья получена: 24.06.2024 **Статья принята к печати:** 28.06.2024 **Опубликована онлайн:** 30.06.2024

DOI: 10.24075/vrgmu.2024.027

Epidemiological significance

The incidence of SLE varies from 4 to 250 cases per 100,000 population, the incidence rate depends on the region, ethnic composition of the population, gender and age [1, 23].

The risk of SLE in women is 8–10 times higher than in men; women have a higher risk during their reproductive years of 16–25 years, while SLE activity is expected during pregnancy and the puerperium [1–3].

Mortality in SLE is 4–5 times higher than in the the population at all; Possible causes of death in patients with SLE include infection (30%), neuropsychiatric disorders (15%), renal failure (14%) and cardiopulmonary damage (8%) [1].

Brief overview of the disease

Systemic lupus erythematosus (SLE) is an autoimmune disease in which the human immune system perceives host connective tissue cells as foreign [4]. The central mechanism of the immunopathology of SLE is a violation of immunological tolerance, leading to uncontrolled activation of B-cells response, the development of which is determined by a combination of genetic and epigenetic predisposition, environmental factors (ultraviolet radiation, viral infections, etc.) and intestinal dysbiosis [5].

Dendritic cells play a central role in the production of type I interferon and influence the clearance and sensitivity of nucleic acids (NAs) and immune complexes, known autoantigens in lupus (Fig. 1). In fact, endogenous and extrinsic nucleic acids are the major antigenic stimulus in SLE. Autoantibodies targeting nucleic acid-bound antigens are one of the hallmarks of the disease. Apoptosis and NETosis (NET) may be the main source of such antigens. Excessive and impaired NET degradation is associated with lupus severity, lupus nephritis, anti-dsDNA antibodies, and complement consumption.

The strategic goal of SLE treatment is to achieve a state of remission or low activity [6–8].

Despite the increase in life expectancy of patients with SLE, associated primarily with improved tactics of using glucocorticoids (GCs) and immunosuppressive drugs, the incidence of deaths remains high, and adequate control of inflammatory diseases is observed in no more than half of patients [9]. Progress in basic research contributes to a better understanding of the pathogenesis of SLE and provides a conceptual basis for the development of new approaches to the pharmacotherapy of SLE [8, 10–11].

Diagnostics

In clinical practice, clinical complaints and manifestations in combination with hematological and immunological disorders are assessed to diagnose SLE [12]

In 2012, the SLICC/ACR diagnostic criteria for SLE were developed: the diagnosis is considered established if 4 criteria are present, of which one criterion must be clinical and the other immunological.

The EULAR/ACR (2019) [13] criteria are used to classify SLE, with sensitivity and specificity ranging from 96.1% to 93.4% [14].

The role of T and B lymphocytes in the pathogenesis of SLE

The causes of SLE in adults can vary, including genetics, hormonal imbalances, past infection, and environmental factors. Often in SLE there is an increased circulation of apoptotic bodies formed after cell death. The engulfment of cell bodies by dendritic or B cells can result in the presentation of self-antigens on their surface in the MHC class II complex, leading to T cell destruction and increased inflammation.

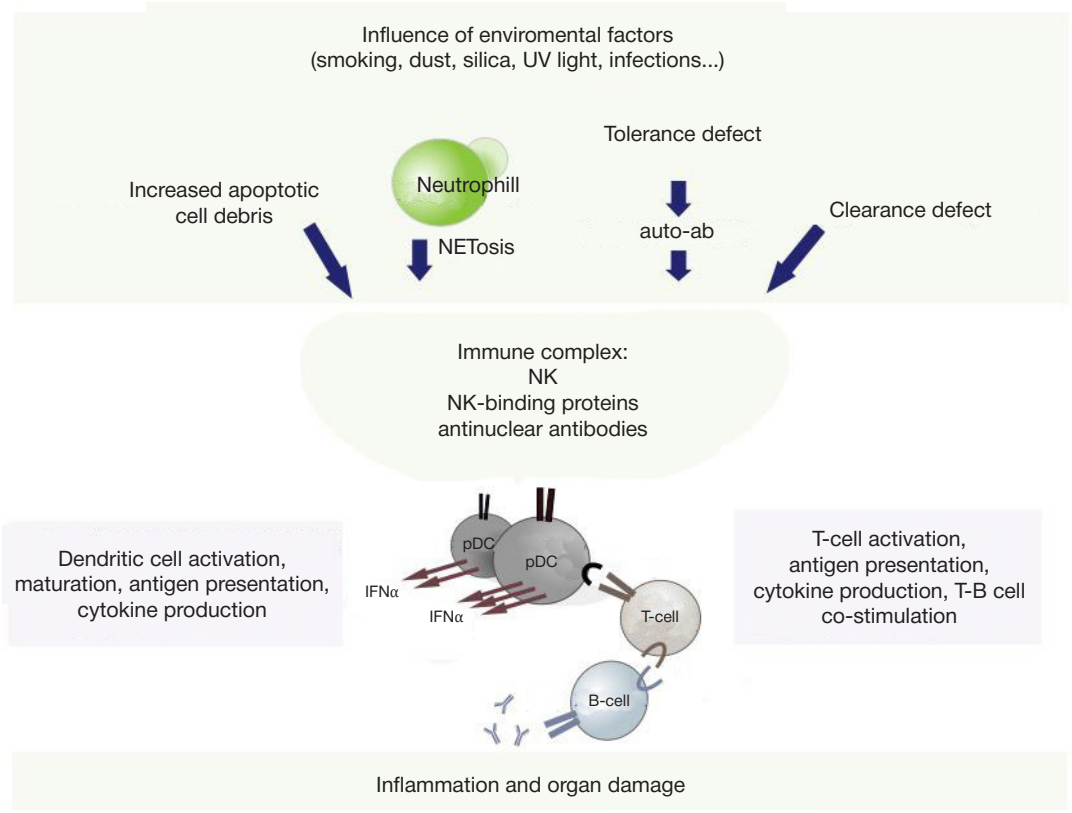

Fig. 1. Development of systemic lupus erythematosus

A study of 41 patients with active SLE and 101 healthy donors showed that the proportion of both $CD4^+CD28^-$ and $CD8^+CD38^+HLADR^+$ T cells with effector activity was significantly increased in SLE [15]. There is a shift toward a Th1716 inflammatory response with a decrease in the proportion of Tregs [16, 17].

Increased levels of expression of interferon-induced genes or type I interferon "signature" are found in the mononuclear fraction of blood, which confirms the key role of the innate immune system in the pathogenesis of SLE [18,19]. An increased type I interferon (IFN) signature has been reported to be found in approximately 75% of adult patients and 90% of pediatric patients [20].

The role of interferons in the development of the disease

Recently, special attention has been paid to the regulation of interferon production in SLE [12, 21, 22].

Interferon is a cytokine produced under normal conditions in response to viral infection and has effects such as regulation of immunity, antiviral and antitumor activity. Depending on the sequence of the first protein, the cognate receptor, the gene locus and the cell type responsible for its production, IFN is mainly classified into three types.

– Type I IFN (IFN-α and IFN-β, -ϵ, -κ and -ω);
– IFN type II (IFN-γ);
– IFN type III (IFN-λ).

Many studies have shown the phenomenon of IFN-α dominance in SLE, but there is evidence that the IFN-γ signature may occur in the early stages of SLE and play an important role in the development of lupus nephritis [23] and, in general, IFN-γ levels are higher in the serum of patients with SLE than in healthy people [24, 25], and the pattern is that there is an abnormal accumulation of IFN-γ long before the diagnosis of SLE and before the appearance of autoantibodies and IFN-α.

IFN-γ levels and their gene boundaries have also been shown to increase with type I IFN activation in SLE patients [26, 27].

IFN-γ is a pleiotropic type II IFN that is primarily produced by effector Th1 $CD4^+$ T cells, cytotoxic $CD8^+$ T cells and NK cells and to a lesser extent by other cell types such as dendritic cells (DCs), macrophages and B cells [28]. IFN-γ binds to IFN-γ receptors (IFNG-R), which is expressed in most cells and activates Janus kinase 1 (JAK1) and JAK2 in canonical pathway, that lead to phosphorylation of STAT1 homodimers and binding to the IFN-γ activation site (GAS) for gene transcription [29]. Moreover, IFN-γ may also play a role in signal transduction through non-canonical pathways. There is overlap (crosstalk) between type I and type II inducible genes, and signaling pathways may be shared between them. Each type of interferon induces the production of the other, which ultimately leads to stimulation from the other side and a mixed signature [29].

IFN-α is a pleiotropic cytokine related to type I IFN that is widely used in patients with certain risk factors and viral diseases. IFN-α can influence tumor cell functions through several principles. In addition, these cytokines can mediate the differentiation and activity of host immune cells.

Type I IFN is critical. At least 10% of the genes of the human body take part for rergulation IFN type I, the expression of which depends on the cell type, cellular distribution of receptors and the nature of activation stimuli [30]. At the same time, against a viral background, the controlled synthesis of type I IFN is important in maintaining immune homeostasis by inducing the differentiation of B cells into plasma cells, synthesizing antiviral antibodies and generating B regulatory cells.

Plasmacytoid dendritic cells (pDCs) are the focus of attention in SLE [31]. Although almost all cells containing a nucleus are capable of synthesizing and activating type I IFN, its main source is pDCs, which generate it 1000 times more powerfully than other cells. Each pDC can produce up to 10^9 IFN-α molecules in 12 hours. This fact, as well as the unevenness of IFN-α compared to IFN-β in the blood in SLE, confirms that pDCs are the main cellular source of IFN-α in SLE. Accordingly, pDC deficiency has been shown to ameliorate disease in mouse models [32, 33]. Although other cell types, including macrophages and fibroblasts, are also known types of IFN I, these cells exclusively synthesize IFN-β. However, isolation of IFN-α-producing PDCs from the blood and tissues of SLE patients remains stringent.

The leading mechanism of activation of type I IFN synthesis in SLE is associated with impaired nucleic acid (NA) clearance.

Type I IFN production primarily triggers the activation of NK-binding receptors, which are released from medical apoptotic and non-totic (NET) cells. The NK-binding receptor group includes endosomal toll-like receptors (TLRs) 3, 4, 7, and 9, cytosolic sensor cyclic GMP-AMP synthase (cGAS), and RNA sensor RIG-I-like receptors (RLRs)-MAVS[34]. Under normal conditions, these NK sensing pathways are tightly regulated and create the requirement for a normal antiviral response [34, 35], but many patients with SLE have chronic hyperactivity of these pathways. NCs themselves are capable of producing IFN, and can also be included in the so-called "interferonogenic" immune complexes (IC). Interferonogenic IC means complexes consisting of NK, NK-binding proteins and antinuclear antibodies.

Impaired clearance and the formation of complexes in the form of the NETs method is very typical of SLE, as well as a weakening of the function of extracellular DNase I. In turn, NK and IR, binding to TLR7 and TLR9, localized in the endosomes of the PDK and induce the synthesis of type I IFN (Fig. 2). The role of TLR7 in SLE is well conserved, as its overexpression is associated with a hard form of lupus in mice, and inhibition of TLR7 is protective [36].

Additional stimuli for the synthesis of type I IFN are mitochondrial DNA, a complex consisting of the cationic antimicrobial peptide LL37 and DNA, and the HMGB1 protein (chromosomal high mobility group block protein 1).

Study of interferons and interferon signatures in the clinic

Highly sensitive methods for determining IFN-α itself in blood serum have been developed, the results of which generally correlate with the parameters of gene expression of IFN type I [34, 37].

In all studies, the effectiveness of type I IFN overproduction is based on the analysis of interferon signals [38] by the expression of various genes (IFI27, IFI44, IFI44L, RSAD2, etc.) using PCR recently. Other approaches to measure the interferon signature include microarray technologies and the highly sensitive NanoString system using probes, which allow analysis of modern genes [39].

Since hyperactivation of the type I IFN signaling system is a feature not only of SLE, routine development of assessments of IFN signaling is also being carried out in Russia [40–42]. A multiparameter diagnostic test system has been patented, which can be used to determine the levels of mRNA of the human RIG-1, IFIT-1, IFIH-1 genes in a biological sample [43].

The question of the necessity and sufficient set of genes remains unresolved; expression should be assessed, as well as a unified method for calculating the interferon index.

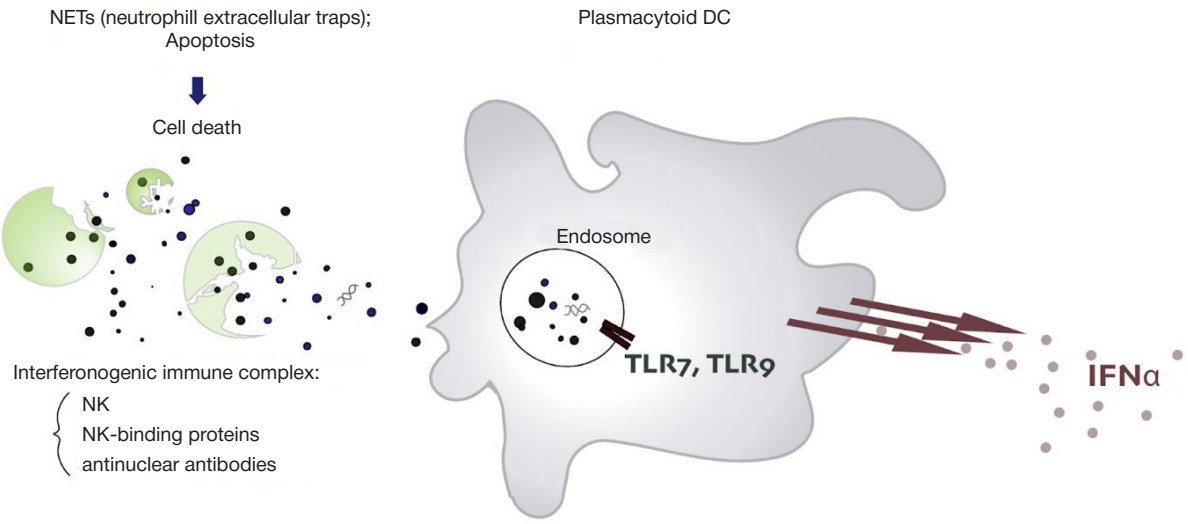

Fig. 2. Pattern of type I interferon production

Should be emphasized that the results of methods for determining the interferon signature depends on material (whole blood, cell population) and quantitative type I IFN genes. In addition, patterns of gene expression induced by IFN types I, II and III are observed. There is evidence that the expression of some IFN-responsive genes may reflect SLE activity [44, 45], but this has not been confirmed in more recent studies [46]. It has been established that overproduction of IFN-α is associated with the detection of "lupus" autoantibodies, primarily to RNA-containing antigens [47–51], but their level does not correlate with the activity of SLE and does not change during therapy.

It is noteworthy that in SLE, natural autoantibodies to type I IFN are present, as a rule, in patients with low disease activity [52], and in COVID-19, on the contrary, in patients with severe infection [53]. These data reflect the primary role of type I IFN in the development of an effective antiviral immune response in patients with COVID-19 and provide the basis for deciphering the relationship between viral infection and autoimmunity in general.

It is noteworthy that hyperproduction of type I IFN in SLE is associated with the development of a wide range of diseases, on the one hand, observed during viral infections, and on the other, characteristic of SLE. These include fever, weakness, myalgia, arthralgia, headaches, pleurisy, as well as hematological disorders (anemia, neutropenia, lymphopenia, thrombocytopenia), damage to the skin, joints, lower extremities and nervous system (CNS). For example, when study target organ biopsy obtained from patients with SLE, an increased IFN signature was found to correlate with skin lesion activity [54, 55], exist in synovial tissue from patients with arthritis [56], in kidney tissue from lupus nephritis [57], and in the cerebrospinal fluid in patients with central nervous system lesions [58].

The recent SPOCS study (SLE Prospective Observational Cohort Study) characterized patients with high disease activity and/or elevated type I interferon levels. As shown, patients with high levels of IFN symptoms are, firstly, younger in age and diagnosed later.And secondly, in such patients there was a predominance of cutaneous, immunological and hematological manifestations compared with patients with low levels of type I IFN [59].

Interferons in the pharmacotherapy of SLE

A body of evidence obtained from basic and medical research provides grounds for the development of new pharmacotherapy options for SLE using monoclonal antibodies (mAbs) that block the activity of type I IFN or its receptors [60–62] (Fig. 3).

Several biologics are currently used to block type I IFN in SLE. The main ones include:

1. Anakinra: A recombinant interleukin-1 (IL1) receptor antagonist that may inhibit interferon-activated signaling pathways. Clinical data on its effectiveness in SLE are limited, but some studies show improvement in patients with refractory forms of the disease. The response to therapy may be variable.

2. Anaxifumab (Anifrolumab): Monoclonal antibody that blocks type I interferon receptors (IFNAR1 (Interferon receptor alpha and beta subunit 1)). Anaxifumab is intended to reduce the activity of the type I interferon signaling pathway. In phase III studies (TULP-1 and TULP-2), anaxifumab demonstrated improvement in patients with SLE. In TULIP-2, improvement in SRI-4 (systemic lupus erythematosus responder index) was observed in 47.8% of patients receiving anaxifumab, compared with 31.5% in the placebo group.

3. Belimumab: Although belimumab is primarily aimed at inhibiting B cells, it also affects signaling pathways associated with interferons and may reduce their activity. Belimumab has received widespread acceptance and approval for the treatment of SLE. In phase III clinical trials (BLISS-52 and BLISS-76), approximately 43–58% of patients experienced further improvement compared to 34–44% in the placebo group.

Among these drugs, anifrolumab (AFM) [63, 64] and belimumab [65] occupy a special place.

AFM induces the internalization of IFNAR1, thereby reducing its membrane expression, which is necessary for the creation of a multifunctional IFN receptor consisting of two subunits — IFNAR1 and IFNAR2. The APM molecule is specially designed with a triple mutation L234F/L235E/P331S in the immunoglobulin chain gene, which leads to a decrease in the connection of APM molecules with membrane cellular Fc receptors. As a result, when introduced into the human body, APM does not have the ability to induce antibody-dependent and complement-dependent cellular cytotoxicity, which reduces the risk of developing infusion phenomena.

When studying the principle of action of APM, it was shown that blockade of IFNAR1-mediated signaling is associated with a wide range of molecular and cellular effects: suppression of the expression of IFN-induced genes; phosphorylation of STAT I (signal transducer and activator of transcription); synthesis of type I IFN and inflammatory cytokines; overexpression of costimulatory molecules on the pDC membrane; pDC and B cell differentiation [66]. There was a decrease in TRAIL (TNF-related apoptosis-inducing ligand) load, which was previously found to increase in SLE [67], as well as IP-10 (interferon gamma-

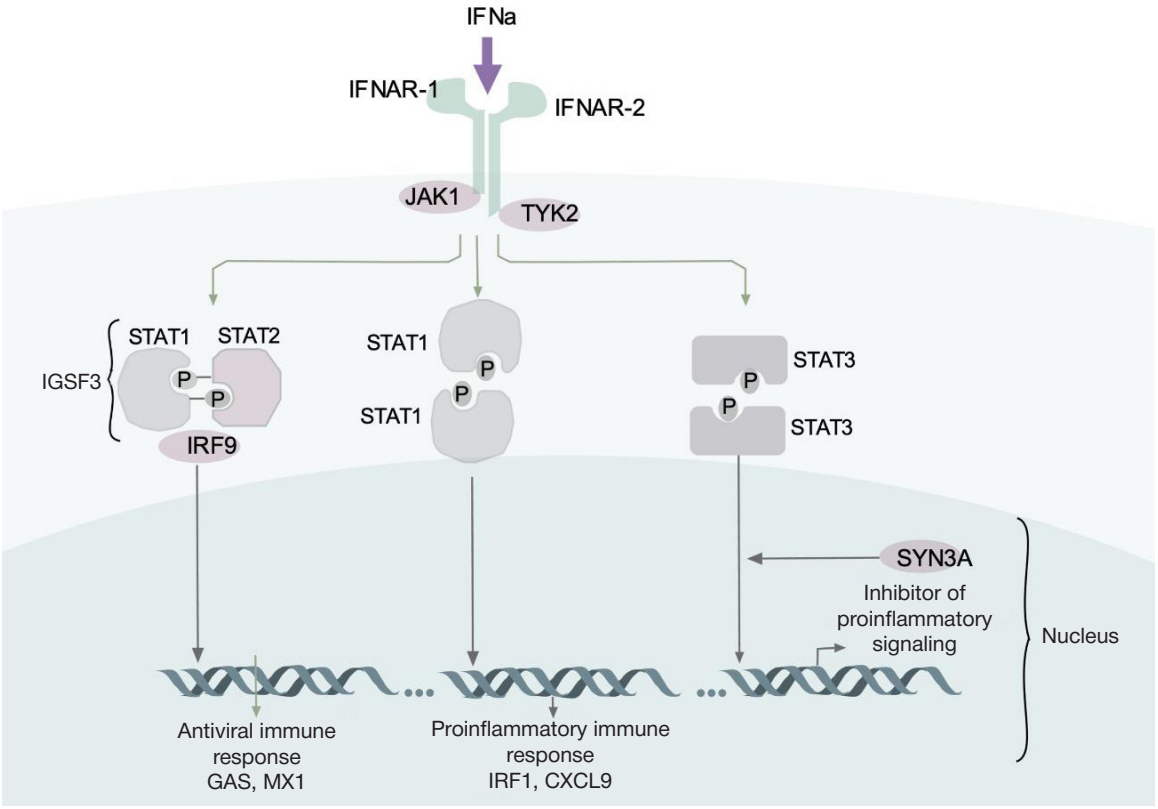

Fig. 3. Type I IFN signaling pathway: IFGF3 — IFN-stimulated factor 3, ISRE — IFN-stimulated response, IFR9 — IFN regulatory factor 9, GAS — gamma-activated sequence, SIN3A-SIN3 — transcription regulator homolog, CXCL9 — ligand 9 CXC chemokines, JAD — 2`5`-oligoadenylate synthesis, MX1 — IFN-induced GTP-binding protein 1, P — phosphate [66]

induced protein 10) and progranulin (regulate the recruitment of immune cells in the zone), associated with activity SKV [68, 69].

Other consequences of AFM include normalization of the B cell cytokine chain, such as BAFF (B cell activating factor belonging to the TNF family), the synthesis of which is altered by IFN type I [70]. When treating AFM in patients with SLE, rapid normalization of the level of lymphocytes, neultrophy, monocytes and platelets, circulating $CD4^+$ and $CD8^+$ T cells and memory B cells is observed. Notably, the anti-BAFF monoclonal antibody (belimumab) used in SLE [65] causes a decrease in naïve and switched B cells but does not affect B cell memory [71]. There was a tendency towards normalization of the level of antibodies to double-stranded DNA (anti-dsDNA) and complement components (C3, C4, CH50).

CONCLUSION

In addition, in the vast majority of patients treated for SLE, AFM was associated with suppression of basal expression of interferon signatures. Thus, after 24 weeks, the average level of suppression index of 21 genes characteristic of the signature was 89.7% at a dose of APM 300 mg after 4 weeks and 91.7% with a dose of AFM 1000 mg in women for 4 weeks. However, suppression of type I IFN signatures in patients with initial overexpression of these genes was detected after 12 weeks and persisted for 52 weeks [72] In addition to standard individual therapy, AFM reduces the need for corticosteroids and reduces the activity of lupus, especially skin and musculoskeletal diseases, and has an acceptable safety profile [73].

The data obtained to date indicate the expediency of studying the level of expression of IFN-induced genes, for example, using PCR test systems, both in the case of SLE and in some other systemic inflammatory diseases. Such a study should improve the stratification of patients with SLE, prompt replacement of other therapeutic approaches with targeted blockade of IFN type I for patients with a high IFN signature, and expand the range of interventions for the use of such therapy.

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IDENTIFICATION OF MICROGLIA AND MACROPHAGES USING ANTIBODIES TO VARIOUS SEQUENCES OF THE IBA-1 PROTEIN

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The Iba-1 protein is traditionally considered a highly selective marker of microglia because of the specific expression of the gene in this particular population of the CNS cells. Alternative splicing creates several isoforms of the Iba-1 protein, which may cause discrepancies in the results of immunohistochomic reactions depending on which epitopes of the immunogen the antibodies selected for the study were developed. In this connection, and with the aim at identifying reliable variants of antibodies to Iba-1 available to researchers in the Russian Federation, we organized with study, seeking to evaluate the results of detecting microglia and macrophages using antibodies to different protein sequences produced by different manufacturers. As material, we used samples of the brain and testis of mature (3–5 months) male Wistar rats (n = 8). Polyclonal and monoclonal (clone JM36-62) antibodies to Iba-1 were used as primary reagents. We found that monoclonal antibodies of the JM36-62 clone enable more selective antigen detection with a better signal-to-background ratio; they can be used as replacements for reagents that are currently not available commercially. Polyclonal antibodies enabled not only immunospecific imaging of microglia and macrophages, but also the identification of cells of the epithelial-spermatogenic layer of the testis. It is assumed that germinal epitelium contains the Iba-1 isoform devoid of an epitope that corresponds to the sequence of the immunogenic antibody clone JM36-62 fragment of the native protein. Functionally, various isoforms of Iba-1 should be investigated further.

Keywords: Iba-1, AIF-1, microglia, macrophages, immunohistochemistry

Funding: the study received financial support from the Russian Science Foundation, project No. 24-15-00032, https://rscf.ru/en/project/24-15-00032/

Author contribution: Razenkova VA — literature analysis, interpretation of the results, manuscript authoring; Kirik OV — analysis of the results, text editing, preparation of figures; Pavlova VS — histology of biological material, immunohistochemical reactions setup; Korzhevskii DE — study conceptualization, planning, text editing.

Compliance with ethical standards: the study was approved by the Ethics Committee of the Institute of Experimental Medicine (Protocol № 2/24 of April 25, 2024), and conducted in full compliance with the provisions of the Declaration of Helsinki (2013).

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Received: 17.05.2024 **Accepted:** 05.06.2024 **Published online:** 29.06.2024

DOI: 10.24075/brsmu.2024.026

ВЫЯВЛЕНИЕ МИКРОГЛИИ И МАКРОФАГОВ С ИСПОЛЬЗОВАНИЕМ АНТИТЕЛ К РАЗЛИЧНЫМ ПОСЛЕДОВАТЕЛЬНОСТЯМ БЕЛКА IBA-1

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Белок Iba-1 традиционно считают высокоселективным маркером микроглии благодаря специфической экспрессии гена именно в этой популяции клеток ЦНС. В результате альтернативного сплайсинга формируется несколько изоформ белка Iba-1, что может служить причиной расхождений результатов иммуногистохомических реакций в зависимости от того, к каким эпитопам иммуногена выработаны выбранные для исследования антитела. В связи с этим, а также с необходимостью определения надежных вариантов антител к Iba-1, доступных для исследователей в Российской Федерации, целью работы было оценить результаты выявления микроглии и макрофагов с использованием антител к различным последовательностям белка, выпускаемых разными производителями. Материалом для исследования служили образцы головного мозга и семенника половозрелых (3–5 месяцев) крыс-самцов Wistar (n = 8). В качестве первичных реагентов использовали поликлональные и моноклональные (клон JM36-62) антитела к Iba-1. Установлено, что моноклональные антитела клона JM36-62 позволяют добиться более высокой селективности выявления антигена при лучшем соотношении сигнал/фон и пригодны для замены реагентов, не доступных в настоящее время к приобретению. Использование поликлональных антител привело не только к иммуноспецифичной визуализации микроглии и макрофагов, но и к выявлению клеток эпителио-сперматогенного слоя семенника. Предполагается, что в клетках эпителио-сперматогенного слоя присутствует изоформа Iba-1, лишенная эпитопа, соответствующего последовательности иммуногенного для антител клона JM36-62 фрагмента нативного белка. Функциональное значение различных изоформ Iba-1 на настоящий момент остается неясным и требует дальнейших исследований.

Ключевые слова: Iba-1, AIF-1, микроглия, макрофаги, иммуногистохимия

Финансирование: исследование выполнено при финансовой поддержке Российского Научного Фонда, проект № 24-15-00032, https://rscf.ru/project/24-15-00032/

Вклад авторов: В. А. Разенкова — анализ литературы, интерпретация результатов, подготовка рукописи; О. В. Кирик — анализ результатов, редактирование текста, работа с иллюстрациями; В. С. Павлова — гистологическая проводка биологического материала, постановка иммуногистохимических реакций; Д. Э. Коржевский — концепция, планирование исследования, редактирование текста.

Соблюдение этических стандартов: исследование одобрено этическим комитетом ФГБНУ «ИЭМ» (протокол № 2/24 от 25 апреля 2024 г.), проведено в полном соответствии с положениями Хельсинкской декларации (2013 г.).

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Статья получена: 17.05.2024 **Статья принята к печати:** 05.06.2024 **Опубликована онлайн:** 29.06.2024

DOI: 10.24075/vrgmu.2024.026

Neuroglia of the central nervous system includes a set of non-neuronal cells that support normal functioning of neurons: nutrients transportation, removal of decay products, myelin formation, signal transmission, etc. [1]. Historically, microglial cells (microglia or microglyocytes) are considered neuroglia, but they have different origin than other glial cells of the central nervous system [2]. Microglia's primary function is development of the brain's immune system [3]. Microglial cells are capable of rapid activation in response to even minor pathological changes in the central nervous system, and in this connection, functional activity of microglia is a widely used indicator of the nervous system's response to infection, trauma, ischemic brain damage, or neurodegeneration [4, 5].

