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## DEPUTY EDITOR-IN-CHIEF'S PREFACE

## DEPUTY EDITOR-IN-CHIEF'S PREFACE TO ISSUE 3, 2026

Lyubov V. Rychkova

Dr. Sc. (Med.), Professor,  
Corresponding Member  
of the RAS

Dear colleagues!

What is a scientific article? It is not only a logically complete, structured authorial work devoted to a specific scientific problem, phenomenon, research method, etc. It is a means of conveying scientific knowledge, experience, and the efforts of entire groups of scientists, presented in a concise form and intended for familiarization, critical analysis, and application in further work by the scientific community and practicing specialists. The summer issue of the journal *Acta Biomedica Scientifica* includes experimental studies to which we would particularly like to draw the readers' attention.

Two papers are devoted to tuberculosis research, a pressing problem for many regions. A team of authors from Moscow (**Reshetnikov M.N. et al.**) presented a new perspective on the alimentary route of tuberculosis infection using a rabbit model. The second study, also conducted by a team from Moscow (**Zaitseva A.S. et al.**), performed a comparative analysis of the characteristics of macrophage interactions with antigens of tuberculous and non-tuberculous mycobacteria *in vitro*. More detailed results can be found in the relevant section of the journal.

Emotional overeating has long been considered one of the foremost problems of the 21<sup>st</sup> century. However, some neurohormonal mechanisms in the pathogenesis of this pathological process remain obscure. Authors from Saint Petersburg (**Dedanishvili N.S. et al.**) investigated the dose- and time-dependent effects of the dual GIP/GLP-1 receptor agonist Tirzepatide on emotional overeating in a rat model of intracranial self-stimulation. The study demonstrated that the drug exerted an effect upon repeated administration, manifesting as a reduction in food intake on days 3 and 7 of the experiment.

The pediatrics section proved to be equally interesting. A team of authors from Novosibirsk (**Izyumov A.O. et al.**) analyzed the course of chronic adenoiditis in children aged 3-6 years who received probiotic support. Probiotics demonstrated efficacy in alleviating breathing difficulties and sleep disturbances.

In Voronezh (**Bavykina I.A. et al.**), the dynamics of intestinal fatty acid-binding protein (I-FABP) and Claudin-2 in the blood serum of children during the first 24 months of life were studied. According to the results, the interpretation of these markers requires consideration of age and gestational age.

A team of researchers from Irkutsk (**Gomellya M.V. et al.**) presented an interesting clinical case of hereditary hypofibrinogenemia in a pediatric outpatient setting. The case highlights the importance of more thorough investigation into inherited coagulopathies in patients with abnormal coagulation tests, even when there are no clinical symptoms present.

Fields such as oncology and COVID-19 continue to enrich the medical knowledge base with new methods and data. The authors from Irkutsk (**Shcherbatykh A.V. et al.**) investigated the use of low-intensity photodynamic therapy for stage I basal cell carcinoma. This development not only improves the efficacy of cancer treatment, but also significantly reduces healthcare costs for certain types of cancer.

The impact of the COVID-19 pandemic on the dynamics of pediatric hospitalizations for acute intestinal infections, including rotavirus infection, in Chita, was studied by a team from the Trans-Baikal Territory (**Andreeva E.V. et al.**), and the prediction of risk of post-COVID disorder using NLRP3 and GSDMD markers

in patients with bronchial asthma who recovered from COVID-19, was presented by authors from Vladivostok (**Lobova T.G. et al.**). Both studies are of interest to a wide readership.

We would like to highlight two particularly significant studies. The first study was conducted in cooperation between several leading educational institutions in Penza, Grozny, Astrakhan, and Palermo, Italy (**Zenin O.K. et al.**), who compared the outcomes of predicting the diameters of daughter segments of coronary artery bifurcations under normal conditions using graph neural networks and well-known numerical modeling methods. The second study was conducted by three prominent research institutions in Yakutsk and Moscow (**Prokopyev E.S. et al.**) with regard to implementing a two-component model for the clinical and radiological screening of pulmonary tuberculosis in the thinly populated area of the Republic of Sakha (Yakutia). Both studies will contribute significantly to the quality and efficiency of data processing, even in remote areas.

*Another significant event occurred during the preparation of this issue of the journal. On May 31, Professor **Oleg L. Lakhman**, a Corresponding Member of the Russian Academy of Sciences, Doctor of Medical Sciences, Professor, and Director of the East Siberian Institute of Medical and Environmental Research, turned 60 years old! On behalf of all colleagues and the editorial board, we would like to extend our heartfelt congratulations to Oleg Leonidovich on this milestone occasion. We wish him excellent health and continued success in all his professional pursuits.*

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## ПРЕДИСЛОВИЕ ЗАМЕСТИТЕЛЯ ГЛАВНОГО РЕДАКТОРА К № 3 (2026)

**Рычкова  
Любовь Владимировна**

**д.м.н., профессор,  
член-корреспондент РАН**

Уважаемые коллеги!

Что такое научная статья? Это не только логически законченное, структурированное авторское произведение, посвященное конкретной научной проблеме, явлению, методу исследования и т.д. Это способ передачи научных знаний, опыта, усилий целых групп ученых, изложенных в сжатой форме и предназначенных для ознакомления, осмысления и применения в дальнейших работах научным сообществом и практикующими специалистами. В летний выпуск журнала "Acta Biomedica Scientifica" вошли экспериментальные исследования, на которые особенно хотелось бы обратить внимание читателей.

Это две работы по исследованию туберкулеза — актуальной проблеме для многих регионов. Коллектив авторов из Москвы (**Решетников М.Н. и соавт.**) представил новый взгляд на алиментарный путь заражения туберкулезом с использованием кроличьей модели. Второе исследование также представил коллектив из Москвы (**Зайцева А.С. и соавт.**), проведя сравнительный анализ особенностей взаимодействия макрофагов с антигенами туберкулезных и нетуберкулезных микобактерий *in vitro*. Более подробно с результатами можно ознакомиться в соответствующем разделе журнала.

Эмоциональное переживание уже давно считается одной из первостепенных проблем XXI века. Но некоторые нейрогормональные механизмы в патогенезе данного патологического процесса остаются в тени. Авторы из Санкт-Петербурга (**Деданишвили Н.С. и соавт.**) выяснили дозо- и времязависимое влияние двойного агониста рецепторов GIP/GLP-1 Тирзепатида на эмоциональное переживание в модели электрической самостимуляции мозга у крыс. В результате исследования препарат оказал влияние при повторном введении, проявляющемся снижением потребления пищи на 3-и и 7-е сутки эксперимента.

Не менее интересным получился раздел педиатрии. Коллективом авторов из Новосибирска (**Исюмов А.О. и соавт.**) проанализировано течение хронического аденоидита у детей 3–6 лет на фоне пробиотической поддержки. Препарат показал свою эффективность в части облегчения затрудненности дыхания и нарушения сна.

В Воронеже (**Бавыкина И.А. и соавт.**) изучили динамику интестинального белка, связывающего жирные кислоты и Клаудин-2 в сыворотке крови у детей в первые 24 месяца жизни. Согласно результатам, интерпретация маркеров требует учета возраста и срока гестации.

А вот коллективом авторов из Иркутска (**Гомелля М.В. и соавт.**) представлен интересный клинический случай наследственной гипофибриногенемии в амбулаторно-поликлинической практике врача-педиатра. Как показал описанный случай, требуется более тщательное изучение наследственных коагулопатий у пациентов с изменениями коагулограммы даже при отсутствии клинической симптоматики.

Такие направления как онкология и COVID-19 стабильно пополняют базу медицинских разработок новыми методами и данными. Авторы из Иркутска (**Щербатых А.В. и соавт.**) изучали применение способа низкоинтенсивной фотодинамической терапии при базальноклеточном раке кожи I стадии. Данная разработка не только повышает эффективность лечения

раковых заболеваний, но и значительно сокращает расходы на лечение некоторых его видов.

Влияние пандемии COVID-19 на динамику госпитализаций детей в Чите с острыми кишечными инфекциями, в том числе с ротавирусной инфекцией, изучал коллектив авторов из Забайкалья (**Андреева Е.В. и соавт.**), а прогноз риска развития постковидных нарушений с использованием маркеров NLRP3 И GSDMD у пациентов с бронхиальной астмой, перенесших COVID-19, представили авторы из Владивостока (**Лобова Т.Г. и соавт.**). Оба исследования будут интересны широкому кругу читателей.

Отдельно хотелось бы отметить два немаловажных исследования. Первое проведено в коллаборации нескольких крупных образовательных учреждений г. Пензы, Грозный, Астрахани и Палермо (Италия) (**Зенин О.К. и соавт.**), ученые которых сравнили результаты прогнозирования диаметров дочерних сегментов бифуркаций венечных артерий в норме, полученных с использованием графовых нейронных сетей и известных методов численного моделирования. А второе проведено тремя крупнейшими научными учреждениями Якутска и Москвы (**Прокопьев Е.С. и соавт.**) о внедрении в практику двухкомпонентной модели клинико-рентгенологического скрининга туберкулеза легких в условиях малонаселенной территории Республики Саха (Якутия). Оба исследования внесут значительный вклад в качество и скорость обработки данных даже в отдаленных регионах.

*Еще одно знаменательное событие произошло за период подготовки данного номера журнала – 31 мая профессору РАН, доктору медицинских наук, профессору, директору ФГБНУ «Восточно-Сибирский институт медико-экологических исследований» **Олегу Леонидовичу Лахману** исполнилось 60 лет! От лица всех коллег и редакции журнала еще раз поздравляем Олега Леонидовича с этой знаменательной датой и желаем крепкого здоровья и дальнейших успехов в его профессиональной деятельности!*

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К 60-летию со дня рождения Лахмана Олега Леонидовича

## INFECTIOUS DISEASES

### RISK PREDICTION OF POST-COVID CONDITIONS USING NLRP3 AND GSDMD MARKERS IN PATIENTS WITH BRONCHIAL ASTHMA AFTER COVID-19

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#### ABSTRACT

**Background.** One of the key factors in triggering the hyperinflammation cascade in patients with bronchial asthma and COVID-19 is the activation of NLRP3 cytosolic complexes and the GSDMD proteins by SARS-CoV-2, which are effector molecules for the lytic and inflammatory forms of pyroptosis.

**Aim.** To determine prognostic criteria for worsening of mild bronchial asthma following an infectious disease caused by SARS-CoV-2.

**Materials and Methods.** In patients with mild, partially controlled bronchial asthma ( $n = 86$ ), who were divided into 2 groups: asthma without COVID-19 ( $n = 34$ ) and asthma following mild COVID-19 ( $n = 52$ ), peripheral blood inflammatory markers were evaluated. The patients were examined immediately after recovery and confirmation of a negative SARS-CoV-2 test result, and six months post-infection. The control group included conditionally healthy volunteers ( $n = 15$ ) with no history of COVID-19. Pulmonary function was monitored by measuring FVCL, OFV1, and OFV1/FVCL. NLRP3 and gasdermin D inflammasomes were measured by enzyme-linked immunosorbent assay.

**Results.** The study revealed that in a number of patients with mild asthma, who had post-COVID conditions 6 months after COVID 19, there were elevated levels of NLRP3 inflammasome and gasdermin D (GSDMD), as well as a decreased pulmonary function, indicating a hyperinflammatory process. A predictive model for worsening of mild asthma after COVID-19 was developed: concurrent elevation of NLRP3  $> 0.04$  ng/ml and GSDMD  $> 0.38$  ng/ml levels during the convalescence period serves as a marker of a high risk of post-COVID complications.

**Conclusion.** The role of NLRP3 and GSDMD in exacerbating the course of asthma after COVID-19 has been established, and predictive threshold values of biomarkers in this patient population have been identified.

**Keywords:** bronchial asthma, post-COVID syndrome, inflammatory markers, NLRP3, gasdermin D

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## ПРОГНОЗ РИСКА РАЗВИТИЯ ПОСТКОВИДНЫХ НАРУШЕНИЙ С ИСПОЛЬЗОВАНИЕМ МАРКЕРОВ NLRP3 И GSDMD У ПАЦИЕНТОВ С БРОНХИАЛЬНОЙ АСТМОЙ, ПЕРЕНЕСШИХ COVID-19

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### РЕЗЮМЕ

**Обоснование.** Одним из ключевых факторов запуска каскада гипервоспаления при сочетании бронхиальной астмы и COVID-19 предполагается активация вирусом SARS-CoV-2 цитозольных комплексов NLRP3 и белка GSDMD, являющихся эффекторными молекулами для литической и воспалительной формы пироптоза.

**Цель.** Определение прогностических критериев утяжеления течения легкой бронхиальной астмы (БА) на фоне перенесённого инфекционного заболевания, вызванного SARS-CoV-2.