Earlier, antibodies to proteins of class II main histocompatibility complex were used as immunohistochemical markers of microglia. In 1996, scientists isolated the Iba-1 protein (ionized calcium-binding adapter molecule 1) [6], which is considered a highly selective marker of microglia because the *iba1* gene is specifically expressed in this cell population in the brain. Several authors consider this protein to be identical to AIF-1 [7–10]. Currently, Iba-1 is the most common protein in microglia studies. However, adequate interpretation of the results of immunohistochemical reaction is problematic, because the *iba1* gene can undergo alternative splicing, which yields several isoforms of the protein [11]. Since these isoforms spread in slightly different ways [12], study to study, immunohistochemical reactions can yield different results, which depend on the epitopes of the immunogen that triggered production of the antibodies. Alternative splicing and various protein isoforms may be why occasionally antibodies to Iba-1 enable detection of not only microglia and monocyte-macrophage cells, but also some of other origin. For example, a positive reaction to the Iba-1 protein was detected in smooth myocytes [13], breast ductal tumor epithelium [14] hepatocellular carcinoma [15], and in the germinal epithelium of the seminiferous tubules [6, 16]. Thus, the alleged high specificity of the respective antibodies to the Iba-1 protein, considered a microglia marker, is doubtful.

One of the most common antibodies used for microglia labeling are goat antibodies to Iba-1 (Abcam, UK; catalog number: ab5076), which, according to the manufacturer's website, are mentioned in 1145 publications. They are produced against a synthetic peptide that mimics the C-termini of the Iba-1 protein. However, currently, antibodies to Iba-1 are (Huabio, China; catalog: ET-1705-78) are easier to procure in the Russian Federation. The immunogen for these primary reagents is a peptide with a sequence similar to the protein's N-termini. These primary antibodies delivered good results in the context of studies employing tissue samples from various laboratory animals [17–19].

Identification of more reliable variants of antibodies to Iba-1 that can be used for microglia labeling, and determination of their interchangeability are urgent tasks, therefore, this study aimed to evaluate the results of detection of microglia and macrophages that relied on antibodies to different sequences of the Iba-1 protein produced by different manufacturers.

METHODS

Samples of brain and testis tissues of mature (3–5 months old) male Wistar rats weighing 350–400 g (n = 8) were used as material in this study. The animals came from the Rappolovo laboratory animal nursery (Leningrad Region, Russia); they were kept in a vivarium at room temperature under standard conditions, with free access to food and water. Before sampling, the animals were euthanized by an ethyl ether vapors overdose. The sampled material was fixed in zinc-ethanol-formalin and embedded in paraffin in a standard way. Five-micron thick sections were cut from the paraffin blocks on a Leica RM 2125RT rotary microtome, and mounted on adhesive slides HistoBond®+ (Paul Marienfeld; Germany). Brain slices were made at –2.12 mm from bregma.

After deparaffinization (standard technique), we inhibited endogenous peroxidase by incubating the sections in a 3% aqueous solution of hydrogen peroxide for 10 minutes. Before application of the primary antibodies, the slices were treated with normal equine serum (008-000-121; Jackson Immunoresearch, USA) or blocking solution (ab64226; Abcam, UK) at room temperature for 10 minutes. For microglia and macrophages detection, we used antibodies to the calcium-binding protein Iba-1: goat polyclonal antibodies diluted 1:1000 (ab5076; Abcam, UK), and recombinant rabbit monoclonal (clone JM36-62) antibodies diluted 1:1200 (ET-1705-78; Huabio, China). Incubation with primary antibodies took place in a humid box for three days at a temperature of 27 °C.

UltraVision Quanto Detection System HRP (TL-060-QHL; Fisher Scientific, USA), which reacts to mouse and rabbit primary reagents, and a VECTASTAIN Universal Quick HRP kit (PK-8800; Vector Laboratories, USA), which detects primary reagents of mice, rats, and goats, were used as the secondary reagents. In addition, we took secondary reagents from the Cell & Tissue Staining Kit (CTS005 and CTS008; R&D Systems, USA) with monoreactivity to rabbit and goat antibodies, respectively. The incubation modes for secondary reagents were as per the manufacturer's recommendations. For both antibody variants, we performed negative control tests using the appropriate sets of secondary reagents and an HRP detection system. For this purpose, the slices were treated with antibody diluent instead of the primary antibodies solution. Normal rat serum was used to block non-specific binding of anti-mouse secondary antibodies to the closely related rat immunoglobulins.

To visualize the product, we used chromogen 3'3-diaminobenzidine from the DAB+ kit (K3468, Agilent; USA). Once the immunohistochemical reaction was set up, some of the sections were stained with alum hematoxylin or alcyan blue. After dehydration and clearing in orthoxylene (Vecton; Russia), the preparations were put into the Cytoseal 60 medium (23-244257; Richard-Allan Scientific, USA). The resulting preparations were analyzed using a Leica DM750 microscope (Leica; Germany) fit with an ICC50 digital camera (Leica; Germany). To capture images, we used the LAS EZ image capture program (Leica; Germany).

Using various antibodies to Iba-1, we digitally assessed the optical density of the stained product of the immunohistochemical reaction. The data were collected in the striatum area; its processing relied on the Fiji morphometric analysis program (ImageJ). The Region of Interest feature allowed pre-selecting preferred areas (10 × 10 microns). We evaluated the average brightness per unit of area of microglyocytes, blood vessel lumen, and striatum neuropile. The results of the study were given in the optical density units. The resulting data were processed in GraphPad Prism 8 (GraphPad Software; USA) and presented as mean ± SD, with 7 values used to calculate that mean.

We used the UniProt service to cross reference the amino acid sequences of the isoforms of Iba-1 (AIF1 in another classification) [20], and compared sequences P55008-1, P55008-2, and P55008-3.

RESULTS

The preliminary analysis of the preparations confirmed the sections and the tissues of the samples were well-preserved.

A high-intensity immunohistochemical reaction to Iba-1 was observed on brain and testis preparations with a minimum level of background staining, as opposed to rabbit primary reagents (none) and goat primary antibodies (insignificant).

Iba-1-positive cells were found in all the studied rat tissue preparations. In brain, they were typical microgliocytes with a large number of thin branched processes (Fig. 1A–D). Spindle-shaped cells with flattened large processes were seen near gray matter's blood vessels. Monoclonal rabbit antibodies forced the processes of Iba-1-containing cells to grow numerous spike-like outgrowths. Moreover, monoclonal antibody reaction was found in the meninges (oval cells, no processes, intensely colored cytoplasm).

The use of goat polyclonal antibodies to Iba-1, despite the high intensity of the specific reaction, yielded an insignificant background on the preparations: minor staining of the neuropile, and a more pronounced nonspecific background staining of the serum of large blood vessels (Fig. 1A). The intensity of staining of the neuropil and serum of large blood vessels was 0.61 ± 0.03 and 0.75 ± 0.03, respectively. Monoclonal rabbit antibodies did not trigger noticeable background staining: the optical density of the stained chromogen in the neuropile and lumen of blood vessels was 0.57 ± 0.02 and 0.59 ± 0.01, respectively. The average value of the microglyocyte staining intensity for both antibody variants was similar: 0.87 ± 0.09 for polyclonal antibodies and 0.87 ± 0.08 for antibodies of the JM36-62 clone.

The use of both variants of primary antibodies on rat testis tissue samples allowed detecting two types of immunopositive stroma cells of convoluted seminiferous tubules (Fig. 1E–F). In the first case, these are cells found close to the vessels. They have a small, rounded circumnuclear region, and thin non-branching processes. The second type are large oval shape with few or no processes. The latter can be isolated or group (3–4 cells per group).

The third type of cells was detected only with the help of polyclonal goat antibodies to Iba-1. In this case, cytoplasm of cells of the germinal epithelium of the seminiferous (seminiferous epithelium) tubules contained accumulation of the product of immunohistochemical reaction. Morphologically, they are similar to early (rounded) and late (elongated) spermatids (Fig. 1E, double arrow). No Iba-1 immunopositive cells were found in the seminiferous epithelium on any of the studied preparations treated with rabbit primary reagents.

Cell & Tissue Staining Kit, which relies on avidin-biotin amplification, produced immunohistochemical reaction results similar to those registered for VECTASTAIN Universal Quick HRP kit and UltraVision Quanto Detection System HRP.

Comparison of the amino acid sequences of three isoforms of Iba-1/AIF1 (sequences P55008-1, P55008-2, and P55008-3 from the UniProt) has shown that this or that isoform may be lacking such sequences of immunogenic fragments of various antibodies. For example, N-terminus of the immunogenic fragment for monoclonal rabbit antibodies of the JM36-62 clone was found only in P55008-1 (with a sequence of 147 amino acids), and the shorter isoforms P55008-2 and P55008-3 (with sequences of 93 and 132 amino acids, respectively) did not have this fragment. In contrast, the region of C-terminus of the immunogenic fragment for polyclonal goat antibodies Abcam is present in the sequences of P55008-1 and P55008-2 (two isoforms of the three studied).

DISCUSSION

Both primary antibodies used in this work yielded good results of the immunohistochemical reaction, and the ratio of immunospecific signal's intensity to nonspecific background staining was high. Nevertheless, the protocol that involved goat primary reagents produced a higher level of background staining of the brain neuropile and testicular parenchyma compared to the protocol that uses the JM36-62 clone antibodies. Figure 1 shows a seemingly lower background-to-specific signal ratio compared to the case of polyclonal antibodies. An additional quantitative study of the digital images has shown that both variants of antibodies trigger specific immunohistochemical reactions of almost similar intensity. However, the intensity of staining of neuropil and serum from the large blood vessels was indeed higher for preparations stained with polyclonal goat primary reagents. Such reagents are known to impose certain limitations on immunohistochemical studies employing indirect immunolabelling (by Coons) and modifications thereof. Firstly, the most common secondary reagents are mono- or bivalent, i.e., they counter immunoglobulins of one or two animal species (typically — rabbit and mouse), which significantly complicates selection of secondary antibodies and increases the cost of the study. Secondly, non-specific background is more pronounced when goat antibodies are used, while the intensity of reaction increases only slightly because such antibodies react with bovine immunoglobulins, too, and reagents containing bovine serum albumin can contain trace amounts of those [6]. The results of this immunohistochemical study confirm this.

The Iba-1 protein, which consists of 147 amino acid residues and has the molecular weight of 17 kDa [21], is always present both in the body and in the processes of microglial cells. This is why it is widely used in tests designed to evaluate the morphofunctional status of microglia. Interacting with fimbrin, Iba-1 supports crosslinking of actin filaments and regulation of the configuration of plasma membrane [22], so the its level increases with microglial activation [23]. However, this property of Iba-1 also means it is found in other phagocytic cells of the body. Indeed, as shown in numerous papers, antibodies to Iba-1 are often used to identify monocyte-macrophage cells. Thus, resident macrophages of various organs of laboratory animals and humans are immunopositive to Iba-1, including Kupffer cells [18, 24], alveolar macrophages of the lungs and macrophages of the spleen [25, 26], and Langerhans cells [6, 16]. Therefore, this study was expected to yield data pointing ti the presence of Iba-1 in interstitial testicular macrophages and leptomeningial brain cells. In the context of an immunohistochemical reaction involving various antibodies to Iba-1, a much more interesting result has to do with the cells of the germinal epithelium of seminiferous tubules. With goat polyclonal antibodies, the reaction yielded stained products in cells that morphologically are similar to spermatids. Antibodies of the JM36-62 clone did not bind antigens in the germinal epithelium.

Several papers state presence of the Iba-1 protein in spermatids found in the seminiferous tubules [7, 16, 27–29]. However, the results of our study were not consistent therewith for this group of cells with different primary antibodies, which may have been conditioned by properties thereof in the context of detection of epitopes of different specificity, or properties of secondary reagents. Therefore, in order to exclude a false positive reaction from the testicular spermatids, we set up an additional control experiment using Cell & Tissue Staining Kit, identical secondary imaging systems for goat and rabbit antibodies based on avidin-biotin technology, which ensures blocking of endogenous biotin. The results of this reaction were consistent with those registered for the experiments with UltraVision Quanto Detection System HRP (rabbit) and the VECTASTAIN Universal Quick HRP kit (goat). This further emphasizes that the reaction from spermatids is not a

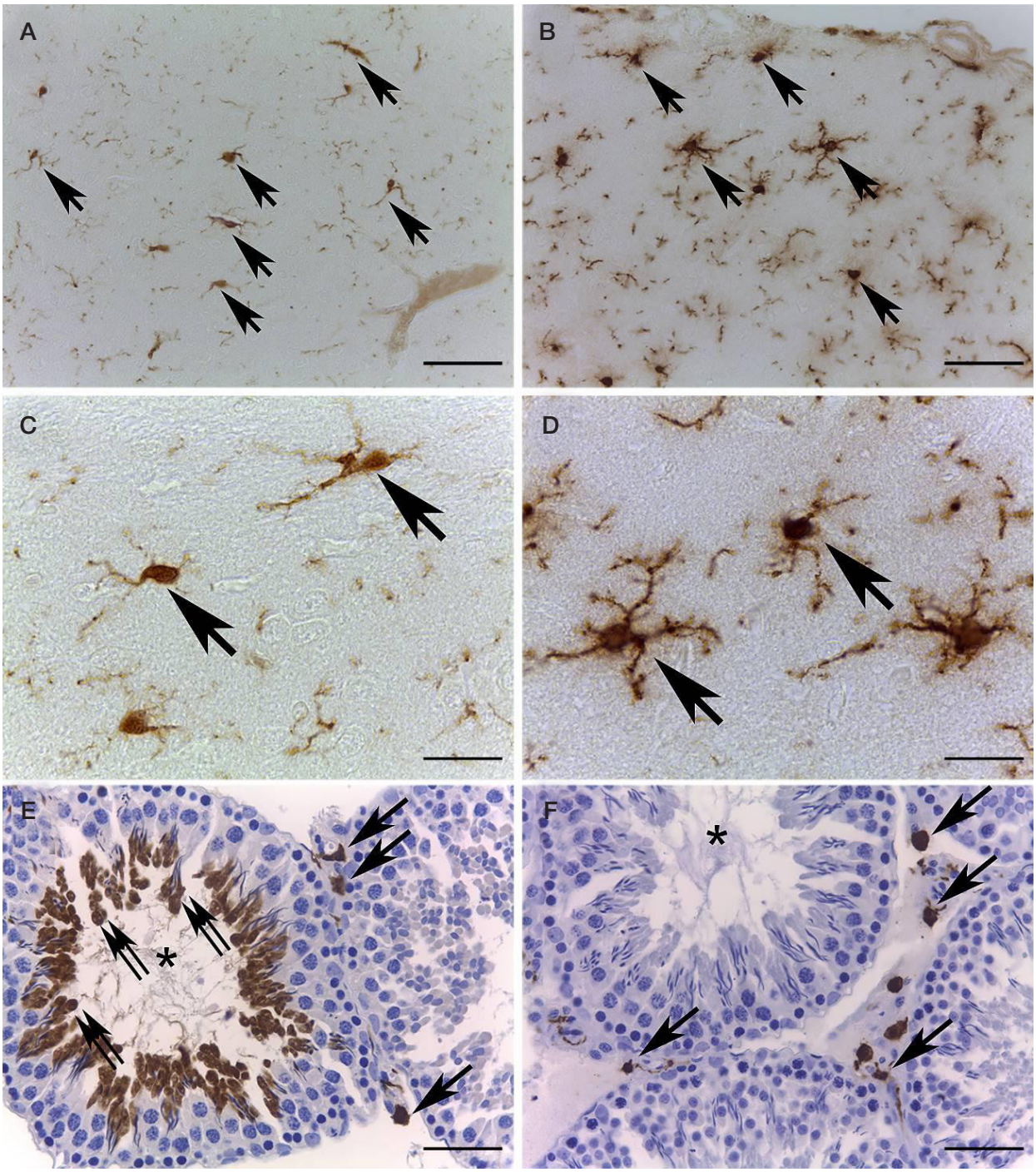

Fig. 1. Iba-1 in rat cells, identified with polyclonal goat (**A**, **C**, **E**), monoclonal rabbit (**B**, **D**, **E**) antibodies. **A–D**. Brain preparations (arrows indicate microglia). **E–F**. Convoluted seminiferous tubules. Arrows point to Iba-1-containing cells in the interstitium, double arrows indicate such in the germinal epithelium, *asterisk* — in the tubule's lumen. Scale: 50 microns (**A**, **B**, **E**, **F**), 20 microns (**C**, **D**)

consequence of non-specific binding of secondary reagents (namely, streptavidin).

This result of the immunohistochemical reaction may come from the differences in antigenic determinants: Iba-1 epitopes in spermatids could have been more like the parotopes of polyclonal, but not monoclonal antibodies. Thus, according to the information provided on the manufacturer's website, a synthetic peptide with the TGPPAKKAISELP sequence localized at the Iba-1 protein's C-terminus was used as an antigen for polyclonal goat antibodies. There is also a note there stating that ab5076 antibodies are capable of detecting at least two isoforms of Iba-1/AIF1. In contrast, the immunogenic peptide for monoclonal rabbit antibodies had the sequence of SQTRDLQGGKAFGL, which corresponds to the N-terminus. A comparison of the sequences of different isoforms of human Iba-1/AIF1 has shown that the sequence of the immunogen for rabbit antibodies corresponds to the protein isoform with a sequence of 147 amino acids, which is accepted as canonical [6]. According to the comparison of the amino acid sequence of protein fragments corresponding to the immunogenic peptide, goat polyclonal antibodies should enable detection of both the canonical isoform and the protein's shorter forms. Thus, our data indirectly indicate that alternative splicing of *iba1* matrix RNAs can exclude from the final transcript the N-terminus detected by monoclonal antibodies of the JM36-62 clone but leave the C-terminus of present isoforms detected

by polyclonal antibodies produced by Abcam. A review of the initial publications that give characteristics of the Iba-1 protein has shown that the authors studied the expression of the gene in various organs [13–16]. Thus, they demonstrated high expression of *iba1* in the testes and spleen, and weak expression in the lungs, kidneys and brain (due to the specific expression of the gene exclusively in macrophages and/or microglia). A large number of other studies covering expression of *iba1* in various tissues [16–18, 24, 25] also suggests that it is not limited to microglia, monocytes, and macrophages. Therefore, it is possible that goat polyclonal antibodies actually detect the Iba-1 protein, in particular, its isoform that is not present in the monocyte-macrophage cells.

CONCLUSIONS

Monoclonal antibodies of the JM36-62 clone enable more selective antigen detection with a better signal/background ratio; they can be used as replacements for a more commonly used reagents. Apparently, higher selectivity of the JM36-62 clone antibodies is the result of the manufacturer choosing a more fitting immunogen. The fact of detection of spermatids with the help of Abcam polyclonal may stem from the presence therein of Iba-1 isoform that is devoid of an epitope corresponding to the sequence of the native protein's N-terminus. Further studies are needed to assess the functional significance of the various isoforms of Iba-1.

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THE IMPACT OF TUBERCULOSIS ON THE DEVELOPMENT OF IMMUNE RESPONSE TO SARS-COV-2

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Given the fact, that adaptive immune response is important for control and elimination of viral infections causing human diseases, estimation of adaptive response to SARS-CoV-2 is extremely important. The neutralizing antibodies and $CD4^+$/$CD8^+$ T cells contribute to the SARS-CoV-2 control. Tuberculosis remains the leading cause of mortality among bacterial infections all over the world. Currently, treatment of tuberculosis is complicated by the COVID-19 co-infection. The aim of the study was to investigate the formation of neutralizing antibodies against SARS-CoV-2 and $CD4^+$ and $CD8^+$ T cells specific for SARS-CoV-2 in patients with pulmonary TB. The levels of neutralizing antibodies against SARS-CoV-2 and the amount of T cells specific for SARS-CoV-2 were estimated at two time points (3 and 6 months after COVID-19) in patients diagnosed with pulmonary tuberculosis (69 individuals: 33 females and 36 males aged 18–70 years). Patients without tuberculosis (35 individuals: 25 females and 10 males aged 18–70 years) who had undergone COVID-19 served as the control group. The study showed equal levels of SARS-CoV-2 neutralizing antibodies in both groups 3 months after COVID-19. The levels of antibodies decreased 6 months after COVID-19 compared to the levels reported 3 months after the disease in both groups. The antibody levels were significantly lower in the group of patients with TB ($p = 0.01$). The amount of SARS-CoV-2 specific T cells was lower in TB patients 6 months after COVID-19 ($p < 0.001$) compared to the control group. Thus, TB co-infection reduces the specific immune response to SARS-CoV-2 6 months after COVID-19.

Keywords: COVID-19, tuberculosis, immunologic memory, $CD4^+$ T lymphocytes, IgG

Funding: research project FURE-2022-0010.

Author contribution: Shepelkova GS — planning the experiments and experimental procedure, analysis of the results, manuscript writing; Chernyh NA — selection of patients for inclusion in the study, primary data analysis; Kosiakova VK — experimental procedure, primary data analysis; Sadovnikova SS — selection of patients for inclusion in the study; Ergeshov A — study design; Yeremeev VV — study design, analysis of the results, manuscript writing.

Compliance with ethical standards: the study was conducted as part of the research project FURE-2022-0010 of the Central Tuberculosis Research Institute and approved by the Ethics Committee of the Central Tuberculosis Research Institute (protocol No. 13/1 dated 28 December 2021). All the patients included in the study submitted the informed consent before enrollment.

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Received: 03.04.2024 **Accepted:** 17.05.2024 **Published online:** 17.06.2024

DOI: 10.24075/brsmu.2024.023

ВЛИЯНИЕ ТУБЕРКУЛЕЗА НА ФОРМИРОВАНИЕ ИММУННОГО ОТВЕТА К SARS-COV-2

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С учетом того, что адаптивный иммунный ответ важен для контроля и устранения вирусных инфекций, вызывающих заболевания у людей, крайне важна оценка адаптивного ответа на SARS-CoV-2. Нейтрализующие антитела и Т-лимфоциты $CD4^+$/$CD8^+$ способствуют контролю SARS-CoV-2. Туберкулез до сих пор остается главной причиной смерти среди бактериальных инфекций в мире. На данный момент лечение туберкулеза осложнено коинфекцией COVID-19. Целью работы было исследовать образование нейтрализующих антител против SARS-CoV-2 и специфичных для SARS-CoV-2 Т-клеток $CD4^+$ и $CD8^+$ у пациентов с ТБ легких. Уровни нейтрализующих антител к SARS-CoV-2 и количество специфичных к SARS-CoV-2 Т-клеток оценивали в двух временных точках (через 3 и через 6 месяцев после перенесенного COVID-19) у больных с диагнозом туберкулез легких (69 человек: 33 женщины и 36 мужчин от 18 до 70 лет). В контрольную группу вошли пациенты, перенесшие COVID-19 без туберкулеза (35 человек: 25 женщин и 10 мужчин от 18 до 70 лет). В результате исследования были зарегистрированы одинаковые уровни нейтрализующих антител к SARS-CoV-2 в обеих группах через 3 месяца после перенесенного COVID-19. Уровни антител снизились в двух группах через 6 месяцев после COVID-19 по сравнению с 3 месяцами. Уровень антител был достоверно ниже в группе больных ТБ ($p = 0,01$). Количество SARS-CoV-2-специфичных Т-клеток было ниже у больных ТБ через 6 месяцев после перенесенного COVID-19 ($p < 0,001$) по сравнению с контрольной группой. Таким образом, коинфекция ТБ снижает специфический иммунный ответ против SARS-CoV-2 через 6 месяцев после перенесенного COVID-19.

Ключевые слова: COVID-19, туберкулез, иммунологическая память, Т-лимфоциты $CD4^+$, IgG

Финансирование: НИР FURE-2022-0010.

Вклад авторов: Г. С. Шепелькова — планирование и постановка экспериментов, анализ результатов, написание рукописи; Н. А. Черных — подбор пациентов для включения в исследование, первичный анализ данных; В. К. Косякова — постановка экспериментов, первичный анализ результатов; С. С. Садовникова — подбор пациентов для включения в исследование; А. Эргешов — дизайн исследования; В. В. Еремеев — дизайн исследования, анализ результатов, написание рукописи.

Соблюдение этических стандартов: исследование проведено в рамках темы НИР ФГБНУ «ЦНИИТ» № FURE-2022-0010 и одобрено этическим комитетом ФГБНУ «ЦНИИТ» Протокол 13/1 от 28.12.2021. Все пациенты, вошедшие в исследование, подписали добровольное информированное согласие до момента включения в исследование.

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Статья получена: 03.04.2024 **Статья принята к печати:** 17.05.2024 **Опубликована онлайн:** 17.06.2024

DOI: 10.24075/vrgmu.2024.023

The ongoing tuberculosis (TB) epidemic was undoubtedly negatively affected by the redistribution of health services during the COVID-19 pandemic. The acute form of COVID-19 and TB may co-occur, and the rate of such co-infection may be underestimated in the regions where the prevalence of TB is high. The prior or current TB is a risk factor of death from SARS-CoV-2. The T-cell response to Mtb can be modulated by SARS-CoV-2, as demonstrated in ex vivo studies of blood cells from individuals with acute infection. In contrast, the activation of innate and adaptive immunity in the presence of concomitant TB may impair the immune response to SARS-CoV-2 and increase inflammation. Although animal studies and epidemiologic data suggest that BCG may protect against SARS-CoV-2, vaccine efficacy has not been confirmed in several large clinical trials [1].

The COVID-19 pandemic has created new problems for patients with pre-existing respiratory infections. Of particular concern is the interaction between COVID-19 and TB, the combination of which presents a challenging clinical landscape [2]. Both respiratory infections are characterized by hyperintense inflammation with high levels of pro-inflammatory cytokines, including TNF, IL6, and IL1. These released cytokines and chemokines attract the immune cells that enhance the pro-inflammatory response and contribute to tissue damage. The data obtained underscore the severity of this interaction, showing that concurrent or sequential lung infection with COVID-19 and TB exacerbates respiratory symptoms and markedly reduces lung function [3].

It has been shown that TB can affect the severity of COVID-19 due to impaired immunity and chronic pneumonia [4]. Dysregulated immune responses are observed in patients with active TB, which can impair the immune response to COVID-19 and result in a more severe disease. In addition, COVID-19 can reactivate latent TB in patients with a history of the disease, further exacerbating the severity of symptoms [5, 6]. Comparison of patients with TB and COVID-19 with the patients having pneumonia showed that 22% of surveyed patients with TB had mild-to-moderate clinical forms of the disease, while other 78% developed more severe COVID-19 forms [7].

The novel coronavirus disease (COVID-19) led to the high morbidity and mortality rates [8]. The adaptive immune response plays an important role in controlling the majority of viral infections. The most important basic components of the adaptive immune system are B cells (as a source of antibodies) and T cells ($CD4^+$ and $CD8^+$). Given the fact that adaptive immune responses are important for control and elimination of almost all viral infections causing human diseases, and both adaptive immune responses and the immunological memory play a key role in developing the vaccine-induced immunity, it is extremely important to understand the adaptive responses to SARS-CoV-2. SARS-CoV-2 specific antibodies as well as $CD4^+$ and $CD8^+$ T cells are produced by the host in response to viral infection [9–11]. Antibodies and T cells play a defensive role in the fight against viral infections. However, the role and importance of each adaptive immunity component vary depending on the type of viral infection. The relationship between the neutralizing antibodies, memory T cells and COVID-19 severity seems to be complex. High titers of neutralizing antibodies are associated with severe disease and possibly extrafollicular B cell responses [12, 13], while memory T cells specific for SARS-CoV-2 show variable associations depending on the study [14–17]. IgG and $CD4^+$ T cells against SARS-CoV-2 antigens are detected in blood of almost all patients having a history of COVID-19. It has been shown that the intensity of Spike-specific $CD4^+$ T-cell response correlates with the Spike IgG levels [9, 16, 18]. However, some individuals have detectable $CD4^+$ T cells against SARS-CoV-2 despite low or negative IgG test results. 1–10% of people infected with SARS-CoV-2 experience this situation [19–21].

The study was designed to evaluate the duration of persistence of cellular and humoral immunologic memory to SARS-CoV-2 in the context of TB co-infection.

METHODS

Patients

A total of 120 people (59 males and 61 females) aged 18–70 years (average age 40 ± 15 years) were included in the study. Inclusion criteria: Healthy individuals (15 persons) — Individuals with no history of COVID-19 and TB; Individuals who had had COVID-19 within 3 months prior to inclusion in the study (without a history of TB) (16 persons); Individuals who had had COVID-19 within 6 months prior to inclusion in the study (without a history of TB) (19 persons); Patients treated at the Central Tuberculosis Research Institute between August 2021 and February 2022 for pulmonary TB who had received COVID-19 within 3 months prior to enrollment in the study (31 people); Patients treated at the Central Tuberculosis Research Institute between August 2021 and February 2022 for pulmonary TB who had received COVID-19 within 6 months prior to enrollment in the study (38 people).