**Материалы и методы.** У пациентов с легкой, частично контролируемой бронхиальной астмой ( $n = 86$ ), которые были разделены на 2 группы: БА без COVID-19 ( $n = 34$ ) и БА после легкой формы течения COVID-19 ( $n = 52$ ) проведена оценка маркеров воспаления в периферической крови. Пациенты были обследованы сразу после выздоровления и подтверждения отрицательного результата теста на SARS-CoV-2, и через шесть месяцев после заражения. В контрольную группу вошли условно здоровые добровольцы ( $n = 15$ ), которые не болели COVID-19. Проводился мониторинг функции внешнего дыхания ФЖЕЛ, ОФВ1, ОФВ1/ФЖЕЛ. Инфламмосомы NLRP3, газдермин D определяли методом иммуноферментного анализа.

**Результаты.** Исследование выявило, что у ряда пациентов с легкой астмой, у которых спустя 6 месяцев после COVID-19 сохраняются постковидные нарушения, отмечено повышение уровней инфламмосомы NLRP3 и газдермина D (GSDMD), а также снижение функции внешнего дыхания, что указывает на гипервоспалительный процесс. Разработан способ прогноза утяжеления лёгкой астмы после COVID-19: одновременное превышение уровней  $NLRP3 > 0,04 \text{ ng/ml}$  и  $GSDMD > 0,38 \text{ ng/ml}$  в периоде реконвалесценции служит маркером высокого риска постковидных осложнений.

**Заключение.** Установлена роль NLRP3, GSDMD в утяжелении течения БА после COVID-19, выделены прогностические пороговые значения биомаркеров у данного контингента пациентов.

**Ключевые слова:** бронхиальная астма, постковидные нарушения, маркеры воспаления, NLRP3, газдермин D

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## INTRODUCTION

Although COVID-19 is no longer classified as a pandemic, it remains a significant clinical problem. The consequences of the infection are particularly serious, especially in immunocompromised individuals, who include patients with asthma [1–3]. According to the WHO, CENTRAL, and Rub Med electronic databases, the overall prevalence of asthma among COVID-19 patients was 8% between December 1, 2019 and July 2021, [4]. In Russia, the proportion of asthma patients who have had COVID-19 reaches up to 15%, depending on the region. This group of patients experiences prolonged recovery [3, 4]. The systematic review “Post-COVID-19 Symptoms in Adults with Asthma” reported post-COVID-19 disorders in asthmatic individuals, including shortness of breath during exercise (75.4%) and at a rest (34.4%), cough (2.1%), and increased fatigue (21.2–65.6%). According to some data, asthmatics have a 1.5–2 times higher risk of these disorders than people without asthma [5].

A pressing issue remains the search for key links in the pathogenesis of persistent systemic inflammation in people with respiratory diseases in the post-COVID period. Current evidence suggests that SARS-CoV-2 directly or indirectly activates polyprotein complexes (inflammasomes) in the intracellular space. NOD-like receptors (NLRs) are a family of intracellular sensors that initiate the assembly of multi-protein complexes (inflammasomes) in response to tissue damage or pathogenic invasion. These structures act as a molecular platform for the activation of caspase-1, a cysteine protease that processes and secretes the active forms of the proinflammatory cytokines IL-1 $\beta$  and IL-18 [6]. NLRP3, an inflammasome, is involved in the recognition of a wide range of viral, bacterial, and fungal agents, as well as endogenous danger signals, and has been characterized in great detail. The high level of NLRP3 expression in the respiratory tract epithelium and immunocompetent cells (monocytes, neutrophils, T-lymphocytes) determines its key role in modulating the respiratory immune response [7]. This mechanism triggers the pyroptotic pathway of inflammation, which may contribute to the formation of post-COVID disorders in patients with asthma [7, 8]. However, currently, there is limited information on the effect of an inflammasome-mediated inflammatory response on the long-term consequences of COVID-19 in people with asthma.

## AIM

To determine prognostic criteria for worsening of mild bronchial asthma following an infectious disease caused by SARS-CoV-2.

## MATERIALS AND METHODS

A total of 101 subjects were enrolled after providing written informed consent. Of these, 52 individuals

had mild persistent, partially controlled asthma and had experienced mild COVID-19 confirmed by PCR testing. Thirty-four patients had mild partially controlled asthma and had not been infected with SARS-CoV-2. The control group consisted of 15 individuals without asthma, chronic bronchopulmonary disease, and a history of COVID-19, as confirmed by PCR and ELISA tests at the time of sample collection. The study groups were comparable in terms of sex and age. 62% of them were women and 38% were men. The average age of the subjects was  $42.0 \pm 5.3$  years. The diagnosis of bronchial asthma was verified based on the criteria of the Global Strategy for the Treatment and Prevention of Bronchial Asthma (GINA), Federal Clinical Guidelines, and in accordance with the International Classification of Diseases 10<sup>th</sup> Revision (ICD-10).

The ACQ-5 (Asthma Control Questionnaire) test was used to determine the level of disease control. A score from 0.75 to 1.5 indicates partially controlled bronchial asthma, and less than 0.75 indicates good asthma control [GINA 2024]. The study was conducted in accordance with the Declaration of Helsinki (1978, revised in 2024) and approved by the Ethics Committee of the Vladivostok branch of the Far Eastern Scientific Center for Physiology and Pathology of Respiration (Protocol No. 9, dated November 24, 2021).

**Inclusion criteria were:** mild partially controlled asthma. Before enrollment, patients with a confirmed diagnosis of mild asthma had been under regular follow-up for at least 1 year and were regularly receiving maintenance therapy with inhaled formoterol + budesonide (6+160 mcg/dose, 1–2 doses twice daily and as needed).

**Exclusion criteria** included acute infectious diseases, chronic internal organ diseases in the acute phase, decompensated chronic heart failure, and uncontrolled asthma.

Patients were enrolled in the first half of 2021 and examined at two time points: early (after recovery and confirmation of a negative SARS-CoV-2 test) and at 6 months. At the time of inclusion in the study and throughout the entire observation period, all subjects underwent PCR testing for SARS-CoV-2 and ELISA testing for immunoglobulins of class G (IgG) to SARS-CoV-2. Participants completed questionnaires on the course and condition of asthma, underwent ACQ testing, and providing information on the timing of COVID-19 infection, symptoms, prescribed medications, and allergic reactions. Post-COVID disorders included asthenic syndrome (increased fatigue, weakness, shortness of breath or palpitations, a feeling of air hunger, sweating; during significant, accelerated or particularly prolonged exertion (effort); impaired concentration, memory loss, sleep disorders, and anxiety). The state of the external pulmonary function (EPF) was assessed using a SPIROLAB III device, with measurement of forced vital lung capacity (FVC), forced expiratory volume in 1 second (FEV1), and the FEV1/FVC ratio. Values were recorded as absolute values and as percentages of predicted values. Disease control was assessed using the ACQ test.