All patients diagnosed with pulmonary TB were prescribed therapy taking into account *M. tuberculosis* drug resistance and individual drug tolerance. Diagnosis of TB in all patients in the study was performed with the use of the BACTEC MGIT 960 (BD; USA) in accordance with the manufacturer's recommendations [22]. All the samples that tested positively were stained by Ziehl-Neelsen and assessed by microscopy in order to identify acid-fast bacteria. Biomaterial was further assessed using the PCR-based method.

All patients enrolled in the study had a history of mild to moderate COVID-19. No patients with severe COVID-19 were enrolled in the study.

Exclusion criteria: age under 18 years; pregnancy; history of diabetes mellitus, polyvalent allergy, bronchial asthma, systemic autoimmune disease, active infection; decompensated chronic heart failure; acute myocardial infarction; continuous intake of corticosteroids.

The study involved the use of peripheral blood samples obtained from the patients and healthy individuals enrolled.

Determination of T cells specific for SARS-CoV-2 antigens

A total of 8 mL whole venous blood was collected from patients on test day. Sodium heparin was used as an anticoagulant. Peripheral blood mononuclear cells were isolated using a Ficoll gradient at a density of 1.077 g/cm^3 (PanEco; Russia). The TigerTest® SARS-CoV-2 kit (GENERIUM; Russia) was used to determine the number of T cells specifically responding to SARS-CoV-2 viral antigens according to the manufacturer's instructions. The S6 Ultra reader (CTL; USA) was used to enumerate spots corresponding to $CD4^+$/$CD8^+$ T cells secreting IFNγ.

Determination of IgG antibodies to the SARS-CoV-2 antigen

A total of 5 mL of whole venous blood was collected from each patient enrolled. Serum was then used for ELISA. The SARS-CoV-2 IgG ELISA kit (FSBI "NMRC for Hematology", Ministry of Health of the Russian Federation) was used for

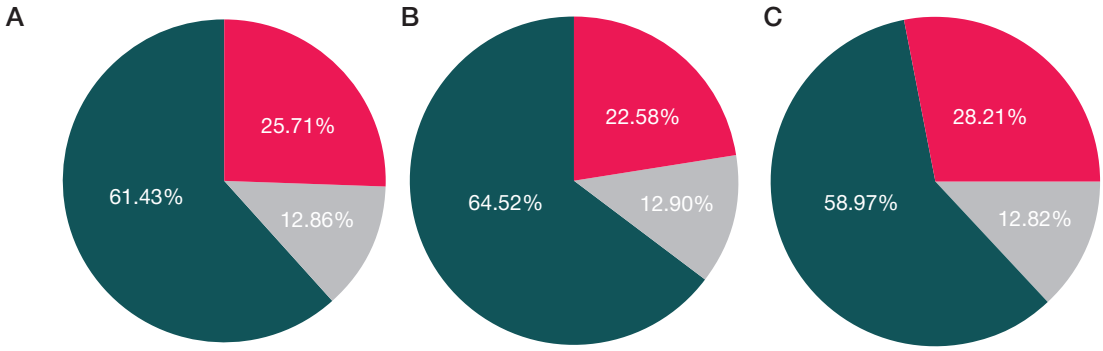

Fig. 1. Distribution by TB process among groups of pulmonary TB patients included in the study. **A**. All the patients diagnosed with pulmonary TB that were included in the study. **B**. Patients diagnosed with pulmonary TB, who had had COVID-19 within 3 months before inclusion in the study. **C**. Patients diagnosed with pulmonary TB, who had had COVID-19 within 6 months before inclusion in the study. Red sector of the diagram — patients with destructive TB; green sector of the diagram — patients with infiltrative TB; gray sector of the diagram — patients with disseminated TB

semi-quantitative determination of IgG to SARS-CoV-2 antigen in serum by solid-phase enzyme-linked immunosorbent assay according to the manufacturer's recommendations. Optical density was measured using the Sunrise™ microplate reader (TECAN; USA).

Statistical analysis

Statistical processing was performed using GraphPad Prism 8.4.3 (GraphPad Software; USA) with Mann-Whitney and χ^2 tests.

RESULTS

Characteristics of enrolled individuals

The study involved 69 individuals (33 females and 36 males) treated in the Central Tuberculosis Research Institute and having a history of COVID-19 within 3 months (31 individuals: 15 females and 16 males; average age 38 ± 15 years) and 6 months (38 individuals: 18 females and 20 males; average age 39 ± 15 years) before enrollment in the study as the experimental groups. The majority of patients had infiltrative TB (up to 64%), up to 28% were patients with destructive pulmonary TB, and 12.9% were patients with disseminated TB (Fig. 1A–C). All the patients were prescribed chemotherapy considering the M. tuberculosis drug resistance and individual drug tolerance. Bacteremia was observed in 58% and 47% of patients with a diagnosis of TB who had COVID-19 up to 3 and 6 months prior to study entry, respectively.

Thirty-five individuals (25 females and 10 males) who had undergone COVID-19 up to 3 months (16 individuals: 14 females and 2 males; mean age 48 ± 13 years) and 6 months (19 individuals: 11 females and 8 males; mean age 40 ± 14 years) prior to enrollment and had no history of TB were included as comparison groups.

Healthy individuals (15 people (7 females and 8 males; average age 49 ± 14 years)) having no history of TB and COVID-19 were included in the study as negative controls.

Determining IgG against the SARS-CoV-2 antigens

In the first phase of the study, we compared the presence and titers of specific IgG against the SARS-CoV-2 antigens in patients from different groups. In the group of patients 3 months after COVID-19, active TB had no effect on the presence of specific IgG. No significant differences between these groups were revealed (Fig. 2).

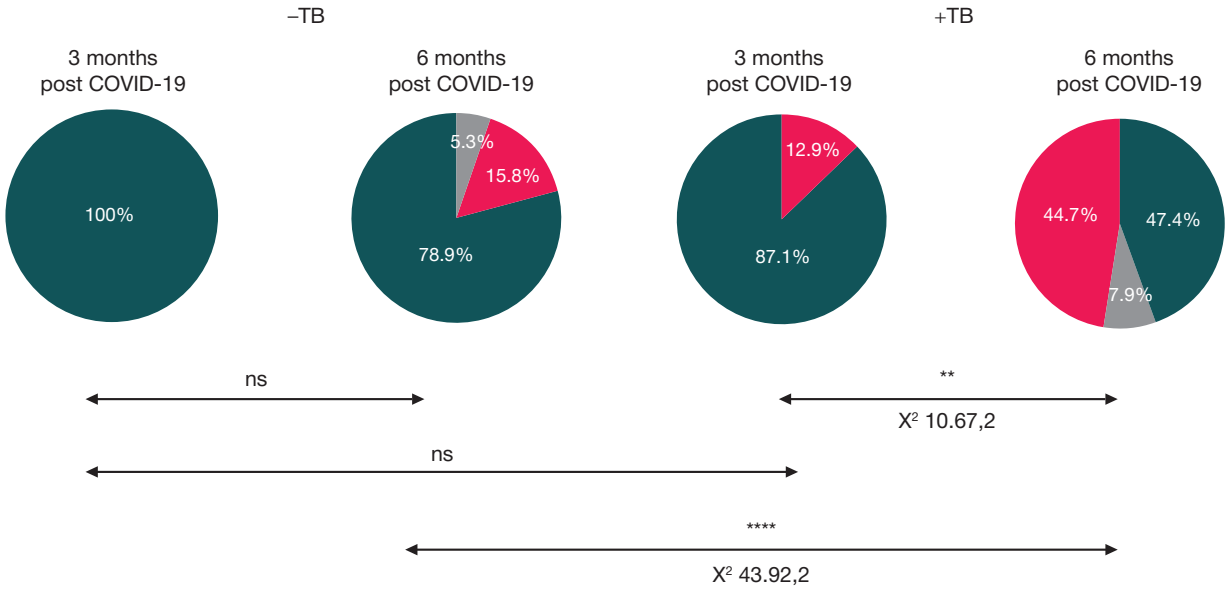

Fig. 2. Differences in serum levels of IgG to SARS-CoV-2 antigens in individuals who had COVID-19 within 3–6 months prior to enrollment, had no history of pulmonary TB (–TB), and were diagnosed with active pulmonary TB (+TB). Green sector of the diagram — patients testing positive for IgG to the SARS-CoV-2 antigens; gray sector of the diagram — patients with indeterminate results of the test for IgG to SARS-CoV-2 antigens; red sector of the diagram — patients testing negative for IgG to the SARS-CoV-2 antigens. ns — non-significant differences; ** — $p < 0.01$; **** — $p < 0.0001$

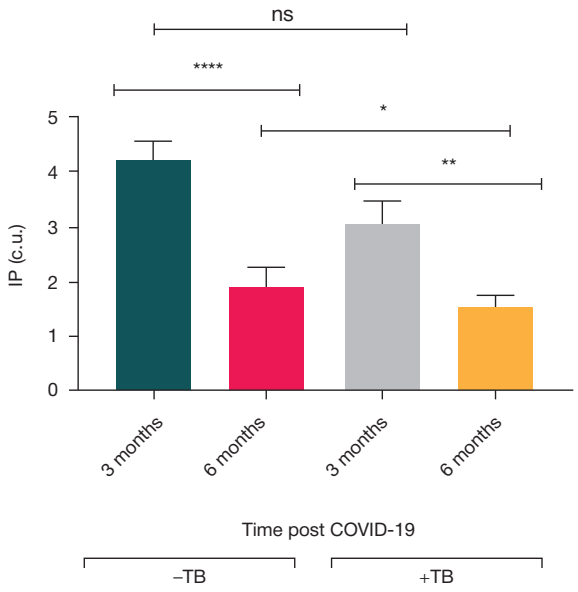

Fig. 3. Titers of IgG to SARS-CoV-2 antigens in blood serum of individuals who experienced COVID-19 within 3-6 months prior to enrollment, had no history of pulmonary tuberculosis (–TB), and were diagnosed with active pulmonary tuberculosis (+TB). Data are presented as a positivity index (PI) calculated according to the manufacturer's recommendations. ns — non-significant differences; * — $p < 0.05$; ** — $p < 0.01$; **** — $p < 0.0001$

Significant differences were observed when comparing the presence of IgG to SARS-CoV-2 antigens in the groups of patients who had a history of COVID-19 within 6 months prior to enrollment in the study. There were also significant differences in the percentage of patients testing positive for IgG against viral antigens (Fig. 2). Thus, positive results of the test for antibodies against the coronavirus antigens were reported in 79% of patients without active TB, while among patients diagnosed with pulmonary TB positive test results were reported only in 47.4%. No IgG antibodies to the SARS-CoV-2 antigens were detected in 12 patients diagnosed with pulmonary TB (44.7%) 6 months after having COVID-19 (Fig. 2).

The titers of IgG to SARS-CoV-2 antigens were different in the groups of patients without diagnosed TB and patients with diagnosed active pulmonary TB. 3 months after COVID-19, the maximum antibody titers to coronavirus antigens were detected in both the group of patients with and without a history of TB (Fig. 3). The IgG titers decreased 6 months after coronavirus infection, and the significantly greater decrease in IgG antibody titers was observed in the group of patients with active TB (Fig. 3).

Thus, titers of SARS-CoV-2 virus-specific IgG and duration of immune memory have been shown to be affected by the TB status.

Assessment of immunological memory to the SARS-CoV-2 antigens

Immunologic memory to the SARS-CoV-2 antigens was assessed using the TigraTest® SARS-CoV-2 kit. The kit is designed to determine the number of $CD4^+/CD8^+$ T cells responding to stimulation with SARS-CoV-2 specific antigens and to enumerate individual activated T cells. Test results were interpreted according to the manufacturer's recommendations. It was shown that, as in the case with antibodies to the coronavirus antigens, the presence of active TB affected the duration of the immunologic T-cell memory to SARS-CoV-2. Thus, in the groups of individuals without TB, there was no change in the percentage of patients with positive test results within 6 months after COVID-19 (Fig. 4), while in patients with active pulmonary TB, the percentage of patients with positive test results decreased by 6 months after viral infection. We also observed significant differences in the number of patients with positive TigraTest® SARS-CoV-2 results in the groups of patients with active TB and no history of TB 6 months after COVID-19 (60.5% and 84.2%, respectively) (Fig. 4).

One of the antigens in the TigerTest® SARS-CoV-2 test system is the S protein of the SARS-CoV-2 virus. This allowed us to study the duration of T-cell immune memory directly against this protein. With this approach, both in the group of patients with active TB and in the group of patients without a history of TB, the number of patients with a negative test result was higher than with the standard assessment of the presence of memory T cells to multiple antigens of the SARS-CoV-2 virus (Fig. 4–5). In the group of patients with lung TB, memory T cells

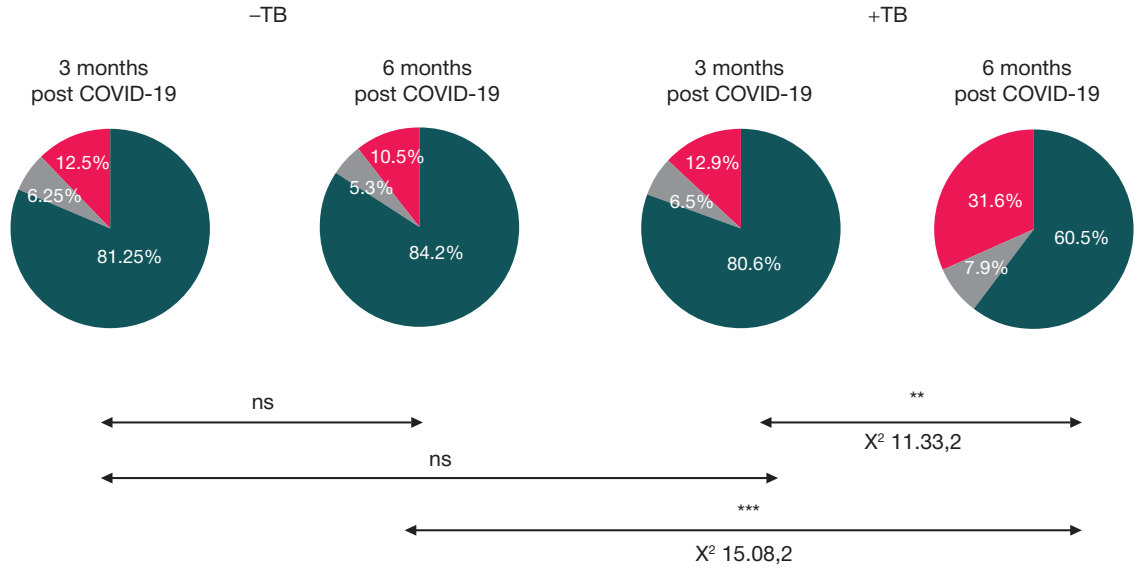

Fig. 4. Differences in the number of positive, negative, or questionable $CD4^+/CD8^+$ T-cell memory test results for SARS-CoV-2-virus antigens in COVID-19 survivors with and without pulmonary TB diagnosed 3 and 6 months before enrollment in the study. Green sector of the diagram — patients testing positive for memory T cells to SARS-CoV-2 antigens; gray sector of the diagram — patients with questionable results of the test for memory T cells to SARS-CoV-2 antigens; red sector of the diagram — patients testing negative for memory T cells to SARS-CoV-2 antigens. (–TB) — patients, who experienced COVID-19, with no history of TB; (+TB) — patients diagnosed with TB, who experienced COVID-19; ns — non-significant differences; ** — $p < 0.01$; *** — $p < 0.001$

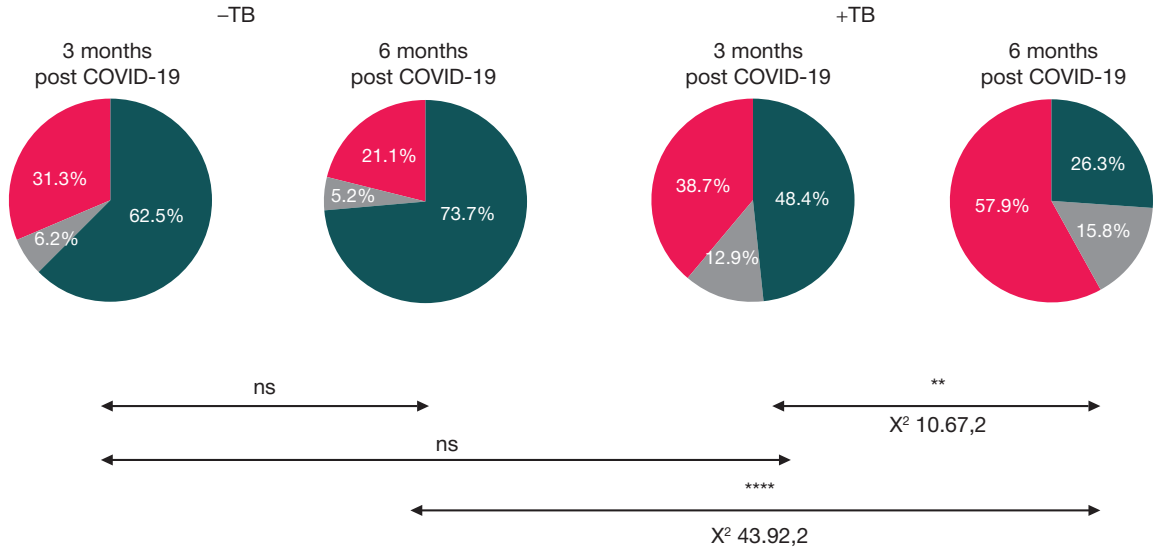

Fig. 5. Differences in the number of positive, negative, or questionable CD4⁺/CD8⁺ T-cell memory test results for S-protein antigens of SARS-CoV-2-virus in COVID-19 survivors 3 and 6 months before inclusion in the study diagnosed with pulmonary TB (+TB) and without pulmonary TB (–TB). Green sector of the diagram — patients testing positive for memory T cells against the SARS-CoV-2 S-protein antigens; gray sector of the diagram — patients with questionable results of the test for memory T cells against the SARS-CoV-2 S-protein antigens; red sector of the diagram — patients testing negative for memory T cells against the SARS-CoV-2 S-protein antigens. ns — non-significant differences; ** — $p < 0.01$; **** — $p < 0.0001$

to the SARS-CoV-2 S protein could not be detected in 57.9% of cases 6 months after the virus infection, while in the group of patients without lung TB, only 21% of such patients could be detected (Fig. 5).

DISCUSSION

The relationship between the COVID-19 virus and TB has been extensively studied to date, with a focus on co-infection and the impact of SARS-CoV-2 infection on latent TB. In this study we assessed the impact of TB on the duration of immunologic memory to viral antigens. Our results suggest a possible decreased effector response and increased regulatory mechanisms of immune response to SARS-CoV-2 antigens under the influence of bacterial infection.

Circulating IgG titers to SARS-CoV-2 were well maintained for 3–4 months in two large studies (>1000 individuals) [19, 23]. Virus-specific memory B cells, antibodies, and memory T cells were detected in mild COVID-19 cases approximately 90 days after infection [24].

One of the studies focused on assessing interaction with latent TB (LTBI) involved examination of seropositive, asymptomatic individuals infected with SARS-CoV-2 in India and comparison of immune responses in the IGRA-positive (LTBI) and IGRA-negative individuals [25]. The authors showed that IGRA-positive individuals had higher levels of humoral, cytokine, and acute phase responses compared to IGRA-negative individuals and thus concluded that LTBI could have a significant effect on the systemic inflammation, as well as on the cytokine response and the increase in the neutralizing antibody potency in individuals infected with SARS-CoV-2.

Based on the analysis of the IFNγ production by T cells in the cohort of participants with co-infection, active TB can have a negative effect on the patient's ability to generate the SARS-CoV-2-specific immune response [26]. The lowest secretion of IFNγ in response to stimulation with the SARS-CoV-2 peptide compared to patients with COVID-19 and patients with LTBI/COVID-19 was detected in the whole blood of patients with TB/COVID-19. The authors demonstrated that patients with COVID-19 having latent or active TB were still able to respond to the Mtb-specific antigens. However, only 20% of patients with active TB and COVID-19 responded positively versus 64% of patients with COVID-19 and LTBI, suggesting that active TB suppresses the host's COVID-19-specific immune response [26] and confirming results obtained in a previous study [24] for COVID-19 and TB/HIV [27].

119 individuals were studied and the blood plasma immune profile of 14 TB/COVID-19 co-infected patients, COVID-19 co-infected patients, TB co-infected patients and 20 healthy controls was compared by 27-component multiplex analysis. [28]. The authors observed that circulating TNF levels were most strongly correlated with TB/COVID-19 co-infection in comparison to COVID-19. They also showed that patients with co-infection had a reduced SARS-CoV-2-specific response based on the number of pro-inflammatory cytokines and/or chemokines, anti-inflammatory cytokines and growth factors. Furthermore, they concluded that co-infection negatively affected the Mtb-specific response (to a lesser extent).

CONCLUSIONS

While the duration of antiviral immunity monitoring in TB patients with a history of COVID-19 has been limited to 3-4 months in the vast majority of studies [29], we have demonstrated a decrease in virus-specific humoral and cellular immune responses in patients with TB co-infection 6 months after COVID-19. Our data suggest that the duration of protective antiviral immunity is shortened by concomitant TB infection. The long-term consequences of SARS-CoV-2 and Mtb co-infection will be the subject of further studies.

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FIRST LINE THERAPY FOR MULTIPLE SCLEROSIS: CYTOKINE LEVELS AND THE IMPACT OF HERPESVIRUS INFECTION

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The effects of the disease modifying drugs (DMDs) for multiple sclerosis (MS), interferon beta (IFNβ) and glatiramer acetate (GA), on the cytokine levels of individuals with MS are poorly understood. The effects of persistent herpesvirus infection (PHVI) on the cytokine production during treatment with DMDs for MS have not been identified. The role of cytokines and PHVI in the development of the treatment-related adverse events (AEs) has not been determined. The study was aimed to assess serum cytokine levels in patients with MS treated or not treated with DMDs for MS, and to determine the relationships between the cytokine levels, herpesvirus infection, and AEs. A total of 36 patients (12 males and 24 females, median age 38.50 (28.00; 48.50) years) with relapsing-remitting MS (criteria by McDonald, 2010) were examined. PHVI reactivation was observed in 18 individuals; in 10 of them it was associated with the history of the virus-associated exacerbation (VAE) of MS or VAE detected during assessment. A total of 30 patients were treated with DMDs for MS: 16 individuals with IFNβ, 14 individuals with GA. Systemic AEs were reported in 9 individuals. Serum levels of 15 cytokines were determined using the xMAP multiplex technique. Patients with MS showed a significant increase in the levels of IL10 ($p < 0.01$) and IL33 ($p < 0.001$) relative to donors when treated or not treated with DMDs for MS; the increase in IL31 levels was reported only in naïve patients ($p < 0.05$). At the same time, individuals with MS had low levels of IL1β, IL17F, IL22, IL25, IL23, and TNFα ($p < 0.01$). We revealed no differences in cytokine levels in the context of taking IFNβ or GA. Elevated IL10 levels were associated with PHVI reactivation ($p < 0.01$). We revealed significant correlations between high levels of IL31 and VAE ($p < 0.01$), IL33 and PHVI ($p < 0.01$). The IL1β levels were significantly higher in individuals with PHVI reactivation treated with DMDs for MS. There were no differences in cytokine levels associated with the presence or absence of systemic AEs. The latter predominated in individuals with PHVI reactivation and VAE. The cytokine levels of individuals with MS are affected by treatment with DMDs for MS and herpesvirus infections.

Keywords: multiple sclerosis, disease modifying drugs for multiple sclerosis, cytokines, herpes, adverse events

Funding: the study was supported by the Foundation for Assistance to Small Innovative Enterprises in Science and Technology (Innovation Promotion Foundation) within the framework of the program UMNIK: Participant of the Youth Research and Innovation Contest (contracts No. 3560GU1/2014 dated 23.09.2014, No. 8815GU2/2015 dated 17.12.2015).

Author contribution: Baranova NS, Gris MS — study planning and design; Gris MS, Artyuhov AS — data acquisition and research procedure; Baranova NS, Gris MS, Baranov AA — data analysis; all authors — data interpretation; Baranova NS, Gris MS — manuscript drafting; all authors — manuscript editing.

Compliance with ethical standards: the study was approved by the local Ethics Committee of the Yaroslavl State Medical University (protocol No. 1 dated 10 October 2013). The informed consent was submitted by all patients.

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Received: 23.04.2024 **Accepted:** 05.05.2024 **Published online:** 19.06.2024

DOI: 10.24075/brsmu.2024.021

ТЕРАПИЯ РАССЕЯННОГО СКЛЕРОЗА ПРЕПАРАТАМИ ПЕРВОЙ ЛИНИИ: УРОВЕНЬ ЦИТОКИНОВ И ВЛИЯНИЕ ГЕРПЕТИЧЕСКОЙ ИНФЕКЦИИ

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При рассеянном склерозе (РС) недостаточно изучено влияние на уровень цитокинов терапии препаратами, изменяющими течение рассеянного склероза (ПИТРС) — интерферона-бета (ИНФ-β) и глатирамера ацетата (ГА). Не установлено влияние персистирующей герпес-вирусной инфекции (ПГВИ) на продукцию цитокинов на фоне терапии ПИТРС. Не определена роль цитокинов и ПГВИ в развитии нежелательных явлений (НЯ) при лечении. Целью исследования было провести оценку концентрации цитокинов в сыворотке крови у больных РС, находящихся на терапии ПИТРС и без нее, определение связи между уровнем цитокинов, герпес-вирусной инфекцией и НЯ. Обследовано 36 больных (12 мужчин и 24 женщины, медиана возраста 38,50 (28,00; 48,50) года) с ремитирующим течением РС (критерии McDonald, 2010). У 18 человек наблюдали реактивацию ПГВИ, у 10 она сопровождалась развитием вирус-ассоциированного обострения (ВАО) РС в анамнезе или при осмотре. Терапию ПИТРС проводили 30 пациентам: 16 человек — ИНФ-β, 14 человек — ГА. Системные НЯ были у 9 человек. Концентрацию 15 цитокинов в сыворотке крови определяли мультиплексной технологией xMAP. У пациентов с РС по сравнению с донорами были значимо повышены IL10 ($p < 0,01$) и IL33 ($p < 0,001$) при терапии ПИТРС и без нее, уровень IL31 возрос только у наивных больных ($p < 0,05$). Одновременно при РС были низкие значения IL1β, IL17F, IL22, IL25, IL23 и ФНО-α ($p < 0,01$). Не установлено различий в уровне цитокинов на фоне ИНФ-β или ГА. IL10 был повышен при реактивации ПГВИ ($p < 0,01$). Выявлены достоверные связи между высокими значениями IL31 и ВАО ($p < 0,01$), IL33 и ПГВИ ($p < 0,01$). На фоне терапии ИНФ-β при реактивации ПГВИ концентрация IL1β была значимо выше. Уровень цитокинов не различался при наличии или отсутствии системных НЯ. Последние преобладали при реактивации ПГВИ и ВАО. На уровень цитокинов при РС влияют терапия ПИТРС и герпес-вирусные инфекции.

Ключевые слова: рассеянный склероз, изменяющие течение рассеянного склероза препараты, цитокины, герпес, нежелательные явления

Финансирование: работа выполнена при финансовой поддержке Федерального государственного бюджетного учреждения «Фонд содействия развитию малых форм предприятий в научно-технической сфере» (Фонд содействия инновациям) в рамках программы УМНИК: Участник молодежного научно-инновационного конкурса (договоры №3560ГУ1/2014 от 23.09.2014, № 8815ГУ2/2015 от 17.12.2015).

Вклад авторов: Н. С. Баранова, М. С. Грись — планирование и дизайн исследования; М. С. Грись, А. С. Артюхов — сбор данных и проведение исследования; Н. С. Баранова, М. С. Грись, А. А. Баранов — анализ данных; все авторы — интерпретация данных; Н. С. Баранова, М. С. Грись — подготовка черновика рукописи; все авторы — редактирование рукописи.

Соблюдение этических стандартов: исследование одобрено локальным этическим комитетом ФГБОУ ВО ЯГМУ Минздрава РФ (протокол № 1 от «10» октября 2013 г.). Все пациенты подписали добровольное информированное согласие.

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Статья получена: 23.04.2024 **Статья принята к печати:** 05.05.2024 **Опубликована онлайн:** 19.06.2024

DOI: 10.24075/vrgmu.2024.021

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system with the autoimmune inflammatory and neurodegenerative pathogenetic mechanisms [1]. Interferons beta (IFNβ) and glatiramer acetate (GA) are the main first line disease modifying drugs (DMDs) for MS [1–3]. Today, despite the long-term effective use of high-dose IFNβ and GA in clinical practice, their exact mechanisms of action are poorly understood [4, 5]. The effects of IFNβ and GA on the levels of pro-inflammatory (interleukin (IL) IL1β, IL6, IL17, IL23, tumor necrosis factor — (TNFα), interferon-γ (IFNγ)) and anti-inflammatory (IL4, IL10) cytokines are the most thoroughly studied [6–8].There are sporadic papers focused on comparative assessment of the cytokine profiles of untreated patients and patients using IFNβ or GA [9–13]. In foreign literature, there is a number of studies focused on assessing the effects of these drugs on the levels of IL31 and IL33 [10, 11, 12, 14–17], however, no such studies have been carried out in Russia. Furthermore, the contribution of herpesvirus infection representing one of the etiological factors of the disease and the trigger of exacerbation in some patients with MS to the cytokine profile formation during treatment with IFNβ or GA is poorly understood [18, 19].

We have earlier determined the differences in the cytokine levels associated with the disease exacerbation and remission, as well as their correlations with the clinical manifestations of the persistent herpesvirus infection (PHVI) reactivation [20], however, no assessment of the effects of the ongoing therapy, drugs use, and the treatment-related systemic adverse events (AEs) have been carried out. This study is an extension of scientific research on the issue.

The study was aimed to assess serum cytokine levels in patients treated and not treated with high-dose IFNβ or GA, as well as to determine the relationships between the cytokine levels, herpesvirus infection and the treatment-related AEs.

METHODS

A total of 36 patients (12 males and 24 females) were included in the study. Inclusion criteria: reliable diagnosis of MS based on the criteria by McDonald, et al. (2010). Patients were enrolled November 2013 to June 2017. The patients' median age at the time of examination was 38.50 (28.00; 48.50) years, the age of onset was 27.00 (21.50; 38.00) years, and the disease duration was 9.50 (3.50; 12.50) years. All the patients had relapsing-remitting MS (RRMS), 29 individuals (80.6%) had remission, 7 individuals (19.4%) had exacerbation of the disease. All patients with RRMS were divided into patients with active and inactive MS (17 (47.2%) and 19 (52.8%) individuals, respectively) in accordance with the classification by F. Lublin (2013). Furthermore, patients with highly active MS (6 individuals (35.3%) having two or more exacerbations per year) were further counted among patients with active MS. The neurological status clinical assessment was performed using the double scoring system by J. F. Kurtzke: Functional Systems (FS) and Expanded Disability Status Scale (EDSS).

A total of 30 patients (83.3%) were treated with DMDs for MS (16 with high-dose IFNβ and 14 with GA) and 6 individuals (16.7%) received no therapy. A total of 18 patients (50.0%) had reactivation of PHVI, and in 10 individuals (27.8%) PHVI reactivation was associated with the history of virus-associated exacerbation (VAE) or VAE detected during examination. Tolerability of the ongoing therapy with DMDs for MS was estimated in 28 patients, who filled the questionnaire for identification of the treatment-related AEs. We analyzed systemic AEs reported in 9 individuals (32.1%).

To achieve the objectives, we performed comparative analysis of the clinical characteristics of the groups of patients with MS not treated with DMDs for MS and treated with IFNβ or GA only (Table 1).

The groups compared did not differ in terms of gender, age of onset, fact of detecting serological markers of past EBV infection. The patients' age at the time of examination was significantly higher in the group of patients taking any DMD for MS than in the group not taking such drugs ($p < 0.05$). The same trend was reported for the groups using IFNβ or GA ($p > 0.05$).

The disease duration and the total number of exacerbations were significantly higher in the overall group of patients taking DMDs for MS and the patients taking IFNβ or GA, than in untreated patients. The total amount of neurologic deficit and the time to reach disability (EDSS = 3) were higher in the context of taking DMDs for MS, however, the differences were non-significant. The signs of PHVI reactivation and VAE, serological markers of past cytomegalovirus (CMV) infection were slightly more frequent in these groups.

More active course of MS, higher rates of exacerbations and highly active variant of the disease, the emergence of new foci on MRI compared to patients taking DMDs for MS were reported in patients not treated with such drugs. The average annual exacerbation rate and the rates of disease progression and neurologic deficit increase were significantly higher ($p < 0.05$ and $p < 0.01$). There were no differences in the majority of indicators between the groups of patients taking IFNβ or GA. However, the use of IFNβ was associated with the more severe systemic AE severity, than the use of GA.

We assessed 18 generally healthy donors as controls. The control group was matched by gender — 7 males (38.9%) and 11 females (61.1%); age — 39.10 (29.00; 49.60) years with the group of patients having MS. Exclusion criteria: chronic neurologic disorder; exacerbation of somatic disorder. The standard neurologic examination and history taking aimed to exclude the disorders capable of affecting the assessment results were performed in all patients.

Blood serum testing aimed to determine the levels of type-specific IgM and IgG against type 1 and 2 herpes simplex virus (HSV), IgM and IgG against varicella-zoster virus (VZV), IgM and IgG against the VCA capsid antigen of the Epstein–Barr virus (EBV), IgG against early antigens EA and nuclear antigen NA of EBV, IgM and IgG against CMV were carried out by enzyme-linked immunoassay (ELISA) using the standard reagent kits (Vector-Best; Novosibirsk, Russia) at the clinical and diagnostic laboratory, Set' LLC (Yaroslavl), in accordance with the manufacturer's instructions in all patients with MS and members of the control groups.

Serum levels of 15 cytokines (IL1β, IL4, IL6, IL10, IL17A, IL17F, IL21, IL22, IL23, IL25, IL31, IL33, IFNγ, TNFα, sCD40L) were determined by the xMAP multiplex technique using the Bio-PlexTM 200 System (Bio-Rad; USA) and appropriate reagents (Bio-Rad; USA) at the laboratory of the Research Institute of Translational Medicine, Pirogov Russian National Research Medical University.

Statistical processing of the results was performed using the Statistica 10.0 software package (StatSoft; USA) including the generally accepted parametric and nonparametric analysis methods. As for parameters, the distribution of which was non-normal, the Mann–Whitney U test was used to compare two groups, and the Kruskal–Wallis test was used to compare three or more groups (for independent groups). The results were presented as the median (Me) with interquartile range [25th; 75th percentiles], the mean (M) and standard deviation

Table 1. Clinical characteristics of patients with MS (Me (25th; 75th percentiles), *n* = 36)

Indicator	No therapy with DMDs for MS (*n* = 6)	Therapy with IFNβ or GA (*n* = 30)	Therapy with IFNβ (*n* = 16)	Therapy with GA (*n* = 14)
Gender: males, *n* (%)	2 (33.3)	10 (33.3)	6 (37.5)	4 (28.6)
females, *n* (%)	4 (66.7)	20 (66.7)	10 (62.5)	10 (71.4)
Age (years)	24.50 (23.00; 39.00)	39.00* (34.00; 51.00)	38.50 (33.00; 45.00)	39.00 (35.00; 55.00)
Age of onset (years)	22.50 (22.00; 39.00)	27.00 (21.00; 37.00)	28.50 (22.00; 34.00)	27.00 (21.00; 39.00)
Disease duration (years)	1.00 (1.00; 4.00)	11.00** (6.00; 18.00)	11.00* (5.50; 12.50)	11.00* (6.00; 20.00)
Duration of therapy with DMDs for MS (months)		30.00 (9.00; 67.00)	30.00 (14.00; 73.00)	38.00 (29.00; 74.00)
Highly active, *n* (%)	3 (50.0)	3 (10.0)*	2 (12.5)	1 (7.1)
Exacerbation detected (clinically + MRI), *n* (%)	3 (50.0)	4 (13.3)	3 (18.8)	1 (7.1)
Emergence of new foci on MRI, *n* (%)	4 (66.7)	8 (26.7)	6 (37.5)	2 (14.3)*
EDSS at the time of examination (points)	2.50 (1.50; 3.00)	3.50 (2.00; 4.50)	3.50 (2.50; 4.75)	3.50 (2.00; 4.50)
Total number of exacerbations	2.00 (1.00; 3.00)	4.00** (3.00; 6.00)	4.00* (3.00; 6.50)	4.50** (4.00; 6.00)
Average annual exacerbation rate	1.50 (0.75;2.00)	0.42* (0.32; 0.83)	0.58* (0.31; 0.90)	0.37* (0.32; 0.83)
Progression rate (points/year)	1.75 (0.75; 2.00)	0.28** (0.21; 0.50)	0.46* (0.22; 0.66)	0.23** (0.19; 0.50)
Duration of first remission (months)	6.00 (2.00; 15.00)	12.00 (8.00; 24.00)	12.50 (9.00; 24.00)	12.00 (8.00; 24.00)
Rate of neurologic deficit increase (points)	3.00 (1.50; 5.00)	0.53** (0.33; 1.00)	0.73** (0.35; 1.00)	0.39** (0.27; 1.08)
Total amount of neurologic deficit on the FS scale (points)	5.50 (2.00; 6.00)	7.50 (3.00; 9.00)	7.50 (4.00; 9.00)	7.50 (3.00; 9.00)
Time to reach disability EDSS = 3 (years)	0.50 (0.00; 3.00)	3.50 (0.00; 7.00)	2.75 (0.00; 8.50)	4.00 (0.00; 7.00)
Duration of therapy with DMDs for MS (months)		34.50 (20.00; 74.00)	30.00 (14.00; 73.00)	38.00 (29.00; 74.00)
Systemic adverse events associated with using drugs modifying the course of MS (*n* = 28), *n* (%)		9 (32.1) (*n* = 28)	5 (35.7) (*n* = 14)	4 (28.6)
Severity of systemic AEs associated with DMDs for MS (points)		6.00 (1.50; 11.00)	6.50 (5.00; 12.00)	2.00 (0.00; 11.00)
Reactivation of persistent herpesvirus infection (PHVI) detected, *n* (%)	2 (33.3)	16 (53.3)	10 (62.%)	6 (42.7)
Virus-associated exacerbation (VAE) detected, *n* (%)	1 (16.7)	9 (30.0)	5 (31.3)	4 (28.6%)
Serological markers of past EBV infection detected, *n* (%)	6 (100)	30 (100)	16 (100)	14 (100)
Serological markers of past CMV infection detected, *n* (%)	4 (66.7)	27 (90.0)	15 (93.8)	12 (85.7)

Note: * — $p < 0.05$, ** — $p < 0.01$ compared to the group not treated with DMDs for MS.

(σ). Fisher's exact test was used to compare samples based on the qualitative traits and to assess the occurrence of traits. Spearman's rank correlation was used for correlation analysis. The differences were considered significant at $p < 0.05$.

RESULTS

Cytokine levels in patients with MS and donors

Table 2 provides the results of assessing cytokine levels in the groups of patients, not treated with DMDs for MS, treated with DMDs for MS, using high-dose IFNβ or GA, and donors.

A significant increase in the levels of IL10 ($p < 0.01$) and IL33 ($p < 0.001$) relative to controls was reported for all groups of patients with MS. The IL31 levels significantly higher relative to donors was reported only for the group of patients not treated with DMDs for MS ($p < 0.05$). The upward trend of the IL4 levels associated with MS was reported ($p > 0.05$).

In contrast, the levels of IL1β, IL17F, IL22, IL25, and TNFα were significantly higher in donors, than in patients. There were almost no differences in the levels of IL6, IL17A, IL21, IL23, IFNγ, and sCD40L between groups.

Cytokine levels in patients not treated and treated with DMDs for MS

We revealed a significant increase in the levels of IL10 in the naïve patients compared to patients treated with DMDs for MS. The untreated patients showed a significant increase in the IL31 levels compared to the overall group of patients taking DMDs for MS and GA. These differences were a trend in individuals taking IFNβ ($p = 0.06$). There were no differences in the levels of other cytokines between the groups compared ($p > 0.05$).

We also revealed no differences in the tested cytokine levels in the groups of patients taking IFNβ or GA.

The increase in the levels of IL31 (by more than 15.08 pg/mL; M + 3σ in the group of donors) was found in 5 individuals (13.8%), more often in the group of patients not taking DMDs for MS. High IL33 levels (exceeding 3.40 pg/mL; M + 3σ in the group of donors) were reported in 20 patients (52.8%), the

Table 2. Serum cytokine levels (Me (25th; 75th percentiles)) of patients with MS and donors

Indicator (pg/mL)	Donors (n = 18)	No therapy with DMDs for MS (n = 6)	Therapy with IFNβ or GA (n = 30)	Therapy with IFNβ (n = 16)	Therapy with GA (n = 14)	p
	1	2	3	4	5	
IL1β	1.45 (0.16; 2.18)	0.04 (0.01; 0.05)	0.04 (0.00; 0.08)	0.04 (0.00; 0.09)	0.05 (0.00; 0.06)	$p_{1-2} < 0.05$ $p_{1-3} < 0.001$ $p_{1-4} < 0.01$ $p_{1-5} < 0.01$
IL4	0.01 (0;73; 3.24)	2.39 (2.29; 2.89)	4.71 (2.22; 11.34)	4.88 (2.59; 8.30)	4.40 (1.75; 12.33)	n/s
IL6	1.36 (0.27; 3.68)	0.70 (0.44; 0.96)	0.52 (0.30; 1.11)	0.59 (0.37; 1.30)	0.52 (0.15; 0.74)	n/s
IL10	0.01 (0.00; 0.01)	3.52 (2.73; 5.25)	1.80 (0.90; 2.73)	2.26 (0.90; 2.73)	1.80 (0.60; 2.10)	$p_{1-2} < 0.01$ $p_{1-3} < 0.01$ $p_{1-4} < 0.01$ $p_{1-5} < 0.01$ $p_{2-3} < 0.01$ $p_{2-4} < 0.05$ $p_{2-5} < 0.01$
IL17A	0.58 (0.00; 1.74)	0.64 (0.42; 0.99)	0.57 (0.28; 0.85)	0.54 (0.28; 0.82)	0.57 (0.14; 0.92)	n/s
IL17 F	6.76 (4.02; 10.6)	0.01 (0.00; 0.93)	0.01 (0.01; 0.62)	0.01 (0.01; 0.62)	0.01 (0.00; 1.25)	$p_{1-2} < 0.001$ $p_{1-3} < 0.001$ $p_{1-4} < 0.001$ $p_{1-5} < 0.001$
IL21	0.01 (0.00; 0.49)	0.00 (0.00; 0.00)	0.00 (0.00; 0.00)	0.01 (0.00; 0.89)	0.00 (0.00; 0.01)	n/s
IL22	47.43 (38.42; 72.64)	0.00 (0.00; 0.00)	0.24 (0.00; 0.32)	0.08 (0.00; 0.32)	0.32 (0.00; 0.63)	$p_{1-2} < 0.001$ $p_{1-3} < 0.001$ $p_{1-4} < 0.001$ $p_{1-5} < 0.001$
IL23	80.11 (0.00; 114.44)	0.00 (0.00; 2.94)	5.51 (0.00; 8.81)	4.41 (0.00; 10.63)	5.51 (0.00; 8.81)	n/s
IL25	13.73 (6.10; 28.99)	0.27 (0.11; 0.32)	0.11 (0.00; 0.32)	0.11 (0.00; 0.32)	0.11 (0.00; 0.32)	$p_{1-2} < 0.001$ $p_{1-3} < 0.001$ $p_{1-4} < 0.001$ $p_{1-5} < 0.001$
IL31	6.28 (2.87; 8.62)	11.61 (8.81; 15.73)	5.71 (3.00; 8.19)	6.95 (3.85; 10.37)	5.09 (2.63; 7.57)	$p_{1-2} < 0.05$ $p_{2-3} < 0.05$ $p_{2-4} = 0.06$ $p_{2-5} < 0.05$
IL33	0.52 (0.17; 0.78)	3.63 (2.51; 9.55)	4.46 (1.12; 6.67)	5.43 (1.95; 9.14)	4.05 (1.12; 5.84)	$p_{1-2} < 0.001$ $p_{1-3} < 0.001$ $p_{1-4} < 0.001$ $p_{1-5} < 0.001$
IFNγ	0.45 (0.00; 5.33)	0.49 (0.49; 1.48)	0.49 (0.49; 1.23)	0.99 (0.49; 1.11)	0.49 (0.00; 1.48)	n/s
TNFα	17.38 (13.65; 31.61)	0.82 (0.49; 1.17)	0.52 (0.44; 0.74)	0.53 (0.45; 0.88)	0.51 (0.44; 0.68)	$p_{1-2} < 0.001$ $p_{1-3} < 0.001$ $p_{1-4} < 0.001$ $p_{1-5} < 0.001$
sCD40L	110.81 (83.58; 122.55)	83.58 (34.36; 158.24)	76.77 (39.27; 112.69)	81.23 (44.29; 122.56)	65.97 (25.41; 95.32)	n/s

Note: n/s — non-significant differences between groups.

rate was almost the same in all the groups compared. Isolated hyperproduction of IL31 was found in only one patient out of five, while in other cases (80.0%) a simultaneous increase in the IL31 and IL33 levels was observed. The IL17A, IL17F and IL21 levels rarely exceeded the normal reference values (in 2.8%, 5.6% and 5.6% of cases, respectively) and always accompanied the increase in the IL33 levels. The levels of other cytokines exceeded the upper limit of normal range in none of the cases.

Herpesvirus infection, therapy with DMDs for MS, and cytokine levels

Reactivation of PHVI took place in 16 patients taking DMDs for MS, IFNβ or GA put of 30 (53.3%). In 9 cases (30.8%), it was associated with the history of MS exacerbation (VAE+) or the MS exacerbation detected during examination. The IL10 levels were significantly higher in patients with PHVI reactivation, than in patients without it (Table 3). These patients also showed an upward trend of the IL1β, IL23 and IL33 levels ($p > 0.05$). No differences in the levels of other cytokines between the groups compared were revealed. The cytokine levels of patients with or without VAE were the same.

Significant correlations between high IL31 levels and VAE+ ($r = 0.51$; $p < 0.01$), IL33 levels and PHVI ($r = 0.40$; $p < 0.05$) were revealed when using all DMDs for MS.

The levels of IL1β were significantly higher in patients treated with IFNβ with PHVI reactivation, than in patients with no PHVI reactivation (n = 10, 0.07 ± 0.06 pg/mL and n = 6, 0.02 ± 0.04 pg/mL, $p < 0.05$, respectively). In this group, the IL17A and IL33 levels were higher in patients with VAE+, than in individuals without VAE (IL17A — 0.92 ± 0.42 pg/mL, n = 5; 0.49 ± 0.52 pg/mL, n = 11; IL33 — 10.70 ± 5.79 pg/mL, n = 5; and 5.63 ± 7.83 pg/mL, n = 11; $p < 0.05$ in both cases). We also revealed a significant correlation between VAE+ and high levels of IL31 and IL33 ($r = 0.56$ at $p < 0.05$ and $r = 0.52$ at $p < 0.05$, respectively).

Table 3. Serum cytokine levels (Me (25th; 75th percentiles)) of patients with MS treated with DMDs for MS, who had clinical manifestations/no clinical manifestations of PHVI

Indicator (pg/mL)	MS with clinical manifestations of PHVI (*n* = 16)	MS with no clinical manifestations of PHVI (*n* = 14)
IL1β	0.06 (0.02;0.08)	0.01 (0.00; 0.05)
IL4	4.88 (2;63;10.95)	4.61 (1.75; 13.11)
IL6	0.74 (0.23;1.81)	0.44 (0.30; 0.74)
IL10	2.57 (1.65;2.73)**	1.05 (0.30; 1.95)
IL17A	0.64 (0.35;0.96)	0.43 (0.14; 0.57)
IL17 F	0.01 (0.00;0.62)	0.01 (0.00; 1.25)
IL21	0.01 (0.00;2.38)	0.00 (0.00; 0.00)
IL22	0.32 (0.00;0.48)	0.00 (0.00; 0.32)
IL23	8.80 (0.00;11.36)	2.57 (0.00; 5.87)
IL25	0.22 (0.06;0.53)	0.06 (0.00; 0.11)
IL31	6.33 (4.47;8.81)	4.78 (2.63; 7.57)
IL33	5.43 (3.21;9.14)	2.23 (1.12; 5.84)
IFNγ	0.74 (0.49;1.36)	0.49 (0.49; 0.99)
TNFα	0.53 (0.44;1.04)	0.52 (0.45; 0.68)
sCD40L	80.53 (41.85;111.52)	65.97 (25.41; 117.39)

Note: ** — $p < 0.01$ between groups.

In patients treated with GA, there were no differences in the tested cytokine levels between the groups of patients having or not having clinical manifestations of PHVI reactivation or VAE. Furthermore, in contrast to IFNβ, no correlation of high IL31, IL33 levels with the PHVI reactivation or VAE was revealed when using GA.

In patients not treated with DMDs for MS, no analysis of the cytokine levels depending on the fact of PHVI reactivation/no PHVI reactivation or VAE was performed due to small number of patients in each group.

Therapy with DMDs for MS, adverse events, herpesvirus infection, and cytokine levels

Systemic AEs were reported in 9 patients out of 28 (32.1%) treated with DMDs for MS. When treated with IFNβ, 5 individuals (35.7%; *n* = 14) experienced a flu-like syndrome (FLS), and 4 patients taking GA (28.6%; *n* = 14) showed the systemic vasomotor response.

In individuals taking IFNβ and GA having or not having systemic AEs, no differences in the tested cytokine levels and the rate of increased levels of some cytokines were revealed. The systemic AE severity also was not correlated to the cytokine levels.

Predominance of systemic AEs in the groups of patients with PHVI reactivation and VAE+ was reported. Thus, during treatment with IFNβ or GA AEs were observed in 7 patients with PHVI out of 15 (46.7%) and 2 patients without PHVI out of 13 (15.4%) ($p = 0.08$). In individuals with VAE, the AE rates were 44.4% (4 individuals out of 9) and 26.3% (5 individuals out of 19) ($p > 0.05$).

During treatment with IFNβ, the AE rates in the groups of patients showing and not showing PHVI reactivation were 44.4% (4 individuals out of 9) and 20.0% (1 individual out of 5), respectively (in 5 individuals (35.7%; *n* = 14); as for VAE, these were 40.0% (2 individuals out of 5) and 33.0% (3 individuals out of 9) ($p > 0.05$). When using GA, the AE rates in the groups of patients showing and not showing PHVI were 50.0% (3 individuals out of 6) and 12.5% (1 individual out of 8) ($p = 0.17$); as for VAE, these were 50.0% (2 individuals out of 4) and 20.0% (2 individuals out of 10).

During treatment with all the DMDs for MS the systemic AE severity was also higher in cases of PHVI reactivation, than in cases of no PHVI reactivation. Thus, during treatment of the groups compared with IFNβ or GA it was 8.00 (1.00; 12.00) and 5.00 (2.00; 6.00) points, when using IFNβ it was 8.00 (6.00; 15.00) and 6.00 (5.00; 6.00) points, and when using GA it was 6.00 (0.00; 11.00) and 2.00 (0.50; 7.00) points ($p > 0.05$ in all groups). In individuals with VAE, such trend was reported for GA only: 5.50 (0.00; 11.00) and 2.00 (1.00; 85.00) points ($p > 0.05$).

DISCUSSION

Cytokines in the naïve patients with MS treated with DMDs for MS and the donors

The literature contains only a few studies involving comparison of the cytokine levels in patients with MS not receiving DMDs for MS and healthy individuals. High levels of the key pro-inflammatory cytokines (IL1β, IL17A, IL17F, IL23, TNFα, and IFNγ) are usually reported in naïve patients with MS [8, 12, 21–23]. However, according to the data provided by other authors, serum TNFα levels of the patients taking DMDs for MS were the same [9] or lower [10, 13] compared to that of the control group. The results of recent studies are consistent with our data.

In patients with MS not treated with DMDs for MS, the increase in the serum levels of other inflammatory cytokines, IL31 [11, 12, 14] and IL33 [10, 15–17], relative to donors was reported. However, no significant differences in the IL33 levels between naïve patients and the controls were revealed [12].

In contrast, the serum levels of anti-inflammatory IL4 and IL10 in the untreated patients with MS were lower than in donors [9, 13, 22, 23] or were the same as in controls [21, 24]. However, according to the data provided by other researchers, the IL10 levels were higher in naïve patients with MS, than in donors [10, 12], which was also found in our study.

In general, our patients with MS not treated with DMDs for MS showed a significant increase in the levels of IL10, IL31, IL33 relative to controls, along with the upward trend of IL4 levels. At the same time, low levels of IL1β, IL17F, IL22, IL25, IL23, and TNFα were reported. There were no differences in the levels of other cytokines (IL6, IL17A, IL21, IFNγ, and sCD40L) between the comparison groups. Similar patterns were

reported when comparing the serum cytokine profiles of the patients treated with DMDs for MS and healthy controls, except for the IL31 levels. Such results are to some extent consistent with the above data provided by certain authors.

Cytokine levels in patients with MS treated and not treated with DMDs for MS

According to the literature data, the significantly higher levels of IL1β, IL17A, TNFα, IFNγ and lower levels of IL4, IL10 relative to the patients taking IFNβ or GA were revealed in naïve patients [6, 9, 21, 23]. It was noted, that therapy with IFNβ or GA resulted in the significant decreased concentrations of IL17, IL23, TNFα, IFNγ and the increased IL4 and IL10 levels [6, 11,12, 22].

The decrease in the IL31 levels was also found during treatment with DMDs for MS [14] that was considered to be associated with the decrease in the $CD3^{+}CD45RO^{+}$Th2 memory cells being the major IL31 producers [25]. We have also revealed a similar pattern.

The plasma IL33 levels of the patients taking IFNβ1a were significantly lower than that of the untreated patients [15, 17]. However, according to some date, therapy with GA or DMDs for MS did not affect the plasma IL33 concentrations [16]. In our study, there were no differences in the IL33 levels between the groups of patients treated and not treated with DMDs for MS.

In general, our untreated patients showed a significant increase in the IL10 levels compared to all the groups of patients treated with DMDs for MS. The IL31 levels were significantly higher in the naïve patients compared to the patients treated with IFNβ or GA and GA. We had earlier shown the increase in the IL10 concentration associated with the MS exacerbation [20]. In this phase of the disease, high IL31 levels and combined hyperproduction of IL33 and IL17A, IL17F, IL21 and IL31 were reported significantly more often, than during remission.

The identified differences in the cytokine profiles of the naïve patients and patients treated with DMDs for MS associated with remission and exacerbation were confirmed by the genetic test results [26]. The whole transcriptome analysis performed in patients with MS revealed impaired expression of 8800 genes in the patients, who had not previously received therapy, compared to the patients treated with IFNβ. The authors believe that in naïve patients the products of dysregulated genes contribute to inflammatory damage to the CNS and impede restoration of the brain. Furthermore, the groups of patients with the complete (no exacerbation) and partial clinical responses to IFNβ therapy showed differences in expression of 277 genes. During remission or exacerbation, the state of mononuclear cells in the patients not treated with DMDs for MS is characterized by extreme instability and the development of "cytokine storm" [26]. Furthermore, the long-term (longer than 5 years) IFNβ therapy adjusts this phenomenon through the gene expression modulation, which results in the state of cytokine harmony.

It is believed that despite similar clinical efficacy of the high-dose IFNβ and GA, the mechanisms underlying their effects on the immune systems can be different [4, 8, 9, 14]. However, we revealed no differences in the levels of almost all tested cytokines between the groups of patients taking IFNβ or GA, except for IFNγ, the levels of which were significantly higher during treatment with IFNβ, than when receiving GA.

Cytokine and herpesvirus infection in patients treated with DMDs for MS, adverse events

The data on the differences in production of IL10, IL31 and IL33 associated with using DMDs for MS obtained in our study can result from the herpesvirus infection reactivation.

It is well-known, that IL10 having a potent anti-inflammatory effect takes an active part in the immune response associated with the infectious, autoimmune, and autoinflammatory diseases [27]. High levels of IL10B produced by plasmablasts and plasma cells were observed in the MS foci [28]. In individuals with viral infections, the long-term antigen persistence accompanied by the increase in IL10 production results in the antiviral T cell phenotype switched mainly to the IL10-producing T cells [29]. Production of both viral homologue of human IL10 and common IL10 associated with the EBV infection has been revealed [30]. We have earlier more thoroughly discussed the possible mechanisms underlying involvement of these cytokines in the MS pathogenesis [20]. Apparently, these are involved in the IL10 production in our situation as well, especially in naïve patients.

Our findings about the simultaneous decrease in the anti-inflammatory IL10 and pro-inflammatory IL31 concentrations during treatment with DMDs for MS relative to the naïve patients, the correlations between IL31 and VAE+, IL33 and PHVI reactivation confirm an important role of herpesviruses in the MS pathogenesis. It is possible that the larger amount of the common IL10 is synthesized during treatment with IFNβ or GA, rather than its homologue. It has been found that treatment with these DMDs for MS increases systemic activity of the non-viral IL10 [31, 32].

Furthermore, the Th17 phenotype is switched under exposure to DMDs for MS, the number of IL17-secreting cells is reduced, and the number of IL10-producing cells and the double cells secreting IL10 and IL17 is increased [33]. The T cell phenotypic shift to the type 1 regulatory T cells, the decrease in the number of memory B cells and the levels of IL10 they produce have been detected [24]. This is partially confirmed by the decrease in the levels of IL10 during treatment with IFN and GA we have detected, associated with the MS clinical manifestations' relief and almost the same detection rates of EBV markers.

All the above can be associated with certain antiviral effects of DMDs for MS, mostly IFNβ. IFNβ reduces the EBV latent membrane protein 2A expression in the patients receiving treatment, inhibits antigen presentation to T cells, induces memory B cell apoptosis [34, 35]. However, in our study a significant increase in the IL1β concentration was revealed only in patients with PHVI reactivation treated with IFNβ. Furthermore, when using IFNβ, the IL17A and IL33 levels were significantly higher in the group of patients with VAE+, than in the group without VAE+. We also revealed a significant correlation between VAE+ and high levels of IL31 and IL33. These differences are likely to result from the higher prevalence of PHVI among individuals treated with IFNβ, than among those treated with GA.