Venous blood was collected in the morning on an empty stomach into tubes with a clot activator

for determination of the inflammatory markers NLRP3 and gasdermin D. Blood samples were centrifuged and serum was collected and frozen at  $-20^{\circ}\text{C}$ , then stored at  $-80^{\circ}\text{C}$ . ELISA was performed using a BEP 2000 analyzer (Siemens, Germany) with commercially available kits for NOD-like receptor protein 3 (NLRP3) (BIOMATIK ELISA Kit, Israel) and gasdermin D (GSDMD) (Human GSDMR Simple Step ELISA Kit Abcam, Great Britain) according to the manufacturers' instructions. The study of SARS-CoV-2 was conducted by quantitative ELISA method using Vector-Best JSC (Russia) on an ALISEI analyzer (Italy).

SARS-CoV-2 viral RNA was detected using the PCR and the SARS-CoV-2 AmpliTest test system. The DTprime detection amplifier (NPO DNA Technology LLC, Russia) and Rotor-Gene Q (Qiagen, Germany) were used. The study for the presence of antibodies to SARS-CoV-2 was carried out using the quantitative ELISA method, following the instructions for the reagent kits (Vector-Best JSC, Russia) on an ALISEI analyzer (Italy).

Statistical processing was performed using Statistica 10. Data was presented as medians and quartiles. Statistical significance was set at  $p < 0.05$ . Between-group differences were assessed using the Mann – Whitney and Kruskal – Wallis tests. The Bonferroni correction was used to solve the problem of multiple comparisons. Asthma patient values were compared with corresponding values from healthy individuals (control group), taken as 100 %.

ROC analysis was used to determine the cutoff thresholds for the investigated biomarkers. The ROC curve is a graphical representation that allows for the evaluation of the quality of a diagnostic test. It reflects the ratio between the proportion of true-positive test results [sensitivity of the test] and the proportion of false-positive test results [test specificity] with varying separation thresholds. The quantitative interpretation of the ROC curve

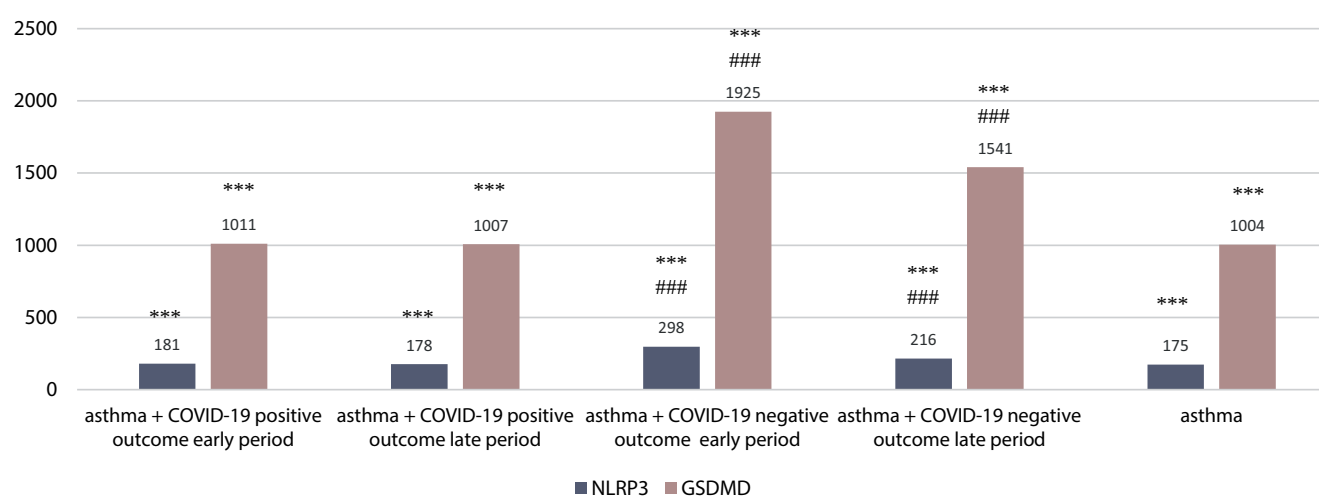
is provided by the AUC indicator. The higher the AUC value, the better the classifier. A value of 0.5 indicates the unsuitability of the chosen classification method [9].

## RESULTS

Based on six-month follow-up results, the main group was divided into 2 subgroups of patients based on their clinical and laboratory status. In the subgroup with positive outcomes ( $n = 34$ , 67 %; no post-COVID disorders), normalization of clinical and functional parameters and a decrease in hyperinflammatory rates were observed during this period, reaching values comparable to those in the asthma group without SARS-CoV-2.

The following dynamics of inflammatory markers were observed in patients with a positive outcome: normalization of clinical and functional parameters and a decrease in hyperinflammation indicators to values comparable to those found in the group of individuals with A, who did not have SARS-CoV-2.

High levels of hyperinflammation markers (Fig. 1) were detected in the subgroup with negative outcomes ( $n = 18$ , 33 %; post-COVID disorders) at 6 months after COVID-19. These patients had symptoms indicative of persistent post-COVID disorders (asthenic syndrome with increased fatigue, weakness, shortness of breath or palpitations, a feeling of air hunger, sweating during significant, accelerated or particularly prolonged exertion; impaired concentration, memory loss, sleep disorders, and anxiety). In patients with a negative outcome, the following marker dynamics were observed: at the initial stage, they were significantly (nearly twice) higher than in the comparison group. At the late stage, there is a decrease in both indicators (NLRP3 by 82 % ( $p \leq 0.001$ ), and gasdermin D by 384 % ( $p \leq 0.001$ )) relative to the initial values. However,



**Note.** Hyperinflammation markers are expressed as a percentage relative to the control group. \* – significant difference compared to the control group; # – significant difference between favorable and unfavorable outcome subgroups. \*\*\* –  $p < 0.001$ , ### –  $p < 0.001$ .