FLS was the most common systemic AE associated with the IFNβ therapy, while systemic vasomotor response was the most common one associated with the GA therapy [2, 3]. It is believed that FLS results from the temporary increase in plasma levels of IL6 and TNFα following the drug administration, as well as from their direct pyrogenic effect on the hypothalamus [36, 37]. The emergence of systemic AEs during treatment with GA is also considered to be associated with the increase in the IL6 and IL4 levels [30]. Our findings have shown that the presence and severity of the systemic AEs associated with the IFNβ or GA therapy in patients with MS are not related to any of the studied cytokines. However, higher prevalence of systemic AEs in the groups of patients with PHVI reactivation and VAE was reported during treatment with any DMD for MS. The systemic AE severity was also higher in individuals with herpesvirus infection, especially in cases of PHVI reactivation. In general,

our findings are consistent with the data we have acquired earlier [38]. The lack of significant differences is likely to be related to the small number of patients in the studied groups.

It is believed that excess activation of the innate immunity, neuronal death and the neurodegenerative processes based on the impaired type 1 IFN pathway regulation predominate at the late stages of MS [26]. Disrupted expression of RNA and proteins in the pathways controlled by the type 1 IFN precedes the Th1, Th2, Th17 cell pathway disruption; this can impair the adaptive and innate immunity and contribute to neuronal death. It is well known that the DNA damage, necrosis, necroptosis, autophagy, and pronounced innate immunity activation are observed in case of viral invasion and HSV1 replication, while the type 1 IFN signaling pathway occupies a central place in the human body protection and induces a broad spectrum of antiviral proteins and control over the incoming pathogens [39–41]. These processes lead to production of TNFα and IL1β by microglia; TNFα and IL1β, in turn, promote IL33 transcription [42, 43]. The latter initiates the synthesis of IL31 by the Th-2 cells via IL4 [44]. The joint production of these cytokines is likely to enhance the pro-inflammatory potential of each of them [45], which is in line with the correlations between IL33 and PHVI, IL31 and VAE found in our study, as well as with the simultaneous increase in the levels of IL1β, IL31, IL33 in the group of patients with the herpesvirus infection reactivation during treatment with IFNβ.

CONCLUSIONS

Currently, MS is considered not only as an inflammatory disease of the CNS, but also as a consequence of the immune regulation disorders. The IFNβ and GA immunomodulatory properties are targeting multiple pathways of the body's innate and acquired immune response. In our opinion, PHVI reactivation accompanied by the disease exacerbation and the lack of the timely prescribed adequate first line therapy with a DMD for MS represent one of the epigenetic factors causing the enhanced innate immune response and neurodegeneration in individuals with MS. In general, the cytokine profiles of patients with RRMS are affected by not only the fact of receiving or not receiving treatment with DMDs for MS and the disease phase, but also infections, especially herpesvirus ones (EBV, type 1 and 2 HSV, VZV). Their contribution may vary depending on the DMD for MS used (IFN or GA). Our study has a number of limitations related to the small number of participants, however, the study results complement the possible immunological mechanisms involved in the MS pathogenesis, the effects of the ongoing first line therapy with DMDs for MS (high-dose IFNβ and GA), as well as concomitant herpesvirus infection.

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EEG COHERENCE IN CHILDREN WITH CEREBRAL PALSY AGAINST THE BACKGROUND OF REHABILITATION EMPLOYING A BRAIN-COMPUTER-HAND EXOSKELETON NEUROINTERFACE

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Neurorehabilitation courses employing a non-invasive brain-computer-hand exoskeleton interface in combination with traditional balneotherapy have been shown to reduce spasticity of hand muscles and improve motor skills in children with cerebral palsy. However, the coherence of the electroencephalogram (EEG) parameters have never been analyzed during such sessions. This study aimed to analyze the coherence changes in the bands of θ, α and β rhythms recorded in the EEG as part of balneotherapy combined with a course of neurorehabilitation prescribed to children with cerebral palsy, and to investigate the relationship of these changes with the indicators of motor activity. The study involved 23 children aged 7 through 15 years, both genders, diagnosed with spastic diplegia; we established coherence coefficients for the intra- and interhemispheric connections of the frontal, central, and parietal regions of the large hemispheres in the context of actions provoking kinesthetic imagery. A significant ($p < 0.05$) growth of the intrahemispheric connections coherence was registered for α rhythms, decline thereof — for θ, β1 rhythms, the fluctuations accompanied by a significant ($p < 0.001$) improvement of the motor functions on the Barthel scale. We identified a relationship between — rhythm coherence in the pair of C4–CP4 leads and the value of the Barthel index ($r = 0.52$; $p = 0.04$). The specifics of changes in the coherence of intrahemispheric connections within the studied rhythms can be used as indicators of neuroplasticity in children with cerebral palsy during rehabilitation, and support development of the new versions of the neurointerfaces classifier programs.

Keywords: children, cerebral palsy, brain–computer interfaces, EEG coherence

Funding: the study was supported by the Russian Science Foundation and the Republic of Crimea grant No. 22-15-20035, https://rscf.ru/en/project/22-15-20035/

Author contribution: Pavlenko VB, Chuyan EN — study planning, data analysis and interpretation, manuscript drafting; Vlasenko SV — study planning, data collection, data analysis and interpretation, manuscript drafting; Pavlenko DV — data collection, data analysis and interpretation, manuscript drafting; Birukova EA, Orekhova LS — data collection, data analysis, manuscript drafting.

Compliance with ethical standards: the study was approved by the V.I. Vernandsky Crimean Federal University ethics committee (Minutes #1 of January 25, 2022). Parents submitted signed informed consent forms allowing their children to participate in the experiment.

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Received: 08.05.2024 **Accepted:** 29.05.2024 **Published online:** 23.06.2024

DOI: 10.24075/brsmu.2024.020

КОГЕРЕНТНОСТЬ ЭЭГ У ДЕТЕЙ С ДЦП НА ФОНЕ РЕАБИЛИТАЦИИ С ПРИМЕНЕНИЕМ НЕЙРОИНТЕРФЕЙСА «МОЗГ – КОМПЬЮТЕР – ЭКЗОСКЕЛЕТ КИСТИ»

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Ранее было показано, что сеансы нейрореабилитации с применением неинвазивного интерфейса «мозг – компьютер – экзоскелет кисти» в сочетании с традиционным курортным лечением снижают у детей с детским церебральным параличом (ДЦП) спастичность мышц кисти и улучшают двигательные навыки. Однако когерентность показателей электроэнцефалограммы (ЭЭГ) при проведении таких сеансов не анализировали. Целью работы было провести анализ изменений когерентности в частотных диапазонах θ-, α- и β-ритмов ЭЭГ при проведении комплексного санаторно-курортного лечения детей с ДЦП с курсом нейрореабилитации, а также оценить взаимосвязь этих изменений с показателями двигательной активности больных. Коэффициенты когерентности внутри- и межполушарных связей фронтальных, центральных и теменных областей больших полушарий определяли во время кинестетического представления движений у 23 детей обоего пола в возрасте 7–15 лет, имевших диагноз «спастическая диплегия». Выявлен статистически значимый ($p < 0,05$) рост когерентности внутриполушарных связей в диапазоне α-ритмов и снижение в диапазонах θ-, β1-ритмов, сопровождающийся статистически значимым ($p < 0,001$) улучшением двигательных функций по шкале Бартел. Обнаружена связь между когерентностью α-ритма в паре отведений C4–CP4 и величиной индекса Бартел ($r = 0,52$; $p = 0,04$). Выявленные особенности изменений когерентности внутриполушарных связей в диапазонах исследованных ритмов ЭЭГ могут быть использованы в качестве индикаторов нейропластичности у детей с ДЦП при проведении реабилитационных мероприятий, а также для разработки новых версий программ-классификаторов для нейроинтерфейсов.

Ключевые слова: дети, церебральный паралич, интерфейс мозг–компьютер, когерентность ЭЭГ

Финансирование: исследование выполнено при поддержке гранта Российского научного фонда и Республики Крым № 22-15-20035, https://rscf.ru/project/22-15-20035/

Вклад авторов: В. Б. Павленко, Е. Н. Чуян — планирование исследований, анализ и интерпретация данных, подготовка черновика рукописи; С. В. Власенко — планирование исследований, сбор данных, анализ и интерпретация данных, подготовка черновика рукописи; Д. В. Павленко — сбор данных, анализ и интерпретация данных, подготовка черновика рукописи; Е. А. Бирюкова, Л. С. Орехова — сбор данных, анализ данных, подготовка черновика рукописи.

Соблюдение этических стандартов: исследование одобрено этическим комитетом ФГАОУ ВО «Крымский федеральный университет им. В. И. Вернадского» (протокол № 1 от 25.01.2022). Получено добровольное информированное согласие от родителей на участие детей в эксперименте.

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Статья получена: 08.05.2024 **Статья принята к печати:** 29.05.2024 **Опубликована онлайн:** 23.06.2024

DOI: 10.24075/vrgmu.2024.020

Cerebral palsy is a group of persistent disorders of movement and posture [1, 2]. However, brain plasticity potentiates treatment of this disease. Actively advanced methods of neurorehabilitation revolve around plasticity, when patients are tasked with multiple repetitions of certain movements or imagining making them. The latter, motor imagery, was noted to even more taxing cognitively than movements per se, thus activating the regenerative processes of the nervous tissue [3]. One of the approaches to neurorehabilitation relies on non-invasive brain-computer interfaces (NIBCI) combined with a hand exoskeleton [4]. In children with cerebral palsy, this approach helped improve motor functions of the upper limbs [5, 6] and motor realization of speech [7].

In the context of rehabilitation of cerebral palsy patients, functional state of the brain and dynamics of neuroplasticity are assessed by analyzing the amplitude of sensorimotor rhythms recorded on an electroencephalogram (EEG) [6, 8, 9]. Another promising approach involves study of the specifics of structural and functional connectivity of neural networks of the brain during treatment. Structural connectivity is defined as a set of neural pathways representing the established anatomical imagery [10]. In patients with cerebral palsy, diffusion tensor magnetic resonance imaging (DTI) revealed abnormalities in the corticospinal tract and the somatosensory thalamocortical projections. Additionally, structural integrity of the commissural and associative pathways of the large hemispheres were found altered [11]. Functional connectivity is defined as a set of interconnections between dynamic activities of neurons in different regions of the brain. The repertoire of functional configurations reflects the underlying anatomical connections. Functional MRI (fMRI) and electrophysiological methods, such as electro- and magnetoencephalography, enable studies in this area [10].

Analysis of the fMRI data allows assessing functional connectivity by calculating correlations between the indicators of activity of brain regions at rest. Children with cerebral palsy were found to be widely susceptible to functional connectivity disorders; moreover, depending on the form of the disease and the choice of the analyzed areas, both reduced and increased values of this indicator (compared to the control group) were registered [11]. Of particular interest is the revealed deterioration of the relationship between frontal and parietal regions, which can promote motor and cognitive disorders in cerebral palsy [12].

However, the results yielded by resting-state fMRI are not a reliable indicator of impaired motor abilities in cerebral palsy [2]. Moreover, fMRI does not offer sufficient temporal resolution, since it measures the metabolic (secondary) response to neuronal activation [13]. EEG recordings have high temporal resolution, thus, they are important for describing spatial and temporal dynamic interdependencies of neural activation and connectivity. The process of description includes an analysis of spectral coherence, which is reflects paired cross-correlation in the time domain [10]. Spectral coherence is usually calculated in the bands of EEG rhythms. Its coefficient varies from zero to one depending on the degree of synchronization of the activity of functionally related regions of the cortex [13, 14].

To date, there is only a few works that analyze EEG coherence in children with cerebral palsy. Initially, the coherence of EEG rhythms in such patients was measured only at rest. In children with diplegia (bilateral brain lesions), interhemispheric coherence in the occipital region had a lower α rhythm value than registered in the control group. In the bands of δ, θ, and β rhythms, coherence was elevated for inter- and intrahemispheric lead pairs, which was interpreted as a reflection of the compensatory processes [15]. In children with hemiparesis, interhemispheric coherence values were high in the bands of δ and θ rhythms, and low — in the bands of α and β rhythms [16]. The values of coherence in the affected hemisphere were lower than in the relatively intact hemisphere, which researchers attributed to the consequences of local disruptions in the neocortex and subcortical white matter.

A later study involved patients with unilateral cerebral palsy and investigated interhemispheric coherence (as registered with EEG) above the central region of the cerebral cortex at rest and when extending wrist [17]. In the α rhythm band, the coherence at rest was reduced, and the higher the degree of motor function disorders, the lower the level thereof. Another recent study, which involved children with unilateral cerebral palsy, looked into the intrahemispheric coherence between central and frontal regions of the neocortex in the μ rhythm band when standing and walking calmly [18]. As opposed to the results of the aforementioned studies [14, 15], in a standing position, compared to the control group, patients had higher coherence in the damaged hemisphere and lower in the intact one.

EEG-based coherence was assessed as indicator of neuroplasticity in the context of rehabilitation of stroke patients. The researchers have underscored the importance of its dynamics as a reflection of the process of recovery of neocortical functions [19]. However, to our knowledge, there was only one study that considered EEG-based coherence data in patients with cerebral palsy during treatment. That work involved children with impaired mobility of the lower extremities, and sought to establish functional connectivity of cortical regions in a series of motor imagery sessions [20]. When patients imagined that they moved a limb up, the classifier program detected changes in the EEG pattern and displayed images on the screen that served as visual feedback. The rehabilitation course pushed up the EEG clustering coefficient in the 8–15 Hz band, which was interpreted as an improvement of the neocortex's network capacities. However, data on the results of motor rehabilitation are not presented in the work.

In this connection, this study aimed to analyze the changes of coherence in the θ-, α- and β rhythm bands as recorded in the EEG in the context of balneotherapy combined with a course of neurorehabilitation (enabled by the NIBCI — hand exoskeleton complex) prescribed to children with cerebral palsy, and to investigate the relationship of these changes with the indicators of motor activity.

METHODS

Sample characteristics

The study was conducted in 2022–2023 at the Health and Rehabilitation Technologies Center of V.I. Vernandsky Crimean Federal University, and at Gelilovich Chaika Health Center for Children and Children with Parents. The participants were 30 children aged 6-15 years, undergoing treatment at the health center combined with a course of neurorehabilitation employing NIBCI. The inclusion criteria were: diagnosed cerebral palsy (as per ICD 10); spastic diplegia with motor functions development at levels I-III as per the Gross Motor Function Classification System for Cerebral Palsy (GMFCS). The exclusion criteria were: motor functions development level above III as per GMFCS; aphasic disorders; drug-induced uncorrectable epilepsy; visual impairments disallowing distinguishing instructions on the screen; moderate, severe and profound mental retardation (F71–73 as per ICD 10).

Ultimately, we excluded the children whose EEG contained many artifacts due to excessive motor activity, and had the

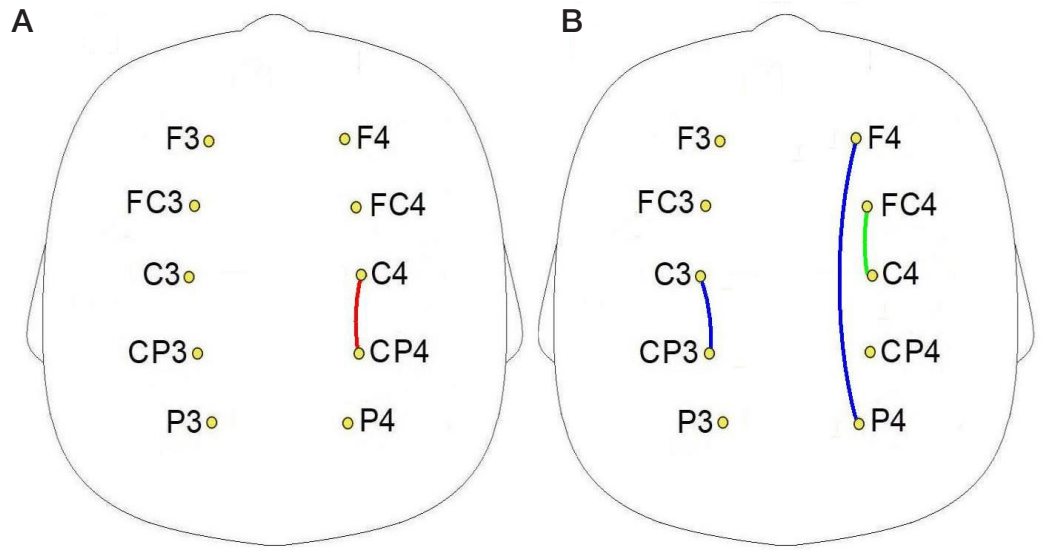

Fig. 1. Changes in coherence associated with imagining movements of left (**A**) and right (**B**) hand. The leads that have shown significant growth of coherence values in the — rhythm band are highlighted red, those with similar growth in the bands of — and —1 rhythms are colored green and blue, respectively.

sample of 23 children (7 girls, 16 boys) aged 7–15 years, with 15 of them suffering mostly right hand movement disorders, 6 — movement disorders of the left hand, and two with such disorders equally pronounced for both hands.

Assessment of indicators of motor functions

To assess the motor functions of the patients, we used the Barthel index, which allows measuring performance in activities of daily living based in a questionnaire [21]. The higher the performance indicators, the higher the index values (range from 0 to 100 points). The Barthel index is applicable to both adult and infant cerebral palsy patients [22]. Children that participated in the study took the Barthel index test on the second day after admission to the health center. They have filled the questionnaire uncovering their motor functions performance the second time once the treatment course was over, on the 14th or 15th day of their stay in the health center.

Rehabilitation procedures

In the health center, the treatment included daily aero- and heliotherapy, hydrokinesiotherapy in thermal mineral water (duration — 10–15 minutes), physical therapy, massage of paretic muscles, electrical stimulation of muscles antagonistic to paretic. Every other day the patients underwent peloid treatment. Botulinum therapy was not used in the health center. Neurorehabilitation employed the brain – computer – hand exoskeleton complex, with the letter being Exokist-2 (Exoplast, Russia; Reg. Cert. RZN 2018/7681). The non-invasive brain-computer interface enabled identification of EEG-registered patterns emerging as part of imagined extension of the hand. A classifier software was used for the purpose; it generates visual feedback and hand exoskeleton movement commands.

The EEG recordings were monopolar, taken with the help of the BMM-52 electroencephalograph (NVX-52 amplifier, Moscow, Zelenograd, MKS) with 32 leads. The electrodes were arranged under the incomplete 10-10 system. An averaged electrode was used as a reference. During recording, the cutoff frequencies for the high and low pass filters were 4 and 30 Hz, respectively. The digitization frequency of EEG signals was 500 Hz.

During the neurorehabilitation, patients sat in a chair in front of a monitor that showed them visual instructions. On their hands, they were wearing mittens of the exoskeleton. In the center of the screen there was a rounded white mark, upon which the patients could fix their gaze; around the mark, there were three arrows, which gave instructions by changing color. The commands were as follows: relax, imagine extension of the left or right hand. To create the specific kinesthetic image, children were given the following instructions: "Imagine that you have a small ball in your hand. You open the hand and drop it. Feel this movement." When the patient successfully executed the command and the classifier program detected certain EEG patterns, the gaze-fixing mark turned green, and the exoskeleton performed the respective movement, i.e., the patient's hand extended passively. Thus was the combined visual and kinesthetic feedback signal generated. The neurorehabilitation course began on the third day of stay at the health center. The patients underwent 10 sessions (daily, except for Sunday) as follows: three takes per session, each take 8 minutes long, at least 5 minutes of rest between the takes. Within a session, the movement imagery task for each hand was repeated 24 times. During the first session, the share of correct responses that triggered the exoskeleton (i.e., were interpreted by the classifier software as correct) ranged from 0.18–0.66, and by the end of the course these figures were 0.30–0.84. Other details of the rehabilitation technique were described earlier [7].

To assess the rearrangement of the cortical regions' interconnections following neurorehabilitation, we studied the EEG to analyze coherence in θ, α, β1 and β2 rhythms in the bands 4–8, 8–13, 14–20 and 20-30 Hz, respectively. Sections of EEG recordings with the amplitude exceeding 250 μV, and those with a large number of artifacts, were excluded. We analyzed at least 10 artifact-free epochs, with the minimum overall duration of EEG being 50 seconds. Since movements proper and imagery thereof rely in the interactions within the frontoparietal network [3], the intrahemispheric coherence of biopotentials was calculated for ten pairs of leads of the frontal, central and parietal regions of the left (F3–C3, FS3–C3, C3–CP3, C3–P3, F3–P3) and right (F4–C4, FS4–C4, C4–CP4, C4–P4, F4–P4) hemispheres; as for the interhemispheric coherence, it was calculated for for five pairs (F3–F4, FS3–FS4, C3–C4, CP3–CP4, P3–P4). For the purpose, we used the Neurosoft software (Russia), which is part of the Neuron-Spektr-5 electroencephalograph kit.

The values of the coherence coefficients were transformed using the natural logarithm function. Numerical values beyond 3 σ were discarded. As a result, distribution of the coherence values was close to normal, which allowed analyzing them using the techniques of parametric statistics.

Statistical data processing

We used STATISTICA v.12 software (StatSoft Inc.; USA) to perform statistical analysis of the data. For each of the rhythms, the coherence values derived from the EEG were subjected to ANOVA with repeated measurements, TRAINING (first and tenth sessions) and PAIRS (10 or 5 pairs of leads) factors. The linear contrast method was used to assess changes in coherence in each of the lead pairs. Since baseline values of the correlation coefficients, which were not subjected to logarithmization, may also be of interest, and their distribution, as shown by the Shapiro-Wilk test, was abnormal, the specific indicators are given in the text as median and interquartile range Me [Q_1; Q_3]. The distribution of the Barthel index was also abnormal, therefore, we applied a similar approach to the presentation of the respective statistical data. To assess the differences in the Barthel index values before and after complex treatment, we used the Wilcoxon test; for correlations, the test of choice was Spearman's. The differences and correlation coefficients were considered significant at $p < 0.05$.

RESULTS

Motor functions indicators before and after the rehabilitation

Before the treatment combined with neurorehabilitation, the mean Barthel index value was was 70 [60; 85] points, after — 77.5 [74.0; 95.0] points (the differences are significant at $p < 0.001$). Thus, the children's capacity for independent movement and self-service have increased significantly.

Coherence dynamics following the rehabilitation

Applied to the changes of intrahemispheric coherence associated with the left hand movement imagining, ANOVA revealed an effect of the TRAINING factor in the α rhythm band: $F_{1, 14} = 7.21$; $p = 0.02$. Compared to the first session, the level of coherence registered during the last session has increased, especially in the right hemisphere, which was contralateral to the imagined movement. The linear contrast method revealed significant differences in the C4–CP4 pair, where the value has grown significantly at $p = 0.004$ (Fig. 1A; red line). The value of the coherence coefficient in this pair increased from 0.38 [0.36; 0.45] to 0.43 [0.39; 0.47].

As detected in the θ rhythm's band, imagining movements of the right hand was affected by the interaction of the TRAINING and PAIR factors: $F_{9, 90} = 2.37$; $p = 0.02$ in the range of the EEG rhythm. In this band, the level of coherence decreased in the pairs of the right, ipsilateral hemisphere. The linear contrast method confirmed the differences in the C4–FC4 pair, where the decrease in coherence was significant at $p = 0.01$ (Fig. 1B; green line). In this pair, the values of the coherence coefficient decreased from 0.41 [0.36; 0.42] to 0.36 [0.33; 0.38]. Also, TRAINING factor was revealed to affect imagining of the right hand movements in the β1 rhythm's band: $F_{1, 7} = 24{,}091$ at $p = 0.002$. In this band, the level of coherence was also decreasing. The linear contrast method confirmed significant differences in the contralateral hemisphere's pair C3–CP3 at $p = 0.03$, and F4–P4 of the ipsilateral hemisphere at $p = 0.01$ (Fig. 1B; blue lines). In these pairs, the values of the coherence coefficients decreased from 0.39 [0.37; 0.42] to 0.37 [0.34; 0.41] and from 0.40 [0.37; 0.43] to 0.38 [0.36; 0.40], respectively.

In the β2 rhythm band, the analysis of dynamics of the intrahemispheric coherence revealed no significant effects of the TRAINING factor nor its interaction with the PAIR factor.

The analysis of the dynamics of interhemispheric coherence in the bands of the θ, α, β1 and β2 EEG rhythms as associated with imagining movements of the left and right hands, as well as analysis of the intra- and interhemispheric coherence in a relaxed state, revealed no effect of the TRAINING factor and its interaction with the PAIR factor.

The relationship between coherence and motor functions indicators

To assess the relationship between coherence and motor functions, we took the values registered with the help of EEG during the final session of the neurorehabilitation course, and incorporated the Barthel index values that reflect the motor functions of children at the end of the course. Only those pairs of leads that yielded significant changes were selected for the analysis (shown above). We have established a relationship between coherence of the α rhythm in the C4–CP4 pair and the value of the Barthel index ($r = 0.52$; $p = 0.04$): the higher the said coherence in the given pair of right hemisphere leads that is associated with imagining movements of the left hand, the better the patient's capacity to move and self-care. There were no significant correlations identified for other EEG-recorded bands.

DISCUSSION

After the course of neurorehabilitation, we identified significant changes in the intrahemispheric coherence of the θ, α and β1 rhythms in individual links of the frontoparietal circuits, which were accompanied by improvements in the patients' motor functions. During the final session, imagining movements of the left hand was associated with a level of the α rhythm coherence significantly higher than registered at the beginning of the rehabilitation. What can reinforcement of interconnections in the band of this rhythm signify? Some researchers suggest that synchronized EEG oscillations enable rapid and selective interaction between regions of the brain [23-25]. In their opinion, it is the α rhythm that supports large-scale synchronization, since α waves are ubiquitous in the cerebral cortex, and they reflect the alternation of periods of inhibition and excitation. Synchronization of such periods in different regions of the brain enhances their interactions, which, inter alia, is a precondition for functioning of the frontoparietal network. It is important to note that the activity in the α rhythm band that we recorded in the central and adjacent leads corresponds to the low-frequency component of the μ rhythm generated in these areas of the neocortex. The dynamics of the μ rhythm reflects the processes of realization or imagining of movements; it is a recommended electrophysiological marker of plasticity of the cortical sensorimotor system [8]. In our study, contrasting revealed significance of growth of the α rhythm coherence in the C4–CP4 pair (Fig. 1A) located above the postcentral sensorimotor cortex and the inferior parietal lobule [26]. In healthy subjects, these regions were activated during movement imagery tasks and were loci of neural networks the coherence of which correlated with the results of such tasks [27].

It is necessary to add that kinesthetic imagery of movements requires actualization of information in the child's memory. Previous studies have reported that phase interactions in the α rhythm band detected in the central and parietal cortex underlie the downward modulation of local oscillation amplitudes in sensory regions, supporting the functions of attention and memory [23]. Thus, the growth of EEG coherence in the α rhythm band improves interaction of brain regions that actualize movement images in memory and keep focus on them. As a result, kinesthetic imagery becomes more successful, as shown by the increased share of correct responses registered by the classifier software. Imagining movements, people activate same neural structures that participate in the actual realization thereof [28, 29], thus, successful kinesthetic imagery enhances the processes of neuroplasticity of cortical and subcortical structures. Consequently, coordination of real limb movements improves, which significantly boosts the Barthel index value.

We have also revealed a decrease in the level of coherence of the θ and β1 rhythms associated with the right hand movement imagining (final session compared to the first one). Previously, children with cerebral palsy have shown elevated coherence of the θ rhythm [15, 16], which was interpreted as an action by the compensatory mechanism triggered by a disruption of functional connectivity in the α rhythm band. Thus, a decrease in the coherence of the θ rhythm can be regarded as a manifestation of certain positive changes in the functioning of the neocortex.

The decrease in the coherence of the β1 rhythm may be caused by the specifics of the task. The band of this rhythm corresponds to the high-frequency component of the μ rhythm. Its dynamics reflects the activity of the primary motor cortex in the process of actual realization of movements [8]. At our sessions, children only needed to imagine, but not realize a movement, which involved mainly the sensorimotor areas of the neocortex. It should be added that the coherence of the β rhythm increases when the movements performed require a higher level of control [30]. Obviously, the task that involved kinesthetic imagery did not call for this sort of control. It can be assumed that during the first session, it was harder for the participants to imagine movements, which necessitated additional activation of the primary motor area. Further on, imagining required less effort, and the activation of the cortex was more local.

In conclusion, we believe it is necessary to note the following. As previously shown, dysfunction of the frontoparietal network in children with cerebral palsy promotes motor and cognitive impairments [12]. The changes in the coherence of EEG-registered rhythms in the links of this network that we have identified can be regarded as indicators of positive changes in the functional state of the neocortex of patients.

The robustness of this study was limited by the lack of a control group that would have included children with cerebral palsy; this group could have had the EEG taken under similar conditions (kinesthetic imagery), but would not have had the neurorehabilitation course. The time of stay at the health center (21 days) disallowed designing this effort as a crossover study, which would be the most appropriate and ethical approach in this case.

CONCLUSIONS

Combination of balneotherapy and a course enabled by the brain-computer-hand exoskeleton interface yielded a significant increase of the Barthel index in children with cerebral palsy ($p < 0.001$), indicating an improvement in mobility and self-service capabilities. We have identified a statistically significant ($p < 0.05$) change in the coherence of intrahemispheric connections in the bands of θ, α and β1 rhythms. The basis of such changes may be reinforcement of the plasticity processes in the neural networks of the neocortex, which control planning, imagining, and execution of complex movements. The data from this study can be used to assess the functional state of brain of the patients in the context of rehabilitation and in development of the new methods for correcting motor function disruptions in children with cerebral palsy. It seems feasible to further analyze EEG of patients in a crossover study in the context of a course of neurorehabilitation at the Health and Rehabilitation Technologies Center of V.I. Vernandsky Crimean Federal University.

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ORPHAN DISEASES IN THE REPUBLIC OF NORTH OSSETIA–ALANIA: STRUCTURE, POPULATION GENETIC FEATURES, ISSUES AND PROSPECTS

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Currently, there are more than 8000–10000 rare disease (RDs), among which 75–80% are hereditary. In the Russian Federation (RF), patients are provided medical care in accordance with two lists: 17 chronic progressive and life-threatening diseases (RLTDs) and 14 high-cost nosologies (HCNs). The study was aimed to assess the range, prevalence, and genetic epidemiological characteristics of the RDs from the lists of RLTDs and HCNs in the Republic of North Ossetia–Alania and RF in general. We determined the number of patients from the RLTD (a total of 18,744 people in the RF, among them 8713 children; 129 and 42 people, respectively, in the Republic of North Ossetia–Alania) and HCN (28727 people/13454 children in the RF; 554 and 64 in the Republic of North Ossetia–Alania) lists and calculated the prevalence per 100,000 population. The global prevalence of RDs was estimated using the Orphanet database. The average prevalence of RLTDs in the whole population of the RF was 11.51 cases and that among children was 25.08. Similar data were obtained for the Republic of North Ossetia–Alania (19.38 and 29.44, respectively). It was found that idiopathic thrombocytopenic purpura, disorder of the complement system, maple syrup urine disease, porphyria were more common in the Republic of North Ossetia–Alania than in the RF in general, while galactosemia was less common. The analysis of disorders from the RLTD list has shown lower prevalence of hemophilia and pituitary dwarfism in the Republic of North Ossetia–Alania compared to the RF and Orphanet, along with the higher prevalence of type VI mucopolysaccharidosis, hemolytic uremic syndrome, and systemic juvenile rheumatoid arthritis. In the Republic of North Ossetia–Alania, the features of the range of genetic variation in the genes *PAH* (phenylketonuria) and *CFTR* (cystic fibrosis) have been identified. Thus, assessment of the RD prevalence in the regions is important and essential for raising awareness of medical personnel, as well as for expansion and improvement of medical care provision to patients with RLTDs and HCNs.

Keywords: rare (orphan) diseases, chronic progressive and life-threatening diseases, high-cost nosologies, prevalence, Republic of North Ossetia–Alania, Russian Federation

Funding: the study was supported as part of the State Assignment of the Research Centre for Medical Genetics and the Ministry of Health of the Republic of North Ossetia–Alania.

Author contribution: Zinchenko RA, Tebieva IS, Gabisova YuV, Khokhova AV — patient examination, making the diagnosis, obtaining the informed consent, and biomaterial collection; Shukan EYu — acquisition of data on the number of patients; Zinchenko RA, Tebieva IS, Kutsev SI — study planning, statistical analysis, manuscript writing; Marakhonov AV — analysis of molecular genetic tests; Tebieva IS, Marakhonov AV, Zinchenko RA — editing; Zinchenko RA, Kutsev SI — general management, editing.

Compliance with ethical standards: the study was approved by the Ethics Committee of the Research Centre for Medical Genetics (protocol No. 5 dated 20 December 2010), it was compliant with the Good Clinical Practice and evidence-based medicine standards. All patients submitted the informed consent to participation in the study.

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Received: 08.05.2024 **Accepted:** 02.06.2024 **Published online:** 26.06.2024

DOI: 10.24075/brsmu.2024.025

ОРФАННАЯ ПАТОЛОГИЯ В РЕСПУБЛИКЕ СЕВЕРНАЯ ОСЕТИЯ – АЛАНИЯ: СТРУКТУРА, ПОПУЛЯЦИОННО-ГЕНЕТИЧЕСКИЕ ОСОБЕННОСТИ, ПРОБЛЕМЫ И ПЕРСПЕКТИВЫ

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В настоящее время известно более 8000–10000 редких болезней (РБ), 75–80% которых наследственные. В Российской Федерации (РФ) пациентам оказывают медицинскую помощь по двум перечням: 17 хронических прогрессирующих и жизнеугрожающих заболеваний (РЖЗ) и 14 высокозатратных нозологий (ВЗН). Целью исследования было оценить спектр, распространенность и генетико-эпидемиологические характеристики РБ из перечней РЖЗ и ВЗН в Республике Северная Осетия — Алания (РСОА) и в РФ в целом. Определено число пациентов из перечней РЖЗ (по РФ всего 18 744 человек, в т. ч. 8713 детей; по РСОА — 129 и 42 соответственно) и ВЗН (по РФ — 28727 /13454 детей; по РСОА — 554 и 64) и рассчитана распространенность на 100 000 человек. Распространенность РБ в мире оценивали по базе Orphanet. Средняя распространенность заболеваний их группы РЖЗ среди всего населения РФ составила 11,51 случаев и среди детей — 25,08. Схожие данные получены для РСОА (19,38 и 29,44 соответственно). Выявлено, что идиопатическая тромбоцитопеническая пурпура, дефект в системе комплемента, болезнь «кленового сиропа», порфирия в РСОА встречаются чаще, чем в среднем по РФ, а галактоземия — реже. Анализ заболеваний из перечня ВЗН показал более низкую по сравнению с РФ и Orphanet распространенность гемофилии и гипофизарного нанизма в РСОА, и более высокую для мукополисахаридоза VI типа, гемолитико-уремического синдрома и юношеского артрита с системным началом. В РСОА выявлены особенности спектра генетических вариантов в генах *PAH* (фенилкетонурия) и *CFTR* (муковисцидоз). Таким образом, изучение распространенности РБ в регионах является важным и необходимым условием для повышения настороженности медицинского персонала, расширения и совершенствования оказания медицинской помощи пациентам с РЖЗ и ВЗН.

Ключевые слова: редкие (орфанные) заболевания, хронические прогрессирующие и жизнеугрожающие заболевания, высокозатратные нозологии, распространенность, Республика Северная Осетия – Алания, Российская Федерация

Финансирование: исследование выполнено при финансовой поддержке Государственного задания ФГБНУ «МГНЦ» Минобр России и Минздрава РСОА.

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Соблюдение этических стандартов: исследование одобрено этическим комитетом ФГБНУ «Медико-генетический научный центр имени академика Н. П. Бочкова» (протокол № 5 от 20 декабря 2010 г.), соответствует стандартам добросовестной клинической практики и доказательной медицины. Все пациенты подписали добровольное информированное согласие на участие в его проведении.

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Статья получена: 08.05.2024 **Статья принята к печати:** 02.06.2024 **Опубликована онлайн:** 26.06.2024

DOI: 10.24075/vrgmu.2024.025

The disease prevalence in the country is the main criterion determining the fact that the disease entity belongs to rare (orphan) diseases (RDs). This indicator varies between 1:1250 and 1:10,000 or more in various countries of the world [1–6]. Among 8000–10,000 currently known diseases, treatment options have been developed for 5% of diseases only, and the treatment effectiveness is directly related to the timing of starting therapy [2, 7, 8].

Efforts are made in different countries of the world to optimize the diagnosis of RDs with the help of various state projects and programs. In 2008, the US National Human Genome Research Institute created the conditions for comprehensive examination of 964 patients with undefined diagnoses for the first time. A network that included a coordination center and seven clinical centers, exome and genome sequencing centers, metabolomics and modeling center, biobank was created in order to implement the project, and the program for undiagnosed disorders was developed by the National Institutes of Health. As a result, the vast majority of patients were diagnosed, numerous new associations between genes and diseases were identified [9–12].

Such a comprehensive approach to the diagnosis of RDs turned out to be successful in Spain [13], Japan [14], Korea [15], and China [16]. High effectiveness of whole-genome sequencing in the diagnosis of RDs was demonstrated during implementation of the 100,000 Genomes Project in the UK [17–21].

Of particular relevance are various genetic tests performed in the format of express diagnosis to detect RDs in the critically ill newborns staying in the intensive care units: whole-genome/exome sequencing. According to the literature data, 25–50% of such tests are informative in terms of RD diagnosis [8].

In recent years, much has been done in our country to optimize the diagnosis of RDs. Such institutions, as the Research Centre for Medical Genetics, Dmitry Rogachev National Medical Research Center of Pediatric Hematology, Oncology and Immunology, Endocrinology Research Center, and some other federal medical centers have the option to conduct expensive genetic tests at the expense of budgetary funds, but the number of such tests is limited. The work of several Orphan centers, in which a multidisciplinary approach to the diagnosis of RDs is used, has been also organized.

Legislative consolidation of the RD treatment was achieved in the Russian Federation (RF). The list of 283 diseases belonging to the groups of RDs was published on the website of the Ministry of Health of the Russian Federation [22], in which 17 disease entities are in the list of chronic progressive and life-threatening diseases (RLTDs) [23] and 14 are in the list of high-cost nosologies (HCNs).

In January 2021, the Circle of Good foundation for support of children with severe life-threatening and chronic diseases, including RDs, was established by the decree of the Russian President to provide such children with pharmaceuticals and medical devices, in some cases with those not registered in the RF, as well as with the rehabilitation equipment not included in the federal list of the rehabilitation measures, rehabilitation equipment and services provided to disabled individuals. The Foundation created an information resource being part of the unified state system in healthcare together with the Ministry of Health of the RF. The information resource contains information about the children, requests from parents (children's legal representatives), requests for medical care provision, information about procurement of pharmaceuticals, pharmaceutical product balances, protocols of the expert and guardianship councils, lists of pharmaceuticals, diseases, and categories of children approved by the Foundation councils.

The study was aimed to assess the range, prevalence, and genetic epidemiological characteristics of the orphan diseases from the lists of RLTDs and HCNs among children and adults in the Republic of North Ossetia–Alania and RF in general, as well as to compare the data obtained with the data provided in the world's literature, determine the issues and prospects of the diagnosis and treatment of this group of diseases.

METHODS

The research object was represented by the pediatric (162,452 people) and adult (680,748 people) populations of the Republic of North Ossetia–Alania and patiens with RDs according to the end of the year 2022: children from birth to 18 years of age and adults.

The population and number of patients in the country were determined based on the data provided by Rosstat and the Ministry of Health of the RF as of 31 December 2022. The population of the RF was 146,447,424 adults and 27,300,000 children.

The Annual Bulletin of the Expert Council on RDs of the State Duma Committee on Health Protection (the so-called White Book) is the only source of information providing the summary of the main analytical materials on the prevalence of RDs from the list of RLTDs in our country. The Bulletin provides information about the number of patients having diseases from the list of RLTDs in the RF. The regional Ministries of Health of the constituent entities of the RF have been providing information about the number of patients annually since 2018 [24] in accordance with the Government Decree No. 403 dated 26 April 2012. The Department of Medical Care for Children, Obstetrics and General Health Services of the Ministry of Health of the Russian Federation is the registry holder. The State Duma Committee on Health Protection obtains the regional data through parliamentary inquiries annually.

The prevalence of certain orphan diseases, diagnosed among pediatric and adult patients in the RF, in the Republic of North Ossetia–Alania was calculated based on the data from the White Book for the year 2022.

The data of the regional registries provided to the Ministry of Health of the RF were used to assess statistical data on the prevalence of orphan diseases from the list of HCNs.

The following formula was used to calculate the prevalence (P) of the disease entities:

$$P = \frac{\text{Number of patients in the RF or Republic of North Ossetia–Alania}}{\text{Population of the RF or Republic of North Ossetia–Alania}} \times 100{,}000$$

The global prevalence of orphan diseases was estimated using the database of the Orphanet international portal on RDs created in 1997 in France to collect scarce data on orphan diseases, improve the diagnosis of such diseases, and provide treatment to patients with RDs. Today, Orphanet is multilingual portal uniting 41 countries in Europe and around the world [25].

We determined the number of adults and children suffering with the diseases from the lists of RLTDs (a total of 18,744 patients in the RF, among them 8713 children; 129 and 42 patients, respectively, in the Republic of North Ossetia–Alania) and HCNs (a total of 28,727 patients in the RF, among them 13,454 children; 554 and 64 patients, respectively, in the Republic of North Ossetia–Alania). A retrospective analysis of the medical records of patients with orphan diseases registered with the medical genetic center at the Republican Children's Clinical Hospital of the Republic of North Ossetia–Alania for the

17-year period was carried out. The whole range of essential laboratory and instrumental tests was provided to patients with hereditary diseases, along with confirmatory diagnostic testing at various branches of the Research Centre for Medical Genetics. Some patients passed confirmatory diagnostic testing in the Genomed LLC on their own.

RESULTS

Analysis of data on the diseases from the list of chronic progressive and life-threatening diseases

Statistical data on the global prevalence of diseases from the list of RLTDs contained in the Orphanet database, the data on the number of patients and the prevalence of these diseases in the pediatric and adult populations of the RF and Republic of North Ossetia–Alania are provided in Table 1.

The analysis considering the molecular genetic diagnosis data was performed for all hereditary RDs. We determined the prevalence of the aromatic amino acid metabolism disorders (classic phenylketonuria (PKU), other hyperphenylalaninemias), which was 1–9/100,000 according to Orphanet, 3.87 in the adult population and 14.2 in the pediatric population of the RF (this corresponded to 1:7142). The prevalence of these disorders in the Republic of North Ossetia–Alania was as follows: 10.28/100,000 in the whole population and 9.23/100,000 among children. The diagnosis of phenylketonuria was confirmed by molecular genetic testing. Seven patients having classic phenylketonuria receive diet therapy in accordance with the federal clinical guidelines [26]. Other patients do not need any therapeutic diet. Predominance of two frequent genetic variants of the *PAH* gene (disease-associated alleles P281L and P211T) was the feature; in ethnic Ossetians, these two variants accounted for 60% of all identified mutations (P281L — 42.11%, P211T — 18.42%). The well-known genetic variants were identified in children with PKU (R408W, R261Q, F33S, M1R (c.2T>G), c.529G>A, c.1222C>T, E390G, c.47_48del (p.Ser16*), c.631C>A (p.Pro211Thr), V230I, c.529G>A (p.Val177Met)) [27].

According to Orphanet, the prevalence of homocystinuria is 1–9/100,000, no data on the prevalence of glutaric aciduria are provided. The prevalence of homocystinuria in the adult/pediatric population of the RF is 0.03/0.10, while the prevalence of glutaric aciduria is 0.05/0.22. One adult patient with homocystinuria has been identified in the Republic of North Ossetia–Alania, the disease prevalence is 0.15 per 100,000 population. The patient was first diagnosed at the age of 24 years. The search for pathogenic mutations was conducted by exome sequencing. The nucleotide sequence variant NM_000071.2(*CBS*):c.209+1G>A in the heterozygous state was identified, that had been earlier reported as pathogenic (HGMD: CS971640); the variant affected an invariant dinucleotide of the donor splice site in the intron 3 of the *CBS* gene encoding cystathionine β-synthase. Furthermore, the nucleotide sequence variant NM_000071.2:c.239T>C in the exon 4 of CBS in the heterozygous state was identified, that had not been earlier reported as pathogenic; the variant led to the nonsynonymous substitution p.(Ile80Thr) of the highly conservative position of this enzyme. No patients with glutaric aciduria were found in the region.

The prevalence of galactosemia in the pediatric population of the Republic of North Ossetia–Alania was 0.88 per 100,000. According to Orphanet, the prevalence of this carbohydrate metabolism disorder is 1–5 cases per 100,000 population, while in the RF the prevalence is 0.34 and 1.70 per 100,000 in the adult and pediatric populations, respectively. The DNA diagnosis revealed Duarte galactosemia resulting from the homozygous N314D mutation in the *GALT* gene (N314D/N314D genotype) in two cases and type 1 galactosemia (GALT enzyme deficiency) with the *GALT* Met142Lys/Lys285Aspn(p.K285N) mutations in one case; type 2 galactosemia (GALK1 enzyme deficiency) resulting from the homozygous genetic variant Q382X in the exon 8 of *GALK1* (Q382X/Q382X genotype) was diagnosed in two cases in sibling patients [28].

Tyrosinemia is a rare disease with the prevalence lower than 1:100,000 according to Orphanet. According to our data, the prevalence in the adult and pediatric populations of the RF is 0.04 and 0.21 per 100,000, respectively. No cases of this disease were reported in the Republic of North Ossetia–Alania during the studied period.

Furthermore, no patients having diseases associated with the fatty acid metabolism disorders were reported in the Republic of North Ossetia–Alania. The prevalence in the adult and pediatric populations of the RF was 0.08 and 0.28, respectively.

According to Orphanet, the prevalence of maple syrup urine disease and other disorders of the branched chain amino acid metabolism (isovaleric academia, methylmalonic academia, propionic academia) can be 1–9/100,000, however, the disease in far less common in the RF: 0.02–0.18/100,000. The prevalence of this disease in the Republic of North Ossetia–Alania was 0.15/100,000. Only one patient diagnosed with leucinosis (maple syrup urine disease) at the age of 2 months was identified for the entire group of disorders. When conducting molecular genetic testing of the target regions of 266 genes, the c.1196>T p.S399F pathogenic nucleotide variant in the homozygous state, that had not been reported earlier, was identified in the DBT gene. The patient is fed with the leucine-, isoleucine-, valine-free formula (Nutrigen 14-leu-val-ile). No patients having other diseases of this group were identified.

The prevalence of sphingolipidoses (Fabry disease, Niemann–Pick disease, and acute intermittent (hepatic) porphyria) in the adult and pediatric populations of the RF was 0.09/0.02, respectively. These disorders were not diagnosed in the region. According to Orphanet, the prevalence of these disorders is 1–5 and 1–9 per 100,000, respectively.

The prevalence of copper metabolism disorder (Wilson's disease) in the adult/pediatric populations of the RF was 0.68/0.49. The disease was not found in the pediatric population of the Republic of North Ossetia–Alania, while the prevalence among adults was 1.03/100,000, which was slightly higher compared to the average value for the country.

The prevalence of osteogenesis imperfecta in the adult and pediatric populations of the RF was 0.56/1.50, respectively, while in the Republic of North Ossetia–Alania it was 1.02/3.08, which was significantly higher compared to the values for the country in general. This feature resulted from the founder effect and the fact that there were 6 Kumyk patients, who were blood relatives in four generations. The NM_000088.3(COL1A1):c.1243C>T, p.(Arg415Ter) mutation in the heterozygous state (HGND:CM960321) was identified in all of them. The variant is associated with type IV osteogenesis imperfecta with the autosomal dominant pattern of inheritance. The patients' clinical manifestations vary between joint hypermobility, blue sclera, connective tissue dysplasia and multiple fractures. Four patients with severe disease were prescribed bisphosphonate therapy.

Five disease entities (paroxysmal nocturnal hemoglobinuria, idiopathic thrombocytopenic purpura, disorder of the

Table 1. Number of patients, data on the prevalence (per 100,000) of orphan diseases from the list of RLTDs in the world, RF, and Republic of North Ossetia–Alania

№	Disease	ICD-10 code	Prevalence per 100,000 according to Orphanet	Russian Federation				Republic of North Ossetia–Alania				*p*-value Russian Federation and Republic of North Ossetia–Alania	
				total 146,447,424/total 27,300,000				total 680,748/ children 162,452					
				Patients		Prevalence per 100,000		Patients		Prevalence per 100,000			
				Total	Children only*	Total	Children only*	Total	Children only*	Total	Children only*	Total	Children only*
1	Paroxysmal nocturnal hemoglobinuria (Marchiafava–Micheli syndrome)	D59.5	1.9	485	19	0.33	0.07	4	0	0.59	0.00	0.247	0.737
2	Idiopathic thrombocytopenic purpura (Evans syndrome)	D69,3	1.9	5638	1224	3.85	4.48	70	14	10.28	9.23	1.9E-17	0.013
3	Disorder of the complement system	D84,1	>1	631	93	0.43	0.34	6	4	0.88	2.64	0.075	5.7E-6
4	Central precocious puberty	E22,8	–	1990	1892	13.59	6.93	10	10	1.47	6.59	0.806	0.708
5	Aromatic amino acid metabolism disorders (classic PKU, other HPA)	E70,0	1.9	5666	3894	3.87	14.26	21	15	3.08	9.89	0.299	0.090
		E70,1											
6	Tyrosinemia	E70,2	>1	64	57	0.04	0.21	0	0	0.00	0.00	0.585	0.560
7	Maple syrup urine disease	E71,0	1.9	28	27	0.02	0.10	1	1	0.15	0.66	0.018	0.045
8	Other branched chain amino acid metabolism disorders (isovaleric academia,	E71,1	1.9	50	48	0.03	0.18	0	0	0.00	0.00	0.630	0.593
	methylmalonic academia		>1										
	propionic academia)		1.9										
9	Fatty acid metabolism disorders	E71,3	–	111	76	0.08	0.28	0	0	0.00	0.00	0.473	0.501
10	Homocystinuria	E72,1	1.9	44	28	0.03	0.10	1	0	0.15	0.00	0.082	0.683
11	Glutaric aciduria	E72,3	–	61	54	0.05	0.22	0	0	0.00	0.00	0.594	0.571
12	Galactosemia	E74,2	1.5	503	468	0.34	1.70	6	5	0.88	3.30	0.017	0.187
13	Other sphingolipidoses: Fabry disease,	E75,2	1.5	251	69	0.17	0.25	0	0	0.00	0.00	0.280	0.522
	Niemann–Pick disease		–										
14	Acute intermittent (hepatic) porphyria	E80,2	1.9	135	5	0.09	0.02	3	0	0.44	0.00	3.1E-3	0.863
15	Copper metabolism disorder (Wilson's disease)	E83,0	–	998	133	0.68	0.49	7	0	1.03	0.00	0.275	0.374
16	Osteogenesis imperfecta	Q78,0	1.5	824	410	0.56	1.50	7	5	1.02	3.08	0.107	0.103
17	Primary idiopathic pulmonary hypertension	I27,0	1.9	1265	216	0.95	0.94	6	1	0.88	0.66	0.961	0.802
Total				18744	8713	11.51	25.08	129	42	19.38	29.44	0	0.172

Note: * — children under the age of 18 years.

complement system, central precocious puberty, and primary idiopathic pulmonary hypertension) are not considered to be genetically determined. According to Orphanet, the prevalence of paroxysmal nocturnal hemoglobinuria is 1–9/100,000, while in the RF it is 0.33 and 0.07 in the whole population and among children, respectively. Four adult patients were identified in the Republic of North Ossetia–Alania: the prevalence was 0.59 and 0, respectively. A total of 70 patients suffering from idiopathic thrombocytopenic purpura were identified in the Republic of North Ossetia–Alania, among them 14 were children; the prevalence was 10.28 and 9.23, respectively, while the in the RF it was 3.85 and 4.48/100,000. According to Orphanet, the prevalence of this disease is 1–9/100,000. A total of 6 patients diagnosed with the disorder of the complement system were identified in the Republic of North Ossetia–Alania, among them four children. The prevalence was 0.88/100,000 in the whole population and 2.64/100,000 among children. Furthermore, the disease prevalence in the RF is 0.43 and 0.4, which is consistent with the global data provided in the Orphanet >1 database. Central precocious puberty was revealed in the RF; the prevalence was 13.59/100,000 in the whole population and 6.93/100,000 among children. A total of 10 pediatric patients were identified in the Republic of North Ossetia–Alania, the prevalence was 1.47 and 6.59, respectively. No Orphanet data

were provided. A total of 6 patients with primary idiopathic pulmonary hypertension were identified in the Republic of North Ossetia–Alania, among them one child; the prevalence was 0.88 and 0.66, respectively. The results obtained are consistent with the data for the RF (0.95 and 0.94 per 100,000 of surveyed individuals, respectively) and the Orphanet data (1–9/1,000,000).

Analysis of data on the diseases from the list of high-cost nosologies

The regional component of the Federal Registry of HCNs for the Republic of North Ossetia–Alania contains information about 554 patients, including 64 children (11.55%).

Information from the patients' genetic records about the diseases included in the list of HCNs is provided below (Table 2). Statistical data on the prevalence without any etiological structure analysis is provided for some diseases/conditions that are not genetically determined, such as organ and tissue transplant, hemolytic uremic syndrome. There are no statistics for some of them provided for the RF or deposited in the Orphanet database. As for diseases of genetic etiology, the more thorough analysis was performed in the Republic of North Ossetia–Alania considering molecular genetic testing, clinical features, and therapeutic interventions. The average prevalence was not calculated for this group of diseases.

Information about 13 patients diagnosed with hemophilia is available from the medical genetic center at the Republican Children's Clinical Hospital of the Republic of North Ossetia–Alania. All the patients receive specific coagulation factor replacement therapy. Currently, molecular genetic testing of children is conducted at the Research Centre for Medical Genetics. Mutations in the F8 gene typical for hemophilia A have been identified: c. 1630G>A (p.Asp544Asn) in two Ingush siblings; del(GRCh37/hg19) in two patients (Russian and Georgian), c.3637del (p.Ile1213Phefs*5), inv22, NC_000023.10:g.(?_154128143)_(154129718_?) del (GRCh37/hg19) deletion that includes exons 20 and 21 of the gene F8 in the hemizygous state, F8 inv 22 mutation in the hemizygous state, c.3637del (p.Ile1213Phefs*5) variant in the hemizygous state, etc. Mutation in the intron 5 of F9 (chrX:138630651G>A) in the hemizygous state affecting the conventional splice donor site that was typical for the disease was identified in patients with hemophilia B.

Twelve patients suffering from pituitary dwarfism are registered in the Republic of North Ossetia–Alania, among them 11 are children; the prevalence in the Republic of North Ossetia–Alania is 5.14 and 8.00/100,000, respectively. According to the all-Russian registry, the prevalence is 7.92 and 12.74 (Table 2). The patients receive hormone replacement therapy. The molecular genetic assessment of the candidate genes in patients with pituitary dwarfism has revealed no genetic variants causing the disease. The results obtained are consistent with the average data for the RF (5.40 and 18.15 per 100,000 surveyed people, respectively) and the Orphanet data (10–50/1,000,000).

The prevalence of cystic fibrosis in the RF is 2.97/100,000 for the whole population and 10.99/100,000 for the pediatric population, which is consistent with the Orphanet data. In the Republic of North Ossetia–Alania, 12 patients were diagnosed with cystic fibrosis during the studied period, among them 11 were children (prevalence of 2.20 and 6.77, respectively). DNA testing revealed well-known mutations in the gene CFTR: W1282X, 1677delTA, F508del, 2184insA, 2118del4, 1248+1G>A, R334W,359insT. It should be noted that the W1282X mutation accounting for 37.5% of all pathogenic alleles in affected individuals was most common in ethnic Ossetians [29]. This mutation is a class 1 mutation associated with cystic fibrosis, for which no target therapy has been developed. The patients receive symptomatic treatment in accordance with the federal clinical guidelines [30, 31].

Mucopolysaccharidoses, the group of glycosaminoglycan metabolism disorders, are represented by two forms in the Republic of North Ossetia–Alania: type I and VI mucopolysaccharidosis. The child was diagnosed with type I mucopolysaccharidosis based on the phenotypic traits, pronounced decrease in α-L-iduronidase activity, high urinary glycosaminoglycan levels, and molecular genetic testing data: the c.1A>C (p.M1?) nucleotide sequence variant in the heterozygous state reported in the HGMD database as pathogenic was found in the exon 1 of the gene *IDUA*; c.510delinsAAGTTCCA (p.His171Serfs*14) in the heterozygous state was found in the exon 5 of *IDUA*. The council decided to prescribe enzyme replacement therapy (Laronidase), treatment was satisfactorily tolerated. In 2022, bone marrow transplant was performed, however, the child died in June 2022 in the intensive care unit due to graft-versus-host disease.

The child born in 2009 was diagnosed with type VI mucopolysaccharidosis based on the phenotypic traits, pronounced decrease in lysosomal arylsulfatase activity, high urinary glycosaminoglycan levels, and DNA testing data: the c.691-1 G>A (IVS as G-A-1; IVS3 — 1g>a) mutation in the homozygous state was found in the *ARSB* gene. The Federal council decided to prescribe enzyme replacement therapy (Galsulfase). Therapy is satisfactorily tolerated.

According to the regional registries, the average prevalence of type I mucopolysaccharidosis in the RF is 0.06/100,000 in the whole population and 0.26 among children, while that of type II mucopolysaccharidosis is 0.09 and 0.38, type VI mucopolysaccharidosis — 0.04 and 0.10, respectively. According to Orphanet, the prevalence of type I and II mucopolysaccharidosis is 1–9/100,000, which is higher compared to the values for the RF and the Republic of North Ossetia–Alania, while the prevalence of type VI mucopolysaccharidosis is similar to the data obtained in our study: <1/100,000.