**FIG. 1.**

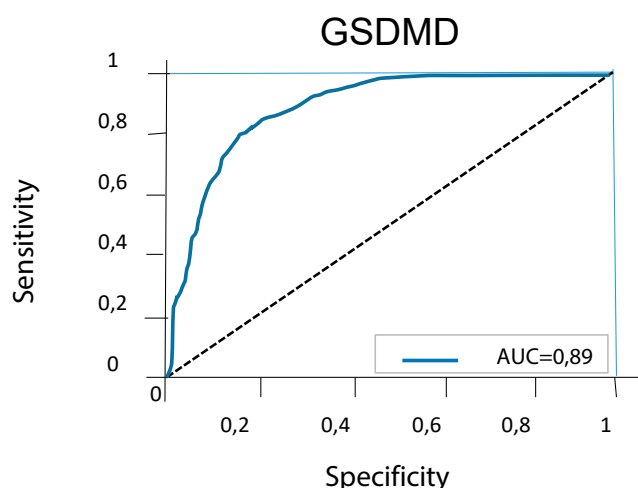
NLRP3 and gasdermin D levels in patients with asthma following COVID-19, stratified by clinical outcome (positive and negative outcome)

NLRP3 levels is 41 % ( $p \leq 0.01$ ) and gasdermin D is 537 % ( $p \leq 0.001$ ) higher than the values of the comparison group. This contributes to prolonged recovery and, in some cases, to the development of post-COVID syndrome.

In the negative outcome group in the late period, asthma exacerbations were recorded 1.5–2 times more frequently than in the positive outcome group during the same period (Table). Pulmonary function parameters also differed between the groups. At an early stage, FVC was reduced by 8.8 %, and by 4.7 % at a late stage compared to the positive outcome group. FEV1 was 76.1 % at an early stage and 82.6 % at a late stage. The FEV1/FVC ratio was 60.4 % at an early stage and 65.5 % at a late stage suggesting mild obstruction (Table).

Based on the data obtained, cutoff values for hyperinflammation markers associated with adverse outcomes were determined for gasdermin D and NLRP3.

The threshold determination algorithm comprised three stages. First, an association between marker levels and post-COVID complication risk was assessed. The analysis showed statistically significant differences ( $p < 0.05$ ) between NLRP3 levels in the positive outcome group and in the COVID-19 negative outcome group ( $p < 0.001$ ). Statistically significant differences ( $p < 0.05$ ) were found between gasdermin D levels in the COVID-19 positive outcome group compared to the negative outcome group ( $p = 0.002$ ). Second, ROC analysis was used to determine the separating threshold for gasdermin D and NLRP3, and performance characteristics of the diagnostic test were evaluated. The optimal threshold value for gasdermin D was 0.38 ng/ml, with a sensitivity (Se) of 85 % and specificity (Sp) of 91 %. The area under the curve (AUC) was 0.89, indicating good quality for the diagnostic model (Fig. 2).



**FIG. 2.** ROC-curve analysis of gasdermin D for predicting adverse outcomes in asthma patients after COVID-19

For NLRP3, the cutoff value was determined at 0.04 ng/ml (Se = 82%, Sp = 87%, AUC = 0.858) (Fig. 3), also indicating good prognostic value of this marker.

## DISCUSSION

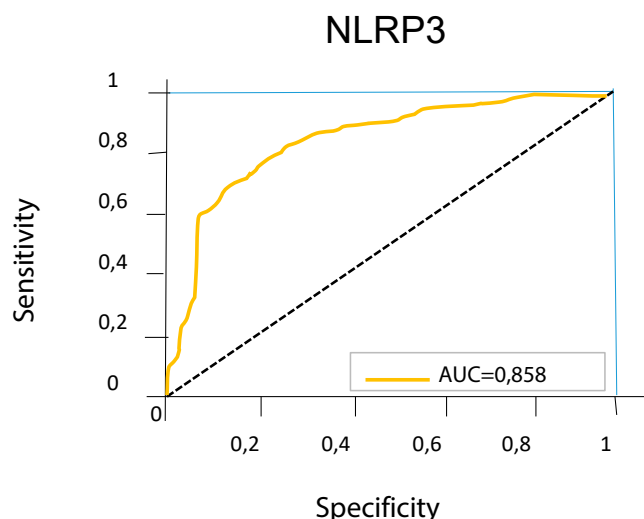
The obtained data indicate the key pathogenetic role of the NLRP3 inflammasome in the initiation and maintenance of hyperinflammatory cascades in patients with asthma following COVID-19. This interaction is of particular interest, as it combines the chronic inflammatory processes characteristic of asthma and the virus-induced

**TABLE**

### COMPARATIVE CLINICAL AND FUNCTIONAL CHARACTERISTICS OF THE STUDIED GROUPS AT DIFFERENT STAGES OF CONVALESCENCE

| Parameter                             | Group with mild partially controlled asthma, positive outcome after COVID-19 (n=34) |                                 | Group with mild partially controlled asthma, negative outcome after COVID-19 (n=18) |                             | Group with partially controlled mild asthma (n=34) |
|---------------------------------------|-------------------------------------------------------------------------------------|---------------------------------|-------------------------------------------------------------------------------------|-----------------------------|----------------------------------------------------|
|                                       | Early stage                                                                         | Late stage (after 6 months)     | Early stage                                                                         | Late stage (after 6 months) |                                                    |
| Number of asthma exacerbations        |                                                                                     | No more than 1 time in 6 months |                                                                                     | 1–2 times in 6 months       | No more than 1 time in 6 months                    |
| ACQ test, points                      | 1.2 ***<br>(1.05–1.34)                                                              | 1.01 ***<br>(0.97–1.14)         | ***1.38 ***<br>(1.38–1.45)                                                          | ***1.43 ***<br>(1.36–1.49)  | ***1.0 ***<br>(0.91–1.06)                          |
| <b>EPF parameters, % of predicted</b> |                                                                                     |                                 |                                                                                     |                             |                                                    |
| FVC % from the proper                 | 94.2 ***<br>(91.3–96.7)                                                             | 95.4 ***<br>(92.7–97.6)         | ***85.4 ***<br>(82.2–89.7)                                                          | ***90.7 ***<br>(86.4–94.9)  | ***95.9 ***<br>(93.8–99.2)                         |
| FEV1 % from the proper                | 88.6 ***<br>(84.5–92.5)                                                             | 90.2 ***<br>(85.2–96.1)         | ***76.1 ***<br>(70.2–79.5)                                                          | ***82.6 ***<br>(76.8–86.2)  | ***92.2 ***<br>(87.5–96.3)                         |
| FEV1/FVC %                            | 71.8 ***<br>(65.9–75.8)                                                             | 75.2 ***<br>(70.4–79.6)         | ***60.4 ***<br>(55.7–67.1)                                                          | ***65.5 ***<br>(61.3–67.6)  | ***77.9 ***<br>(71.5–84.1)                         |

**Note.** Right\* indicates statistical significance compared to the control group; left\* indicates differences between the subgroups with positive and negative outcomes. \*\*\* –  $p < 0.001$ .