In the assessed period the diagnosis of Gaucher disease type 3 was established in a child born in 2016 at the age of 1.5 years in the Republic of North Ossetia–Alania. Molecular genetic testing revealed the p.L444P variant in the homozygous state in the *GBA* gene, which was confirmed by Sanger sequencing. The lifelong enzyme replacement therapy (Imiglucerase in a dose of 60 U/kg — 1200 U) was prescribed. However, the therapy conducted was followed by severe allergic reaction, anaphylaxis and angioedema. Imiglucerase therapy in accordance with the rapid drug desensitization scheme was initiated since the beginning of 2022 due to progression of complications. Furthermore, considering the IgE-mediated mechanism of allergy in this particular patient, the IgE antagonist, omalizumab, was added to premedication. During the period of 10 months physicians managed to administer 800 U with a 2-week interval. However, the child died in October 2022 due to secondary intercurrent disease [32]. The average prevalence in the RF was 0.31/100,000 in the whole population and 0.38 among children, which was lower compared to the Orphanet data (1–9/100,000).

DISCUSSION

Comparative analysis of the data on orphan diseases in the Republic of North Ossetia–Alania was conducted, the previously

Table 2. Number of patients, data on the prevalence (per 100,000) of orphan diseases from the list of high-cost nosologies (HCNs) in the world, RF, and Republic of North Ossetia–Alania

№	Disease	ICD-10 code	Prevalence per 100,000 according to Orphanet	Russian Federation				North Ossetia–Alania				*p*-value Russian Federation and Republic of North Ossetia–Alania	
				Patients		Prevalence per 100,000		Patients		Prevalence per 100,000			
				Total	Children only*	Total	Children only*	Total	Children only*	Total	Children only*	Total	Children only*
1	Hemophilia	D66	1.9	11601	3479	7.92	12.74	35	13	5.14	8.00	0.010	0.091
2	Pituitary dwarfism	E23,0	10.5	7915	4955	5.40	18.15	12	11	1.76	6.77	4.4E-5	6.7E-4
3	Cystic fibrosis	E84,0	10.5	4352	2945	2.97	10.79	15	11	2.20	6.77	0.246	0.127
4	Mucopolysaccharidosis I	E76,0	1.9	90	70	0.06	0.26	1	1	0.15	0.62	0.371	0.369
5	Mucopolysaccharidosis II	E76,1	1.9	139	104	0.09	0.38	0	0	0	0	0.421	0.431
6	Mucopolysaccharidosis VI	E76,2	<1	52	27	0.04	0.10	1	1	0.15	0.62	0.127	0.040
7	Gaucher disease	E75,2	1.9	455	105	0.31	0.38	1	1	0.15	0.62	0.444	0.637
8	Multiple sclerosis	G35,0	–	–	–	–	–	1	1	0.15	0.62	–	–
9	Hemolytic uremic syndrome	D59,3	1.9	502	312	0.34	1.14	6	2	0.88	1.23	0.017	0.916
10	Systemic juvenile rheumatoid arthritis	M08,2	1.9	1846	1148	1.26	4.21	22	11	3.23	6.77	5.3E-6	0.112
11	Aplastic anemia	D61,9	–	1420	142	0.97	0.52	5	1	0.73	0.62	0.534	0.867
12	Organ and tissue transplant	Z94	–	–	–	–	–	84	10	12.34	6.16	–	–
13	Malignant neoplasms of the lymphoid, hematopoietic and related tissues	C81-C96	–	–	–	–	–	372	1	54.65	0.62	–	–
14	Hereditary coagulation factor deficiency	D68,2		355	167	0.24	0.61	2	0	0.29	0	0.786	0.319
	II (fibrinogen),		<1										
	VII (labile factor),		1.9										
	X (Stuart–Prower factor)		1.9										
Total				28727	13454	19.62	49.28	554	64	81.38	39.40	0	0.073

Note: * — children under the age of 18 years.

unpublished data on the diseases from the list of RLTDs in the RF were provided. The average prevalence of RLTDs in the RF was 11.51/100,000 in the whole population (1 : 8688 people), 25.08/100,000 among children (1 : 3987 children). Similar data were obtained for the Republic of North Ossetia–Alania: 19.38/29.44 (1 : 5160 people/1 : 3396 children). No significant differences were revealed (Table 1).

The average prevalence of idiopathic thrombocytopenic purpura in the Republic of North Ossetia–Alania is higher than the average prevalence in the RF, both in the whole population ($p = 1.9 \times 10^{-17}$) and among children (p = 0.013). Comparison with the Orphanet data has revealed no differences considering variability of the prevalence in different countries. The prevalence of the disorder of the complement system turned out to be higher only among children ($p = 5.7 \times 10^{-6}$), which is in line with the data reported for Europe. Maple syrup urine disease is more common in the Republic of North Ossetia–Alania, than in the RF ($p = 0.018$ and $p = 0.045$, respectively), which is consistent with the Orphanet data. The prevalence of galactosemia in in the whole population of the Republic of North Ossetia–Alania is lower ($p = 0.017$), than the average prevalence in the RF and Europe, which can be associated with low detection rate of the disease in the Republic due to milder course. At the same time, acute intermittent porphyria is characterized by higher prevalence ($p = 3.1 \times 10^{-3}$), than in the RF, and similar to the values reported for Europe. Such disease entities, as fatty acid metabolism disorders, glutaric aciduria, tyrosinemia, other amino acid metabolism disorders, have not been reported in the Republic of North Ossetia–Alania.

The differences were also revealed for the group of orphan diseases from the list of HCNs. The prevalence of hemophilia ($p = 0.010$ for the whole population) and pituitary dwarfism ($p = 4.4 \times 10^{-5}$ for the whole population, $p = 6.7 \times 10^{-4}$ for children only) in the Republic of North Ossetia–Alania turned out to be lower compared to the average prevalence in the RF and the Orphanet data. At the same time, the prevalence of type VI mucopolysaccharidosis ($p = 0.040$ for children), hemolytic uremic syndrome ($p = 0.017$), and systemic juvenile rheumatoid arthritis ($p = 5.3 \times 10^{-6}$) is higher than in the RF and is consistent with the Orphanet data.

All other diseases in the groups of RLTDs and HCNs showed the values that were statistically similar to the data for the RF and the Orphanet data.

Attention should be also paid to the specific range of mutations:

– in the *PAH* gene associated with phenylketonuria — predominance of two frequent genetic variants of the *PAH* gene (P281L and P211T), not typical for both RF and all world's populations. In ethnic Ossetians, these two variants together account for 60% of all the mutations identified (P281L — 42.11%, P211T — 18.42%). The P211T variant is a mild genetic variant with the residual phenylalanine hydroxylase activity of 72%, which results in milder clinical features of the disease and no need for replacement diet therapy;

– in the CFTR gene associated with cystic fibrosis — predominance of the W1282X class 1 mutation, which limits the target therapy options available for patients.

CONCLUSIONS

Today, there is no universal approach to improvement of the diagnosis, treatment, and drug provision to patients with

orphan diseases that might be replicated all over the world. At the same time, a rather big experience of dealing with the issues related to RDs has been accumulated. There are also certain achievements in Russia. Thus, the federal registries of various orphan diseases have been compiled and are regularly updated, the budget to support treatment that is often expensive has been determined. In our study we determined the structure and population genetic features of the RDs from the lists of RLTDs and HCNs in the Republic of North Ossetia–Alania and the RF. The features of prevalence were demonstrated for a number of diseases, along with the statistical similarity of values to the data reported for the RF and the Orphanet data for the vast majority of diseases. Despite significant breakthroughs in the field of diagnosis and treatment of orphan diseases, the detection rate of the discussed disease entities in both the region and the country is still inadequate. This results in the need to use the world's experience of organizing transregional orphan centers, wider adopt the confirmation diagnosis methods, exome/genome sequencing at the expense of budgetary funds. A separate problem is the limited range of options of free DNA diagnosis for adults. Establishment of the Circle of Good foundation contributed enormously to increasing the availability of pharmaceuticals and the earlier start of therapy. The system for communication with the Foundation has been worked out in the region. Every year, when drafting the budget, the Ministry of Health of the Republic of North Ossetia–Alania includes the demand for funding of preferential drug provision to the discussed category of patients calculated in accordance with personal prescriptions for each patient. As for the end of 2022, the republican segment of the Circle of Good information resource contains 22 patients suffering from RDs. All the patients are provided with pharmaceuticals and therapeutic food products in full. There are no interruptions of the ongoing therapy in the patients using drugs at the expense of the Foundation. Pharmaceuticals worth 369,165,971.70 rubles have been received since November 2021. A total of 188 prescriptions worth 210,304,075.10 rubles have been dispensed. Expansion of the range of screened diseases will contribute to optimization of the RD detection at the preclinical stage. Certainly, we are at the beginning of the completely new era, when the early diagnosis of such diseases can become a routine, and expansion of the gene therapy capabilities will make it possible to optimize the course of a large number of diseases, that have been earlier considered incurable.

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POTENTIAL OF NON-TRADITIONAL CELL CULTURES FOR PRODUCTION OF BIOTHERAPEUTIC PROTEINS

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Production of biotherapeutic drugs in mammalian cells, recombinant proteins in particular, may be handicapped by the limitations imposed on the cultures by metabolic burden. An alternative solution is to produce proteins in cells of other animals (e.g., Sf9, S2 and High Five insect cell lines, *Caenorhabditis elegans* and *Schistosoma mansoni* cell line) or orthogonal cell systems, including plant-based. In our opinion, non-traditional cell cultures may become promising tool for production of affordable and effective biotherapeutic drugs.

Keywords: biotherapeutic drugs, plant cell cultures, metabolic burden, High Five cell line, Sf9 cell line

Funding: the work was supported by the Russian Science Foundation grant No. 21-74-10152, https://rscf.ru/project/21-74-10152/

Acknowledgements: the authors thank A.D. Barykin from the Department of Bioorganic Chemistry of the Institute of Bioorganic Chemistry of the Russian Academy of Sciences for the fruitful discussion of the idea of this publication.

Author contribution: Dobronos MA — literature search and analysis, manuscript authoring; Osipova ZM — project management, manuscript editing; Markina NM — manuscript idea, literature search and analysis, manuscript authoring.

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Received: 20.04.2024 **Accepted:** 12.05.2024 **Published online:** 13.06.2024

DOI: 10.24075/brsmu.2024.022

ПОТЕНЦИАЛ НЕКЛАССИЧЕСКИХ КЛЕТОЧНЫХ КУЛЬТУР ДЛЯ ПРОИЗВОДСТВА БИОТЕРАПЕВТИЧЕСКИХ БЕЛКОВ

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Производство биотерапевтических препаратов, в частности, рекомбинантных белков в клетках млекопитающих может быть затруднено из-за ограничений используемых культур в связи с метаболической нагрузкой. Альтернативным подходом для решения таких задач является наработка белков в клетках других животных (например, культуры клеток насекомых Sf9, S2 и High Five, культуры клеток червей видов *Caenorhabditis elegans* и *Schistosoma mansoni*) или ортогональных клеточных системах, в том числе растительных. С нашей точки зрения, применение неклассических клеточных культур может стать перспективным направлением для получения более доступных и эффективных биотерапевтических препаратов.

Ключевые слова: биотерапевтические препараты, растительные клеточные культуры, метаболическая нагрузка, клеточная линия High Five, клеточная линия Sf9

Финансирование: работа выполнена при поддержке гранта Российского научного фонда 21-74-10152, https://rscf.ru/project/21-74-10152/.

Благодарности: авторы благодарят А. Д. Барыкина из отдела биоорганической химии ИБХ РАН за плодотворное обсуждение идеи публикации.

Вклад авторов: М. А. Добронос — поиск и анализ литературы, написание рукописи; З. М. Осипова — руководство проектом, редактирование рукописи; Н. М. Маркина — идея рукописи, поиск и анализ литературы, написание рукописи.

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Статья получена: 20.04.2024 **Статья принята к печати:** 12.05.2024 **Опубликована онлайн:** 13.06.2024

DOI: 10.24075/vrgmu.2024.022

Today, one of the key tasks before the pharmaceutical industry is to increase the efficacy of production of biotherapeutic drugs. Depending on the desired composition of the drug, the stages that may present hindering obstacles are the search for a natural source thereof or the development of its artificial analogue, boosting production by the source, or optimization of purification (removal of impurities and ineffective forms) [1, 2]. We would like to present the potential of non-traditional cell cultures as a key part of the solutions to such problems.

Cell cultures are most common in the production of biotherapeutic protein preparations, with monoclonal antibodies being the most significant thereof. The flagship culture is that of Chinese hamster ovary cells (CHO): it is easy to cultivate and grows rapidly, guarantees correct translation, folding and posttranslational modification of the recombinant protein, and releases large amounts of the product into the culture medium, yielding the largest amounts of the target drug among all mammalian cell cultures [3]. The drawbacks of all mammalian cell cultures, including CHO, are high cost and need for special working conditions and equipment, as well as susceptibility to metabolic burden. When the output of the recombinant protein reaches a certain level, this burden prevents standard production boosting technologies from working, including those that involve increasing the number of copies of the recombinant gene, using stronger regulatory sequences, etc. [4]. This happens because recombinant processes begin to compete for resources with the host cell's viability maintenance processes; there are about 8–10 of these in total, including the processes of

Table. Comparison of the most common types of expressing cell cultures [11, 24, 25]

Expression platform	Advantages	Flaws	Post-translational modifications	Cost, complexity of purification	Scaling potential	Safety
Bacteria (*E. coli*)	Low cost Simple genetic engineering process Rapid growth of culture and high yield of the target product Proven expression optimization strategies	Incorrect folding of some proteins and formation of inclusion bodies. Presence of endotoxins	Non-native No glycosylation Difficulties with formation of disulfide bonds	Low	+++	Moderate
Yeasts (*P. pastoris*, *S. cerevisiae*)	Low cost Simple genetic engineering process Rapid growth of culture and high yield of the target product Proven optimization strategies Correct folding of large (> 30 kDa) proteins	Cell wall can handicap purification	Non-native There are strains with limited glycosylation capabilities	Low	+++	Moderate
Plant-based systems (BY-2, NT-1)	Rapid growth of culture and high yield of the target product Possibility of expression of multi-protein complexes	Genetic instability of lines during long-term cultivation Increased risk of culture contamination	Non-native (genetic vectors optimization required)	Moderate / None for edible plants	+++	Very high
Insect cells (Sf21, Sf9, Hi5)	Expression of eukaryotic multi-protein complexes with correct folding Higher product yields	Expression with strong promoters can disrupt folding Non-targeted glycosylation	Simplified *N*-glycosylation	Moderate	+++	Low
Mammalian cells (CHO, HEK293)	Native lipid environment and folding conditions Possibility of inducible expression by transient transfection Possibility of using FACS (Fluorescence Activated Cell Sorting) on stable lines	Low expression level Overexpression of some proteins is impossible due to toxicity Long-term optimization of expression conditions	Native	Moderate	++	Low
Cell-free expression systems	Fast expression method Possibility of producing toxic proteins Detailed control of environmental parameters during expression	High cost Lack of *in vivo* factors enabling folding	Need for additional components (EPR microsomes, etc.)	Low	+	Very high

transcription, translation, post-translational modifications, and protein export [5]. Various metabolism balancing techniques are used to counter the negative effects of metabolic burden, but this is a very labor-intensive process, since it is necessary to identify all the limiting stages [6] and choose the method to overcome them without compromising the overall viability of the producing cell [7, 8]. However, even successful metabolism balancing may not yield a significant boost in recombinant protein production, since in the case of some biotherapeutic proteins, the process is so laborious for a mammalian host cell that all attempts to optimize it are limited by the physiological capabilities of that cell. For example, recombinant production of the blood coagulation factor VIII (F8) in the CHO culture has the approximate "energy cost" of about 10,000 ATP molecules per a functional F8 molecule [5].

As an alternative, protein preparations can be produced in hosts whose physiological resources are initially higher than those of mammalian cells; such hosts are cells from other animal species or orthogonal cellular systems, like plant cell cultures (Table). One of the main advantages of plant-based biotherapeutic compounds is their safety: they cannot be infected with human pathogens, produce no endotoxins, and have reduced immunogenicity, which improves drug tolerance and minimizes side effects. For example, taliglucerase alpha (β-D-glucosyl-*N*-acylsphingosinglucohydrolase) produced in transgenic carrot cells for treatment of Gaucher disease type 1 has shown to not trigger any evident side effects associated with N-glycan residues during clinical trials. Moreover, no antibodies to this drug have been detected [9]. In addition, biological preparations produced in plant cell cultures can be administered orally without purification or with minimal purification. Plant cell walls can protect biological products from enzymatic degradation in the gastrointestinal tract, as well as facilitate the delivery of these drugs to the intestine lymphoid tissue in the active form. Clinical trials have shown that production of oral biopharmaceuticals from edible plant tissues is feasible [10].

Plant cell cultures allow achieving a high level of expression of multiprotein complexes that require complex folding and assembly processes, which is also an important aspect in the context of their use for the purpose. Strategies involving construction of a single vector with a set of recombinant genes and joint biosynthesis of recombinant proteins together with chaperones of the same origin can help increase the output of such complexes [11]. In addition, introduction of an exogenous signal sequence directing the protein along a specific secretory pathway can increase the yield of small proteins weighing less than 30 kDa. We believe that optimization of the fermentation process, including continuous or semi-continuous fermentation, is a universal method of increasing protein output from both plant and insect cell cultures.

Insect cell lines *Spodoptera frugiperda* Sf21, Sf9 and *Trichoplusia* ni BTI- 5B1-4 (High Five), adherent nonpermissive cell cultures obtained from ovarian tissues of the respective insects, are also widely in production of biotherapeutic proteins in baculovirus expression systems [12]. Insect cell cultures

offer similar mechanisms of post-translational modification of proteins, which makes them a cost-effective and scalable tool for the production of vaccine antigens and virus-like particles [13]. Besides, engineered baculoviruses with mammalian promoters (BacMam) possess a significant potential as vectors for gene delivery to mammalian cells [14]. The glycosylation pattern in these expression systems differs slightly from that of humans, but can be humanised through parallel expression of mammalian glycotransferases and the removal of insect-specific alpha-1,3-fucosylated glycans, which can cause allergies in people. Antigen proteins that are core of candidate vaccines against COVID-19 [15–17] and malaria [18] are produced in insect cell cultures.

CRISPR technology can help significantly accelerate the production of stably expressing glycoengineered insect lines. It has been shown that CRISPR can be used to knock out genes in the *Drosophila* and *Bombyx* cell lines, as well as to knock out the *N*-acetylglucosaminidase gene in the S2 cell line, which triggers exponential growth of the number of GlcNAc terminal residues in recombinant human erythropoietin [19]. Another promising direction is modification of the Sf9 and High Five cell lines, e.g., multiple duplication of the mammalian glycotransferase genes, can secure an even higher level of protein expression with correct glycosylation and folding.

Worm cell lines are a viable alternative for a number of biomedical applications. For example, embryonic *C. elegans* cell cultures are used to study the processes of cell differentiation, morphogenesis, and gene expression, opening up a wide range of previously experimentally inaccessible opportunities [20]. Somatic cells from various tissues of *C. elegans* (neurons, muscle cells, hypodermic and intestinal cells, etc.) can be cultured for investigation of tissue-specific interactions and signaling pathways [21]. Cell culture of *Schistosoma mansoni*, a parasitic flatworm, which can be continuously cultivated for 6 months [22], may also become an interesting tool for studying the parasite-host interactions and anthelmintic drugs testing. Many species of marine worms are sources of biologically active compounds, including peptides with antimicrobial, anti-inflammatory, immunomodulatory, antioxidant, and antihypoxic effects [23]. The development and optimization of the technology to isolate and cultivate worm cells (long-term cultivation) could boost screening and subsequent effective development of such biologically active substances.

CONCLUSION

Thus, the use of nontraditional cell cultures is a promising way to increase the efficiency of the production of biotherapeutic drugs. Several features of expression mechanisms in alternative cultures can minimize side effects and improve tolerability of the resulting protein preparations. Moreover, alternative producing organisms also help to circumvent the limitations associated with the increased metabolic burden in mammalian cell cultures. This enables further development and production of more effective and affordable biotherapeutic drugs, contributing to the overall progress in the fields of pharmaceuticals and medicine.

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ASPECTS OF THE LENS CHEMISTRY AND PATHOCHEMISTRY

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The lens (*lens cristalina*) is part of the light conducting and light refracting system of the eye. Transparency and light refraction are the main properties of the lens. Nutrients are supplied to the lens through the capsule by diffusion and active transport. The energy needs of the avascular epithelial structure are 10–20 lower compared to that of other organs and tissues. Such needs are satisfied through anaerobic glycolysis. Currently, there is insufficient information about the fundamental chemical mechanism underlying the existence of the lens in the healthy body, the mechanisms of its functional "survival" against the background of somatic disorder, such as diabetes. The paper reports the authors' view of certain chemical aspects clarifying pathochemical alterations of the lens with the possible mechanisms underlying its "adaptation"/"protection" associated with the systemic disorder at the molecular level. In particular, the view of the involvement of glucose-6-phosphate dehydrogenase/transketolase, the enzymes of the oxidative and non-oxidative phases of the pentose phosphate pathway belonging to the native crystalline fraction protein family, in the mechanisms underlying protection of the lens against the oxidative and osmotic stress, involvement of aldo-keto reductases in pathochemical alterations of the lens, as well as the role the NO, NO_3-, B_6, PQQ small molecules having an antioxidant cytoprotective effect is reported.

Keywords: lens, aldo-, ketoreductase, transketolase, crystallines, key pathochemistry aspects

Author contribution: Smirnov SV — literature review, data acquisition in the field of fundamental bioorganic chemistry; Kuznetsova OYu — research data interpretation, manuscript writing; Postnikov MA — manuscript design.

Acknowledgements: the authors would like to thank F.N. Gilmiyarova, D. Sc. (Med.), Professor at the Department of Fundamental and Clinical Biochemistry with Laboratory Diagnosis, Samara State Medical University, for her help in manuscript writing, editing, and design.

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Received: 28.02.2024 **Accepted:** 06.05.2024 **Published online:** 29.05.2004

DOI: 10.24075/brsmu.2024.019

АСПЕКТЫ ХИМИИ И ПАТОХИМИИ ХРУСТАЛИКА

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Хрусталик (*lens cristalina*) является частью светопроводящей и светопреломляющей системы глаза. Главные свойства хрусталика — прозрачность и светопреломление. Питательные вещества поступают к нему через капсулу путем диффузии и активного транспорта. Энергетические потребности бессосудистого эпителиального образования в 10–20 раз ниже, чем потребности других органов и тканей. Они удовлетворяются посредством анаэробного гликолиза. На сегодняшний день недостаточно сведений, отражающих особенности базового химического механизма существования хрусталика в норме, механизмы его функциональной «выживаемости» на фоне соматической патологии, в частности, диабета. В работе представлен взгляд авторов на отдельные химические аспекты, проясняющие патохимические изменения хрусталика с возможными механизмами его «адаптации»/«защиты» на фоне системной патологии на молекулярном уровне. В частности, на участие ферментов окислительного и неокислительного этапов пентозофосфатного шунта — глюкозо-6-фосфатдегидрогеназы/транскетолазы, относящихся к семейству белков собственной кристаллиновой фракции, в механизмах защиты хрусталика от окислительного и осмотического стресса, участие альдо- и кеторедуктаз в патохимических изменениях хрусталика, а также роль «малых молекул» NO, NO_3-, B_6, PQQ с антиоксидантным цитопротекторным эффектом.

Ключевые слова: хрусталик, альдоредуктаза, кеторедуктаза, транскетолаза, кристаллины, ключевые аспекты патохимии

Вклад авторов: С. В. Смирнов — анализ литературы, сбор данных в сфере фундаментальной биоорганической химии; О. Ю. Кузнецова — интерпретация научных данных, подготовка рукописи; М. А. Постников — дизайн рукописи.

Благодарности: выражаем благодарность за помощь профессору кафедры фундаментальной и клинической биохимии с лабораторной диагностикой ФГБОУ ВО СамГМУ, д. м. н. Ф. Н. Гильмияровой в подготовке и редакции материалов и дизайна рукописи.

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Статья получена: 28.02.2024 **Статья принята к печати:** 06.05.2024 **Опубликована онлайн:** 29.05.2004

DOI: 10.24075/vrgmu.2024.019

The lens (*lens cristalina*) is a component of the light conducting and light refracting system of the eye. Transparency and light refraction are the main lens properties. It is second to the cornea in the degree of light refraction. The optical power of this living biological lens is within 19.00 diopters. The lens has a layered structure. Nutrients are supplied through the capsule by diffusion and active transport. The energy needs of this avascular epithelial structure that are 10–20 lower compared to the needs of other organs and tissues are satisfied mainly through anaerobic glycolysis [1, 2]

Features of chemical composition

Chemical analysis of the lens was first performed in the late 19th century, when U. Mörner extracted a soluble protein referred to as crystalline. In 1950s, V. N. Orechovich and then R. A. Reznik extracted three fractions with different molecular weight referred to as α-, β- and γ-crystallines from the soluble fractions of lens proteins. The lens is made up of about 35% proteins, 1% lipids and 64% water. The lens proteins are divided into water soluble and water insoluble. More than 90% of soluble proteins are represented by α-, β- and γ-crystallines. Heterogeneity of protein composition is typical for the lens: fractions of the α-, β- and γ-crystalline high molecular weight forms predominate in the nucleus, while α- and β_L-crystallines are the main cortical proteins. α-Crystallines (chaperones, molecular weight 160–1000 kDA) have two predominant isoforms (αA-crystallines and αB-crystallines), inhibit aggregation of damaged proteins, thereby maintaining the

lens transparency. β-Crystallines (up to 60%) include the acidic (β_A-crystallines) and alkaline (β_B-crystallines) subgroups, four isoforms per subgroup (designated with Arabic numerals, 1 to 4). And, finally, γ-crystallines are represented by seven isoforms designated with Arabic letters A–F and S. γ-Crystallines exist as 20 kDA monomers only and have a structural function, like the previous group [3, 4]. Of low molecular weight compounds, large amounts of vitamins have been found. Ascorbic acid plays a certain role in the energy production processes: it transports hydrogen to the lens, constitutes part of the lens antioxidant system. Vitamins A, B_1, B_2, B_5 have an impact on the mitotic activity of the lens epithelium; vitamin E is considered as a probable antioxidant factor preventing the lens opacification. Several glutathione analogues have been found in the lens, the content of which is lower; the cysteine residue is substituted in each of these analogues. The leucine aminopeptidase of the lens is an essential member of the crystalline fraction protein family, along with the transketolase [5].

Features of major metabolic pathways

To provide energy supply, glucose breakdown is accomplished through aerobic and anaerobic glycolysis, direct (apotomic) oxidation of glucose (pentose phosphate pathway), and the Krebs cycle. The sorbitol pathway for glucose uptake by the lens is also possible. However, against the background of the disorder, such as diabetic retinopathy, the other pathway for utilization of excess carbohydrates coming from the outside, the polyol pathway realized by means of the key enzyme, aldose reductase, is enhanced. This group of enzymes belonging to the first class of oxidoreductases deals with the nicotinamide coenzyme, specifically with its phosphorylated form essential for the tandem binding to the active center of this reductase [6–9]. Our question is: why does such a basic substance, as pyruvic acid, the final metabolite of glycolytic carbohydrate degradation, that shows high carbonyl activity, exerts no effect on the aldose reductase enzyme present here in the cytosol before entering the mitochondria (pyruvate dehydrogenase complex (PDC) of the inner membrane) in individuals with diabetes and tissue ketoacidosis? Moreover, the enol forms of bioorganic substances showing keto-enol tautomerism predominate under native conditions, and the presence of the conjugated carbonyl group with the multiple bond in the linear molecular structure allows such form to be a potent nucleophilic agent. Or is a certain effect still possible? And how is it realized under conditions of molecular environment in the cytosol? On the one hand, it may be mainly converted to lactate, and on the other hand, binding with the specific cytosolic/mitochondrial transporter is possible. The latter transports it using the proton symport mechanism (the energized, electrically charged inner membrane of the mitochondrion can be de-energized and discharged, which is mediated by the H^+/ATPase activity). Apparently, the pyruvate affinity for this protein is rather high, otherwise a certain pool of this could move to the appropriate cellular compartments and decrease the activity of the first phase of polyol pathway, specifically covalent interaction with the active structural motif of such reductases, such as the tyrosine amino acid, since monocarboxylic acids can be involved in the Friedel-Crafts reactions, in this case occurring in the form of the Fries rearrangement reaction that is most typical for phenols. The phenolic radical of the tyrosine amino acid can also be safely included in this group of compounds. According to the available research data, this reaction sometimes does not require a catalyst, especially when the final product representing an aromatic ketone is produced having lower activation energy of the molecule, which stabilizes the reaction product and makes it less active compared to the initial reagents due to the M- and I-effect of the carbonyl group. Perhaps, it is this mechanism that underlies partial blockage of the cytosolic aldo- and ketoreductases and preserves viability of the eye lens for a long time against the background of chronic diabetes. This issue requires more thorough investigation and conceptualization under the conditions of using NMR spectroscopy and the kinetic isotope effect that will probably allow the researchers to reveal more subtle mechanisms underlying the above pathochemical process.

Effect of NO on the polyol pathway

It is well-known that the aldose reductase inhibitors (ARIs) and sorbitol dehydrogenase inhibitors (SDIs) cause changes in the content of sorbitol and fructose. The aldose reductase activity is decreased under exposure to the nitric oxide (NO). Since superoxide reduces the amount of NO, the aldose reductase activity increase is associated with oxidative stress, and reduction of the reactive oxygen species levels inhibits aldose reductase. Hyperosmolarity induced by sorbitol results in depletion of the organic osmolytes and antioxidants (such as taurine) [10].

Contribution of the nitrate ion as a donor of nitric oxide

Based on the chemical nature, this nitrogen compound is classified as a diatomic neutral molecule, however, it represents a free radical, colorless gas with the half-life of 2–30 s and the average lifetime in biological tissues of 5–6 s. The molecule has an unpaired electron in the outer ϖ orbital, which makes it a high-spin radical. The nitrate ion can well react with other free radicals and is capable of covalent bonding. This very property allows it to both activate and block (chain interruption) the free radical substitution reactions (S_R). Recently, the other source of the compound, other than oxidation of the proteinogenic L-arginine α-amino acid guanidine group supplying this vasoactive metabolite to the body's tissues, has been determined. Thus, it has been found that not only (1–3) NO-synthase (NOS) isoforms can generate NO in the lens and cornea of the eye; NO can be formed non-enzymatically from the other compound, nitrate, in the tissues. In particular, the reaction of direct asymmetric fission or reduction of the nitrate ion (heterolytic mechanism) is possible that results in the formation of the nitrite ion and NO (Fig. 1). Such a process may possibly occur predominantly under conditions of acidification of the environment, i.e. under conditions of ischemia, which can only affect the eye lens function. In our opinion, it is also necessary to consider the fact that breakdown of such one of the most potent oxidizing agents in the organic world, as peroxynitrite ion ($ONOO^-$) yielding nitrogen dioxide under conditions of acidosis (in the form of nitrite ion under native conditions) and the hydroxyl radical ($OH^•$), occurs under the same conditions. In this case the pool of intracellular nitrite ion is increased due to both first (heterolysis of nitrate) and second (breakdown of peroxynitrite associated with acidification of the environment) reactions, which is likely to shift equilibrium of the entire pathochemical process to the side of reagents (precursors) and reduces total NO levels, which is especially important for the process of peroxynitrite accumulation during realization of the lipid peroxidation (LOP) pathways. However, in the course of long evolution, essential NO levels became involved in the process of the lens "rescue" during such reactions. To ensure survival of the lens epithelial tissue, such

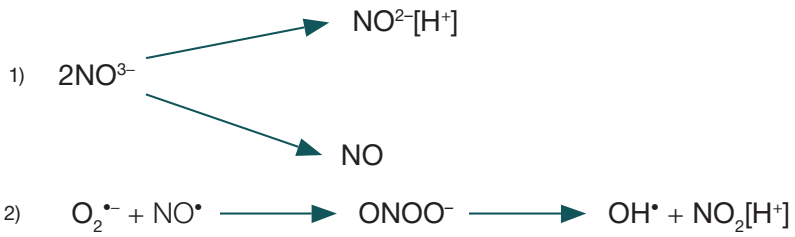

Fig. 1. Pathochemical pathways involving the oxygen-containing nitrogen compounds

levels are achieved through generation of S-nitrosoglutathione. It represents an endogenous long-lived donor of such an active small molecule, as NO. This is especially important in the context of ischemia with subsequent reperfusion, when the function of the lens capsule inflow vessels is impaired and the release of free radicals is enhanced, resulting in the NO biosynthesis limitation.

Effects of vitamins B_6 and PQQ

The aldose reductase inhibitors were extensively studied using multiple compounds that were not chemically related.

Pyridoxamine was first described as inhibitor of production of the advanced glycation end products (AGEs) following the Amadori rearrangement, but it also inhibits formation of the advanced lipoxidation end products (ALEs) on protein during LOP. Administration of 1 g per 1 L of drinking water to rats with diabetes throughout 28 weeks decreased the diabetic capillary regression by 71%. To date, it is unclear whether such treatment inhibits the loss of pericytes, including in the inflow arterioles of the lens capsule, or not.

Vitamin PQQ (former name B_{14}) being a potent cytoprotective agent as an antioxidant is more active as a reducing agent than vitamin C; it is more active as an oxidizing and reducing agent than the vitamin B_2 derivatives; it is more active as a compound showing carbonyl activity than vitamin B_6 due to the fact that it represents a co-enzyme of glucose dehydrogenase (the first key enzyme of the eye lens crystalline family actually being one of the crystalline fractions of proteins). We believe that this is nothing less than bioorganic chemical reduction ("simplification") of the pentose phosphate pathway oxidative phase that has emerged over millions of years of evolution against the background of the lens functional specialization. By analogy with this statement, we can conclude that transketolase, in turn, can be considered as similar reduction of the phosphate pathway non-oxidative phase, which is quite enough for "survival" of the lens tissue when various changes of homeostatic conditions occur associated with the prominent organ (tissue) specialization.

The second key enzyme of the crystalline family: transketolase as the "reduced" pentose phosphate pathway of the eye lens

In mammals, transketolase links pentose phosphate pathway with glycolysis by dispatching excess sugar phosphates to the main carbohydrate metabolic pathways. The presence of transketolase is essential for NADPH production, especially in the tissues actively participating in the biosynthesis, including tissues of the lens. The co-enzyme form of vitamin B_1, thiamine pyrophosphate (TPP), is an important co-factor, along with calcium, and functions in a complex with the latter (this complex is similar to the ATP/ Mg^{2+} complex as an electron-deficient system). The entrance to the active site of this enzyme consists of several side chains of arginine, histidine, serine, and aspartate. Despite the fact that the enzyme is capable of binding various types of substrates, it shows high specificity for stereochemical arrangement of hydroxyl groups in sugars. These hydroxyl groups in the C-3 and C-4 positions of the ketose donor carbon chain should have a D-treo configuration to properly match positions C-1 and C-2 in the aldose acceptor. His263 is used as a donor of proteins for the substrate-acceptor-TPP complex, which can then generate erythrose 4-phosphate. The histidine and aspartate side chains used for effective stabilization of the substrate contribute to the substrate deprotonation. The phosphate group of the substrate also plays an important role in the substrate stabilization when it enters the active center, along with the ionic nature of the bond of the salt bridge connecting Arg359 with the phosphate group. Catalysis is initiated by B_1 deprotonation in the thiazolium ring. Then the carbanion binds to the carbonyl group of the donor substrate, thereby breaking the bond between C-2 and C-3. This keto-fragment remains covalently linked to the TPP C-2. After that the donor substrate is released. The acceptor substrate enters the active site, where the fragment bound to the α-β-dihydroxyethyl-TPP intermediate is transferred to the acceptor [11, 12].

Inhibition of transketolases by the AGE and LOP end products

Protein glycation (glycation) is a process referred to as the Maillard reaction. In the course of the Maillard reaction, intermediate products, such as glyoxal, methylglyoxal and 3-deoxyglucosone, are also yielded that can be formed as a result of both monosaccharide auto-oxidation (for example, glucose in the Wolf reaction) and Schiff base rearrangement (Namiki pathway) or Amadori rearrangement (Hodge's model — he discovered the fact of interaction between glucose and glycine yielding at least 24 compounds as early as in 1953). Since free amino groups are glycated, any protein can be potentially subject to this process, which is especially important for protein fractions of the lens that are mostly basic. AGEs, both free and bound to proteins, are found in plasma of the inflow blood vessels, including in the eye lens. At least 20 different AGEs have been reported, among them N-carboxymethyl-lysine, pentosidine and hydroimidazolones are relatively inert and can be used as biomarkers of the AGE content in the tissues. The Maillard reaction includes several phases. First, glucose (or other reducing carbohydrate, such as fructose, pentose, galactose, mannose, xylulose) react with the free amino groups of amino acids yielding an unstable compound, the Schiff base. The Schiff base (aldimine) is subject to spontaneous rearrangements yielding a relatively stable ketoamine (1-amino-1-deoxy-2-ketose), the Amadori compound [13–15]. Further degradation of these early glycation products yields a heterogeneous group of irreversible compounds, the AGEs. Fig. 2 presents one of possible (in our opinion) mechanisms underlying pathochemical blockage of transketolase involving switching off the enzyme itself. Malondialdehyde as a biomarker of oxidative stress in the epithelial cell of the eye lens plus arginine (2-amino-5-guanidino-pentanoic acid) of the transketolase represent the reaction of condensation and intermolecular cyclization yielding 2-aminopyrimidine. The reaction is based on the mechanism

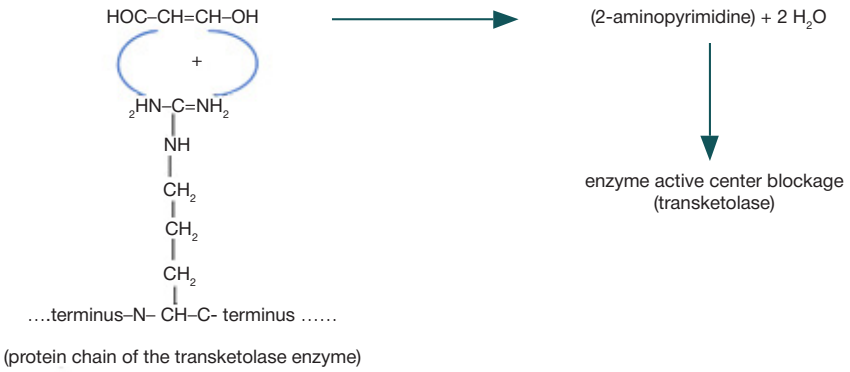

Fig. 2. One of possible mechanisms underlying blockage of the transketolase active center involving the malondialdehyde enol form

of nucleophilic bimolecular addition. Furthermore, due to the presence of malondialdehyde as a carbonyl group in the enol form, along with the conjugated multiple bond, it is converted to the potent nucleophile attacking the arginine electrophilic center in the form of imine nitrogen $=NH^{2+}$ in the first phase to yield pyrimidine derivatives (by analogy with the chalcones used for artificial synthesis of antitumor agents based on the aminopyrimidine framework when assessing molecular docking during production of new dosage forms).

CONCLUSION

We have tried to understand, what chemical substances and how they interact with each other in the context of the lens basic metabolism and pathochemistry. The cataract, representing partial or complete clouding of the lens substance and/or capsule, is among the most common causes of vision loss, that is why we have touched upon the issue of cataracts and fundamental chemical causes of their development against the background of diabetes mellitus, one of the most important medical and social problems. Pathogenesis of diabetic lens disorders is extremely complex and multifactorial. AGEs that realize their potential through the effects on the protein structure and activation of the AGE–RAGE axis contribute greatly to the diabetes progression, which results in a number of pathological changes. Since AGEs have many adverse effects, it is necessary to search for new chemical and pharmaceutical technological strategies, primarily targeted ones focused on reducing the AGE levels. Interruption of the cascade of pathochemical events triggered by the AGE–RAGE interaction can also represent a promising and reasonable direction of developing new approaches to prevention and treatment of the diabetic complications affecting such highly specialized functional structures of the eye, as the lens.

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THIAMINE RESPONSIVE MEGALOBLASTIC ANEMIA (ROGERS SYNDROME) IN A THREE-YEAR-OLD CHILD

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Thiamine responsive megaloblastic anemia (TRMA), or Rogers syndrome, is a rare autosomal recessive disease characterized by the development of megaloblastic anemia, diabetes mellitus, and progressive sensorineural hearing loss. In some cases, the syndrome causes ophthalmological disorders (retinitis pigmentosa, optic nerve atrophy, maculopathy, nystagmus), heart diseases (paroxysmal atrial fibrillation, supraventricular tachycardia, congenital heart defects, intracardiac conduction disorders) and neurological disorders (epilepsy, cerebrovascular accidents). TRMA develops due to a mutation in the *SLC19A2* gene, which encodes ThTr-1 (thiamine transporter protein) expressed in hematopoietic stem cells, pancreatic beta cells, and inner ear cells. The article presents a clinical case of TRMA in a three-year-old child, with the onset in the first year of life, manifesting as anemia and diabetes mellitus. Thiamine therapy ensured a pronounced positive dynamics: the patient's peripheral blood parameters normalized. The clinical description and the literature review herein aim to raise awareness of doctors of all specialties about this syndrome. An atypical clinical picture and lack of knowledge about TRMA often delay the diagnosis and start of therapy.

Keywords: thiamine-responsive megaloblastic anemia, Rogers syndrome, bone marrow, ring sideroblasts, diabetes, sensorineural deafness, gene *SLC19A2*

Author contribution: Trukhina EV — data collection, compilation of the list of references; Konyukhova TV — development of the article's design, manuscript authoring.

Compliance with ethical standards: the study was approved by the local Ethics Committee of Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology and Immunology.

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Received: 16.05.2024 **Accepted:** 02.06.2024 **Published online:** 24.06.2024

DOI: 10.24075/brsmu.2024.024

ТИАМИН-ЗАВИСИМАЯ МЕГАЛОБЛАСТНАЯ АНЕМИЯ (СИНДРОМ РОДЖЕРСА) У РЕБЕНКА ТРЕХ ЛЕТ

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Тиамин-зависимая мегалобластная анемия (ТЗМА), или синдром Роджерса, — редкое аутосомно-рецессивное заболевание, характеризующееся развитием мегалобластной анемии, сахарным диабетом и прогрессирующей нейросенсорной тугоухостью. У части пациентов выявляют офтальмологические нарушения (пигментную ретинопатию, атрофию зрительного нерва, макулопатию, нистагм), поражение сердца (пароксизмальную мерцательную аритмию, наджелудочковую тахикардию, врожденные пороки сердца, нарушения внутрисердечной проводимости) и неврологические нарушения (эпилепсию, нарушение мозгового кровообращения). ТЗМА развивается вследствие мутации в гене *SLC19A2*, кодирующем белок-переносчик тиамина ThTr-1, который экспрессируется в гемопоэтических стволовых клетках, бета-клетках поджелудочной железы и клетках внутреннего уха. В статье представлен клинический случай ТЗМА у ребенка трех лет, с дебютом заболевания на первом году жизни в виде анемии и сахарного диабета. На фоне проводимой терапии тиамином у пациента достигнута выраженная положительная динамика в виде нормализации показателей периферического анализа крови. Описание собственного клинического наблюдения, а также представление обзора литературы направлено на повышение осведомленности врачей всех специальностей о данном синдроме. Атипичная клиническая картина и отсутствие знаний о ТЗМА часто являются причиной задержки постановки диагноза и начала терапии.

Ключевые слова: тиамин-зависимая мегалобластная анемия, синдром Роджерса, костный мозг, кольцевые сидеробласты, сахарный диабет, нейросенсорная глухота, ген *SLC19A2*

Вклад авторов: Е. В. Трухина — сбор данных, подготовка списка литературы; Т. В. Конюхова — разработка дизайна статьи, написание текста рукописи.

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Статья получена: 16.05.2024 **Статья принята к печати:** 02.06.2024 **Опубликована онлайн:** 24.06.2024

DOI: 10.24075/vrgmu.2024.024

Thiamine responsive megaloblastic anemia (TRMA, OMIM 249270), or Rogers syndrome, is an extremely rare autosomal recessive disease with a characteristic medical triad: megaloblastic anemia, progressive sensorineural hearing loss, and diabetes mellitus. Some patients are diagnosed with congenital heart defects, optic atrophy, and cerebrovascular disorders [1–3]. Hematological manifestations include ring sideroblasts in bone marrow; leukopenia and thrombocytopenia may be moderately pronounced or lacking, and pancytopenia is rarely detected [4, 5]. TRMA develops as a result of changes in the *SLC19A2* gene that encodes thiamine transporter 1 (ThTr-1). The *SLC19A2* gene is expressed mainly in hematopoietic stem cells, pancreatic beta cells, and inner ear cells [6]. In other organs, thiamine is brought to cells by ThTr-2 transporter protein, which remains functionally active [7].

There is no reliable information about prevalence of TRMA. There have been described 183 patients from 138 families. The majority of cases (62%) were registered in the families where spouses are closely related to each other (37.7% in the Middle East, 21.9% in South Asia, and 17% in the Mediterranean countries), with the debut during infancy and adolescence [8]. The disease was first described by L.E. Rogers et al in 1969; the patient was an 11-year-old girl with bilateral sensorineural hearing loss, diabetes mellitus, and recurrent megaloblastic

Table 1. Interpretation of the patient's hemogram

Indicators	08.02.2024	Unit of measurement	Normal value
Leukocytes	9.12	10^9/l	6.05–9.85
Erythrocytes	2.55	10^{12}/l	4.2–4.6
Hemoglobin	74	g/l	115–138
Hematocrit	20.8	%	31–40
Average erythrocyte volume	81.6	fL	75–100
Average hemoglobin content in an erythrocyte	29	Pg	25–33
Average hemoglobin concentration in an erythrocyte	356	g/l	322–368
Relative red cell distribution width	15.4	%	11–16
Platelets	103	10^9/l	204–356

anemia. It was assumed that anemia stems from disruptions of metabolism of vitamin B1, a hypothesis later confirmed when the condition was alleviated by oral administration of thiamine [9].

Case description

A three-year-old boy, first pregnancy, first vaginal birth at 38–39 weeks, birth weight — 3280 g, height — 51 cm. The Apgar score was 7/8 points. Boy's ethnicity — ingush. His parents are not closely related to each other. Case history: at the age of 10 months, CBC revealed a drop in hemoglobin count to 85 g/l; the patient received iron preparations for two months, to no effect. Further on, the boy periodically needed transfusions of packed red cells. At the age of one year, he was diagnosed with diabetes mellitus (hyperglycemia — up to 31 mmol/l, glycated hemoglobin — 9.1%). Prescriptions: insulin therapy in the basic bolus mode (aspart and degludec). At the age of two, parents noticed the child's problems with speech and hearing. Examination confirmed stage 4 bilateral sensorineural hearing loss. At the age of three, CBC revealed anemia (hemoglobin — 62 g/l) and thrombocytopenia (platelets — 18 thousand/ ml). The combination of conditions — diabetes mellitus, sensorineural hearing loss, anemia, thrombocytopenia — pointed to TRMA as the most likely reason thereof. On 08.02.2024, the child was admitted to the Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology and Immunology for clarification of the diagnosis and development of further therapy tactics.

Upon admission, the condition is severe per the disease, stable, no fever. Physical development typical for the age. The skin is pale, with individual petechiae on the trunk. No swelling. The tongue is clean and moist. Regular musculoskeletal system. The peripheral lymph nodes not enlarged. Cardiovascular system: heart tones clear, tachycardia up to 128–132 beats per minute. Puerile breathing in the the lungs, even over all fields, no wheezes. The abdomen soft, painless, the liver protrudes 2 cm from under the costal arch, spleen not palpable. Diuresis not factored in. Daily stool without pathological impurities.

Tables 1, 2 present the main indicators of the patient at admission to the Dmitry Rogachev National Research Medical Center. There were signs of anemia, thrombocytopenia, as well as high counts of lactate and glycosylated hemoglobin (7.78%).

The patient underwent a bone marrow puncture from two anatomical points (iliac ridges). Morphology of bone marrow (BM) aspirate was studied using light-optical microscopy (smears stained as per the Pappenheim-Kryukov method). Table 3 presents data on the BM cells differentiation and their percentage ratio.

Both punctates are rich in myelocaryocytes, polymorphic and similar in composition; both include a small amount of neutral fat, clusters of stromal elements.

The content of blast cells is 0.8% and 0.4% (Point 1 and Point 2, respectively).

Neutrophilic lineage preserved, with some cells showing signs of dyspoesis (hypogranularity, annular neutrophils). Monocytic lineage preserved, no significant morphological peculiarities. Lymphoid lineage narrowed. Erythroid lineage expanded, with features of dyspoesis (binuclearity, Howell-Jolly bodies, karyorrhexis). Erythropoiesis with megaloblastoid features in most cells (Fig. 1). Hemoglobinization delayed in basophilic forms. Megakaryocytic lineage expanded, represented by megakaryocytes at different stages of maturation. Part of the megakaryocytes with platelet release. Perls staining gives a positive reaction in 50% of erythrocaryocytes, with 45% of erythrocaryocytes classified as ring sideroblasts (Fig. 2), and 5% having the positive material in the form of a few scattered granules. The reaction was negative in 50% of erythrocaryocytes.

Instrumental studies

Electrocardiography. Normal position of cardiac electric axis. Accelerated atrial rhythm, against this background — frequent supraventricular extrasitolia (single and paired premature contractions), with the average heart rate (HR) of 149 beats/min. Complete blockade of the atrioventricular band's right leg.

Echocardiography. The study had tachyarrhythmia in the background. Both atria enlarged sharply. Left ventricle enlarged slightly, global systolic function at the lower limit of normal, may be slightly reduced. Systemic cardiac output maintained above average. Right ventricle enlarged moderately, global systolic function reduced. Secondary (possibly due to disrupted local kinetics in the left ventricular myocardium) mild mitral valve insufficiency.

Holter monitoring. Tachycardia registered through the day; all average heart rate values above normal for the patient's age. Rigid circadian rhythm profile. Recurrent supraventricular tachycardia. Frequent polytopic ventricular extrasystole. Frequent atrial extrasystole.

Table 2. Interpretation of the patient's biochemical blood assay

Indicators	08.02.24	Unit of measurement	Normal value
Glucose	4.11	mmol/l	2.6–24.9
Lactate	2.1	mmol/l	0.5–1.6
Iron	13.2	mmol/l	9–21.5

Table 3. Patient's BM aspirate examination

Cellular elements	Normal, %	Point 1, %	Point 1, %
Undifferentiated blast cells	1.6–3.4	0.8	0.4
Myeloblasts	1.6–3.0	1.2	0.8
Promyelocytes	2.3–4.0	4	3.6
Myelocytes	7.2–11.3	13.6	24
Metamyelocytes	5.5–8.5	8	7.2
Band neutrophils	14.8–22.4	18	9.2
Segmented neutrophils	9.8–20.5	18.4	20.8
Neutrophils count	40.0–66.7	62	64.8
Eosinophilic myelocytes		–	–
Eosinophilic metamyelocytes		–	–
Band eosinophils		–	–
Segmented eosinophils		2.4	2.4
Eosinophils count	3.3–6.4	2.4	2.4
Basophils	0–0.2	0.8	0.4
Promonocytes		–	–
Monocytes	0.03–3.0	3.2	2.8
Monocytes count		3.2	2.8
Lymphocytes	12.1–17.9	2.4	2.4
Plasma cells	0.03–0.3	–	–
Erythroblasts	1.0–1.9	3.2	1.6
Basophilic normoblasts	1.3–2.4	8.8	8
Polychromatophilic normoblasts	8.2–10.8	13.2	12.4
Oxyphilic normoblasts	5.9–8.8	2	4
Erythrocaryocytes count	16.4–23.9	27.2	26
Neutrophil maturation index	0.5–0.9	0.7	1.16
Hemoglobinization index	0.8–0.9	0.56	0.63
Leuko-erythroblastic ratio	3.3–4.5	2.6	2.8

Cardiologist's opinion. Atrial fibrillation, supraventricular extrasystole. Dilated cardiomyopathy (primary? arrhythmogenic myocardial dysfunction?) with a slightly decreased contractility of the left ventricle's myocardium. Second stage mitral valve insufficiency, first stage tricuspid valve insufficiency. First degree heart failure. Recommendation — antiarrhythmic therapy: amiodarone (200 mg), 100 mg orally in the morning and 50 mg orally in the evening; torasemide (5 mg) half a pill twice a day p.os.; potassium and magnesium asparaginate, one pill twice a day orally; propranolol 5 mg 3 times a day after meals.

Ophthalmologist's opinion. OU-hypermetropia/moderate myopic astigmatism. Partial atrophy of the optic nerves.

Sum total of clinical and laboratory data: megaloblastic anemia concomitant with thrombocytopenia, ring sideroblasts in BM; endocrine disorders (diabetes mellitus); sensorineural hearing loss; cardiac arrhythmia may be attributable to TRMA. Full exome sequencing recommended to confirm diagnosis. Specific thiamine therapy initiated: 150 mg/m^2/day (100 mg/day) IV (from 09.02 to 29.02). From 01.03 — thiamine 150 mg/m^2/day (100 mg/day) orally.

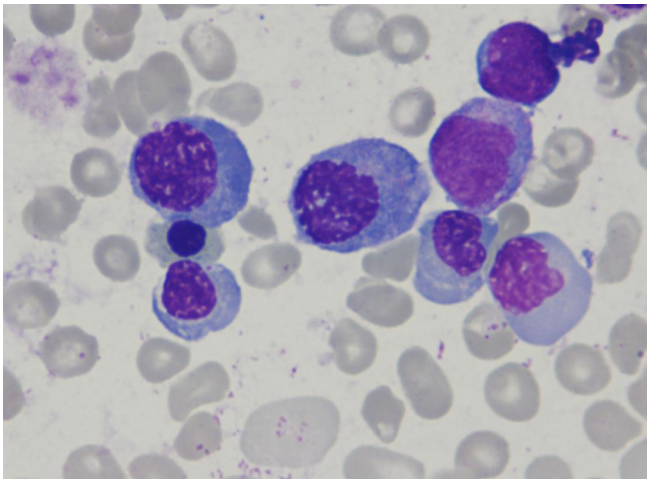

Fig. 1. Bone marrow: megaloblastic type of erythropoiesis. Pappenheim–Kryukov staining. Magnification: ×1000

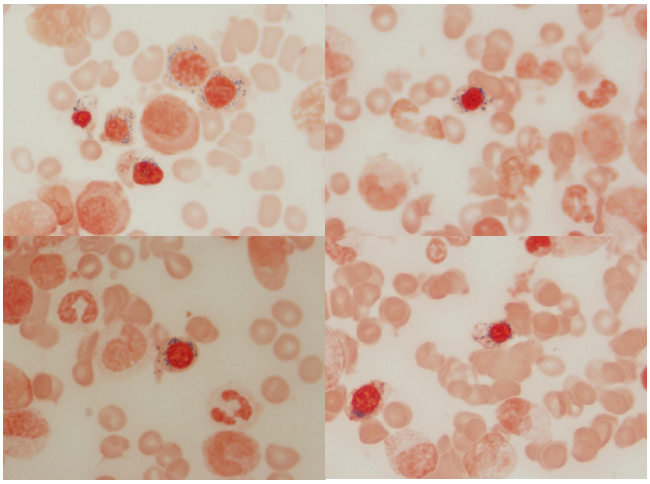

Fig. 2. Bone marrow: ring sideroblasts. Perls Prussian blue staining. Magnification: ×1000

Table 4. Interpretation of the patient's hemogram

Indicators	29.02.24	Units of measurement	Normal value
Leukocytes	10.26	10^9/l	6.05–9.85
Erythrocytes	3.88	10^{12}/l	4.2–4.6
Hemoglobin	112	g/l	115–138
Hematocrit	33.2	%	31–40
Average erythrocyte volume	85.6	fL	75–100
Average hemoglobin content in an erythrocyte	28.9	pg	25–33
Average hemoglobin concentration in an erythrocyte	337	g/l	322–368
Relative red cell distribution width	14.9	%	11–16
Platelets	343	10^9/l	204–356

The study revealed the *SLC19A2* gene to have a nucleotide replacement C.1223+1G > A in the homozygous state. Thiamine responsive megaloblastic anemia diagnosed based on the results of the studies.

Thiamine therapy yielded a pronounced positive effect: peripheral blood parameters normalized and remained stable (Table 4).

Case discussion

The article presents a rare case of TRMA in a three-year-old patient, which manifested as anemia, diabetes mellitus, and sensorineural hearing loss in the first year of life. This complex of symptoms points to TRMA as a possible disease. According to the previously published papers covering patients with TRMA, anemia was detected in 95.4% of cases, diabetes mellitus in 92.7%, hearing loss in 92.7%. Megaloblastic anemia was diagnosed in 70.8% of patients [8]. Further diagnosing efforts revealed the patient to have megaloblastic, sideroblastic anemia in BM, as well as visual impairment and cardiac arrhythmia. Thus, given the presence of the classic TRMA medical triad (megaloblastic anemia, diabetes mellitus, and sensorineural hearing loss) and the effectiveness of thiamine therapy, the clinical diagnosis is beyond doubt.

Thiamine responsive megaloblastic anemia is a very rare disease, which lowers the level of clinical alertness and complicates diagnosing. In our case, the child was diagnosed only when he reached the third year of life.

Currently, the only TRMA treatment strategy is lifelong thiamine therapy, which alleviates anemia symptoms in 97.1% of patients and allows decreasing the dose of insulin or discontinuation of its intake in in 69.9% of patients [8].

CONCLUSION

Thiamine responsive megaloblastic anemia is an extremely rare autosomal recessive disease characterized by a number of pathological conditions such as megaloblastic anemia, diabetes mellitus, loss of hearing and vision. The variety of clinical manifestations requires a multidisciplinary approach to TRMA at both the treatment and the follow-up stages. The diseases has been studied well, yet the only strategy available currently is oral administration of high doses of thiamine to address anemia. Thiamine treatment reduces the need for insulin and can delay the onset of diabetes mellitus, as well as prevent vision loss. Hearing loss in TRMA is irreversible and cannot be prevented with thiamine. Currently, genetic counseling is the most effective approach to prevention of this disease. Relatives of a TRMA patient should undergo a molecular genetic study together with their partners to determine the risk of bearing a sick child. In addition, pregnant women running a high risk of giving birth to a child with TRMA should be screened accordingly, because administration of thiamine during pregnancy can minimize or delay the appearance of symptoms of the disease.

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