**FIG. 3.**  
ROC-curve for NLRP3 in asthma patients with a history of COVID-19

inflammatory response in COVID-19. The combination of these factors creates a unique pathogenetic setting that predisposes to prolonged inflammation and the development of post-COVID disorders [1–3].

Inflammasomes, in particular the NLRP3 complex, are an important element of the innate immune defense, providing recognition of pathogens and triggering inflammatory cascades. NLRP3 refers to receptors that activate intracellular signaling pathways in response to pathogen-induced damage [5, 6].

The inflammatory response arising in this context in patients with asthma and COVID-19 becomes cyclical: virus-induced NLRP3 activation triggers excessive cytokine release, leading to epithelial damage of the respiratory tract, vascular endothelium injury, increased tissue sensitization, and NLRP3 reactivation [7]. Prolonged activation of NLRP3 and GSDMD can lead to persistent hyperinflammation and disruption of tissue repair mechanisms. The present study demonstrates that even six months after COVID-19, patients with bronchial asthma continue to have elevated NLRP3 and GSDMD levels, accompanied by asthenic syndrome, decreased pulmonary function, and impaired asthma control. These findings are consistent with previous studies reporting prolonged activation of proinflammatory pathways after COVID-19 in individuals with chronic bronchopulmonary disease [8–10].

The persistence of elevated concentrations of NLRP3 and GSDMD in patients with asthma after COVID-19 may explain the presence of clinical signs such as increased fatigue, shortness of breath, decreased exercise tolerance, and frequent asthma exacerbations. Hyperactivation of inflammasomes may not only contribute to the persistence of inflammation but also reduce the efficacy of maintenance therapy by decreasing sensitivity to inhaled glucocorticoids [11, 12]. In this study, ROC analysis demonstrated that NLRP3 and GSDMD levels have high diagnostic accuracy for predicting negative outcomes. These markers may serve as potential biomarkers

for the risk of post-COVID disorders and worsening of asthma control.

## CONCLUSION

The results confirm the involvement of the NLRP3 and GSDMD in the formation of the hyperinflammation phenotype in patients with asthma in the post-COVID period. The developed prognosis method demonstrates that a concurrent elevation of NLRP3 above 0.04 ng/ml and GSDMD above 0.38 ng/ml indicates a high risk of worsening of the underlying disease, as well as the need for personalized therapy and long-term follow-up of this patient category. These findings open up prospects for further research aimed at developing new diagnostic and prognostic criteria for post-COVID disorders, as well as the identification of therapeutic strategies capable of modulating the activity of inflammasome-mediated inflammatory pathways in mild asthma following COVID-19.

## Conflicts of interest

The authors declare no conflicts of interest.

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# PHENOTYPIC COMPOSITION OF PERIPHERAL BLOOD MONOCYTES IN YOUNG PATIENTS WITH COVID-19 AND ITS ASSOCIATION WITH THE RISK OF ACUTE MYOCARDIAL INFARCTION

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## ABSTRACT

**Background.** COVID-19 is associated with systemic inflammation, endothelial dysfunction, and coagulopathy, significantly increasing the risk of thrombotic complications—including acute myocardial infarction (AMI) – including in young patients. The role of the monocyte–macrophage system in the pathogenesis of these complications remains insufficiently studied.

**Aim.** To investigate the phenotypic composition of peripheral blood monocytes in young patients with COVID-19 alone and in those with concomitant COVID-19 and AMI.

**Methods.** A comparative cross-sectional study included 223 male patients aged 36.4 (31.2; 41.6) years: 15 with moderate COVID-19 and AMI (main group), 179 with moderate COVID-19 without AMI (comparison group), and 29 healthy individuals (control group). Expression levels of CD14, HLA-DR, CD11b, CD206, CD32, TNFR1, TNFR2, CD68, TRAIL, and TGFβ1 on monocytes were assessed by flow cytometry.

**Results.** In patients with COVID-19, monocyte expression of CD14 and HLA-DR was significantly reduced compared to controls, while expression of TNFR2, TRAIL, TGFβ1, CD206, CD68, CD32, and CD11b was elevated. These alterations were more pronounced in patients with both COVID-19 and AMI, including increased TNFR1 expression relative to controls. Comparative analysis revealed significantly higher expression levels of TNFR1, TNFR2, TGFβ1, CD206, and TRAIL in the main group versus the comparison group. Significant correlations were found between monocyte marker expression (CD14, HLA-DR, CD206, TNFR1, TNFR2, TRAIL, TGFβ1) and the development of AMI in patients with COVID-19. ROC analysis confirmed the prognostic value of HLA-DR, CD206, TRAIL, and TGFβ1 as markers of high AMI risk in this population.

**Conclusion.** COVID-19 induces a phenotypic shift in monocytes toward an immunosuppressive, pro-inflammatory, and pro-fibrotic profile, thereby increasing the risk of AMI.

**Keywords:** COVID-19, acute myocardial infarction, monocytes, peripheral blood, phenotypic composition

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## ФЕНОТИПИЧЕСКИЙ СОСТАВ МОНОЦИТОВ КРОВИ У ПАЦИЕНТОВ МОЛОДОГО ВОЗРАСТА С COVID-19 И СОЧЕТАНИЕМ COVID-19 И ОСТРЫМ ИНФАРКТОМ МИОКАРДА

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### РЕЗЮМЕ

**Обоснование.** COVID-19 ассоциирован с развитием системного воспаления, эндотелиальной дисфункцией и коагулопатией, что значительно повышает риск тромботических осложнений, включая острый инфаркт миокарда (ОИМ), в том числе у пациентов молодого возраста. Роль моноцитарно-макрофагального звена в механизмах развития данных осложнений остается недостаточно изученной.

**Цель.** Исследовать фенотипический состав моноцитов периферической крови у пациентов молодого возраста с COVID-19 и сочетанием COVID-19 и ОИМ.

**Методы.** Проведено сравнительное одномоментное исследование с участием 223 пациентов мужского пола в возрасте 36,4 (31,2; 41,6) лет, из них 15 пациентов со среднетяжелым течением COVID-19 и ОИМ (основная группа); 179 пациентов с COVID-19 средней степени тяжести без ОИМ (группа сравнения); 29 здоровых лиц (контрольная группа). Уровень экспрессии моноцитами CD14, HLA-DR, CD11b, CD206, CD32, TNFR1, TNFR2, CD68, TRAIL и TGFβ1 оценивали методом проточной цитометрии.

**Результаты.** У пациентов с COVID-19 выявлено достоверное снижение экспрессии моноцитами CD14 и HLA-DR относительно контрольной группы. Одновременно отмечено повышение уровней TNFR2, TRAIL, TGFβ1, CD206, CD68, CD32 и CD11b. При сочетании COVID-19 и ОИМ изменения были более выраженными, включая увеличение экспрессии TNFR1 по сравнению с контрольной группой. Сравнительный анализ показал, что у пациентов с COVID-19 и ОИМ уровни экспрессии TNFR1, TNFR2, TGFβ1, CD206 и TRAIL были значимо выше, чем у пациентов с COVID-19. При оценке взаимосвязи изменения параметров моноцитарного ответа с развитием ОИМ у пациентов с COVID-19 были выявлены значимые корреляции с CD14, HLA-DR, CD206, TNFR1, TNFR2, TRAIL и TGFβ1. ROC-анализ подтвердил прогностическую значимость HLA-DR, CD206, TRAIL и TGFβ1 как маркеров высокого риска ОИМ у пациентов с COVID-19.

**Заключение.** COVID-19 инициирует изменение фенотипа моноцитов, приводя к формированию субпопуляции с иммуносупрессивными, провоспалительными и профибротическими свойствами, что повышает риск развития ОИМ.

**Ключевые слова:** COVID-19, острый инфаркт миокарда, моноциты, периферическая кровь, фенотипический состав

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## BACKGROUND

The novel coronavirus disease, COVID-19, caused by SARS-CoV-2, is recognized not only as a respiratory illness but also as a systemic disorder affecting multiple organs and systems, including the cardiovascular system [1]. Although the respiratory tract remains the main target of the virus, cardiovascular complications are increasingly being reported and contribute significantly to both morbidity and mortality, especially among patients with severe disease [2].

Myocardial injury in COVID-19 may arise through direct viral cytopathic effects or indirectly, via immune system hyperactivation, dysregulation of the renin-angiotensin system (RAS), endothelial dysfunction, and microvascular thrombosis [3]. SARS-CoV-2 enters cells, including cardiomyocytes and myocardial vascular cells, through the angiotensin-converting enzyme 2 (ACE2) receptor [4]. Following intracellular entry, the virus induces ACE2 internalization, leading to impaired regulatory function of ACE2 and a predominance of pro-oxidant, pro-inflammatory, and vasoconstrictive effects mediated by angiotensin II via the AT1 receptor [5].

A central role in the pathogenesis of cardiac complications is attributed to the systemic inflammatory response, characterized by excessive secretion of pro-inflammatory cytokines, including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-2 (IL-2), IL-6, IL-7, and other inflammatory mediators [6]. This immune hyperreactivity initiates a cascade of pathological events, including cardiomyocyte apoptosis, microcirculatory dysfunction, and activation of the coagulation cascade. In this process, activated macrophages and monocytes release additional cytokines and procoagulant factors, contributing to atherosclerotic plaque destabilization, atherothrombosis, and ultimately acute myocardial infarction (AMI) [7]. In a significant subset of patients, AMI develops due to an imbalance between myocardial oxygen supply and demand, which can be precipitated by hypoxia, tachycardia, hypotension, or sepsis [8]. Meta-analyses indicate that signs of myocardial injury are detectable in 20–28 % of hospitalized COVID-19 patients and are associated with a significantly increased risk of mortality [9, 10].

Monocytes constitute a heterogeneous population of immune cells capable of polarization in response to the local microenvironment: pro-inflammatory M1 macrophages drive the inflammatory response, whereas anti-inflammatory M2 macrophages promote tissue remodeling and resolution of inflammation [11]. In severe COVID-19, a shift from M1 to M2 macrophage polarization has been observed [12], potentially leading to immune dysregulation and contributing to the development of cardiovascular complications.

The rising incidence of AMI among young adults aged 18–45 years [13] poses a serious clinical challenge and points to a prominent role played by pathogenetic mechanisms that are directly linked to viral-induced

inflammation and coagulation in COVID-19. Investigation of the phenotypic composition of circulating monocytes has particular scientific and clinical relevance in this context. Alterations in immune cell polarization underlie both pro-inflammatory and pro-fibrotic immune responses. Therefore, phenotypic analysis of peripheral blood monocytes in young patients with COVID-19, as well as in those with concomitant AMI, may not only deepen our understanding of the pathogenesis of cardiovascular complications but also identify potential targets for immunomodulatory therapeutic strategies.

## AIM

To investigate the phenotypic composition of peripheral blood monocytes in young patients with COVID-19 alone and in those with concomitant COVID-19 and AMI.

## MATERIALS AND METHODS

### Study design and setting

A comparative cross-sectional case-control study was conducted involving 223 male patients, with a median age of 36.4 (31.2; 41.6) years. The main group consisted of 15 patients with moderate COVID-19 who developed AMI during hospitalization. The comparison group included 179 patients with moderate COVID-19 and no AMI, while the control group comprised 29 healthy individuals with no cardiovascular disease and with a negative PCR test for SARS-CoV-2.

**Inclusion criteria were:** age < 45 years, laboratory-confirmed COVID-19 (positive PCR for SARS-CoV-2 RNA), moderate disease severity, and newly diagnosed cardiovascular pathology during hospitalization.

**Exclusion criteria were:** history of coronary artery disease, prior AMI, coronary artery stents, chronic heart failure (NYHA functional class II–IV), malignancies, HIV infection, and severe COVID-19.

In addition, a retrospective review of medical records (Form No. 003/u) and inpatient charts was conducted. Laboratory analyses were performed at the research facilities of the Far Eastern Scientific Center of Physiology and Pathology of Respiration using equipment from the Shared Resource Center.

### Ethical approval

The study was conducted in accordance with the Helsinki Declaration (World Medical Association, 2013) and it was approved by the local ethics committee of the Far Eastern Scientific Center of Physiology and Pathology of Respiration (Protocol No. 5, dated October 20, 2021). All participants provided written informed consent before participating in this study.

### Study duration and methods

Sample collection was performed between 2021 and 2023 at the Infectious Diseases Department

of the Blagoveshchensk City Clinical Hospital (Blagoveshchensk, Amur Region). Peripheral venous blood was collected in the morning after an overnight fast within the first 72 hours of hospitalization into vacuum tubes containing EDTA (China). Mononuclear cells were isolated by density gradient centrifugation on Ficoll-Paque medium (density 1.077 g/mL; Cytiva, Sweden). Monocyte surface marker expression was assessed by flow cytometry using a BD FACS Canto II cytometer (Becton Dickinson, USA) with fluorochrome-conjugated monoclonal antibodies (BioLegend, USA) against CD14, HLA-DR, CD11b, CD206, CD68, CD32, TNFR1, TNFR2, TRAIL, and TGFβ1. Data were analyzed using BD FACSDiva™ v8.0 software (Becton Dickinson, USA).

#### Statistical analysis

Statistical processing was performed using IBM SPSS Statistics v.23.0 (IBM Corp., USA). Quantitative variables are expressed as median (Me) and interquartile range (Q1; Q3). Normality was assessed using the Kolmogorov – Smirnov and Shapiro – Wilk tests. Between-group comparisons of non-normally distributed continuous

variables were performed using the Mann – Whitney U test. A  $p$ -value  $< 0.05$  was considered statistically significant.

## RESULTS

Phenotypic analysis of peripheral blood monocytes in young patients with moderate COVID-19 revealed statistically significant alterations in cell surface antigen expression, associated both with SARS-CoV-2 infection itself and with the development of AMI in the context of infection (Table 1).

In the COVID-19 group, compared to the control group, there was a decrease in the number of monocytes expressing CD14 – by 1.25 times ( $p < 0.001$ ) and HLA-DR antigen – by 1.18 times ( $p < 0.001$ ). TNFR2 expression levels increased by 3.03 times ( $p < 0.001$ ), TRAIL – by 3.96 times ( $p < 0.001$ ), and TGFβ1 – by 3.08 times ( $p < 0.001$ ). At the same time, an increase in CD206 expression by 1.80 times ( $p < 0.01$ ), CD68 – by 2.29 times ( $p < 0.001$ ), CD32 – by 1.04 times ( $p < 0.05$ ),

TABLE 1

PHENOTYPIC COMPOSITION OF PERIPHERAL BLOOD MONOCYTES IN THE STUDY GROUPS (%)

| Parameters | COVID-19<br><i>n</i> =179 | COVID-19 + AMI<br><i>n</i> =15 | Control group<br><i>n</i> =29 | <i>p</i>                      |
|------------|---------------------------|--------------------------------|-------------------------------|-------------------------------|
| CD14       | 75.2 (73.9; 76.4)         | 76.7 (74.3; 79.9)              | 93.9 (91.5; 94.6)             | $p_{1,2}<0.001$<br>$p_3>0.05$ |
| HLA-DR     | 79.0 (76.0; 83.7)         | 80.6 (77.0; 83.1)              | 93.4 (90.9; 96.6)             | $p_{1,2}<0.001$<br>$p_3>0.05$ |
| CD11b      | 80.4 (72.4; 82.0)         | 82.3 (73.3; 83.5)              | 75.9 (73.6; 77.9)             | $p_1<0.001$<br>$p_{2,3}<0.05$ |
| CD206      | 7.9 (7.1; 8.7)            | 10.7 (10.0; 11.5)              | 4.4 (4.1; 5.5)                | $p_{1-3}<0.001$               |
| CD32       | 93.6 (92.4; 94.5)         | 94.4 (93.8; 95.1)              | 89.8 (89.2; 90.7)             | $p_{1,2}<0.05$<br>$p_3>0.05$  |
| TNFR2      | 41.8 (37.0; 45.7)         | 65.8 (61.7; 69.3)              | 13.8 (10.5; 15.1)             | $p_{1-3}<0.001$               |
| TNFR1      | 62.3 (56.0; 64.2)         | 78.6 (74.7; 84.1)              | 62.3 (56.7; 64.9)             | $p_1>0.05$<br>$p_{2,3}<0.001$ |
| CD68       | 50.4 (46.8; 64.6)         | 48.7 (46.0; 52.4)              | 22.0 (18.0; 24.4)             | $p_{1,2}<0.001$<br>$p_3>0.05$ |
| TRAIL      | 55.5 (53.2; 57.3)         | 59.0 (57.8; 61.9)              | 14.0 (11.7; 14.6)             | $p_{1,2}<0.001$<br>$p_3>0.05$ |
| TGFβ1      | 11.4 (9.3; 12.6)          | 17.9 (14.8; 19.8)              | 3.7 (3.0; 5.2)                | $p_{1-3}<0.001$               |

**Notes.**  $p_1$  – statistical significance of differences between the COVID-19 group and the control group;  $p_2$  – statistical significance of differences between the COVID-19 + AMI group and the control group;  $p_3$  – statistical significance of differences between the COVID-19 group and the COVID-19 + AMI group.

and CD11b – by 1.06 times ( $p < 0.05$ ) was noted. TNFR1 expression levels in the COVID-19 group did not differ significantly from those in the control group ( $p > 0.05$ ).

In patients with COVID-19 and AMI, compared to the control group, there was a decrease in CD14 expression – by 1.22 times ( $p < 0.001$ ) and HLA-DR – by 1.16 times ( $p < 0.001$ ). TNFR1 expression levels increased by 1.26 times ( $p < 0.001$ ), TNFR2 – by 4.77 times ( $p < 0.001$ ), TRAIL – by 4.21 times ( $p < 0.001$ ), TGF $\beta$ 1 – by 4.84 times ( $p < 0.001$ ), CD206 – by 2.43 times ( $p < 0.001$ ), CD68 – by 2.21 times ( $p < 0.001$ ), CD32 – by 1.05 times ( $p < 0.05$ ), and CD11b – by 1.08 times ( $p < 0.05$ ).

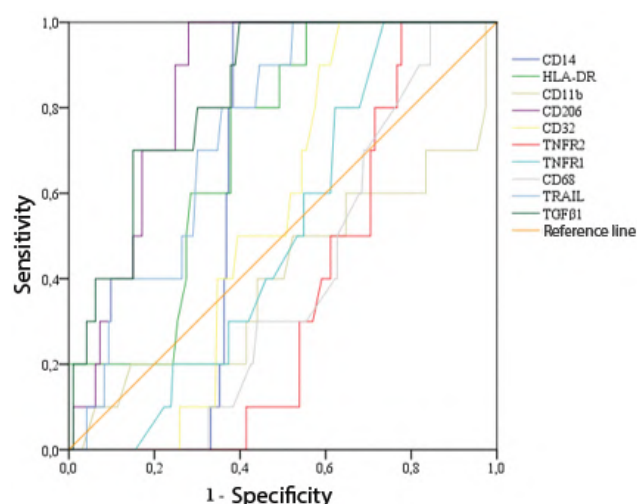
Further comparative analysis showed that TNFR1 expression levels in the COVID-19 + AMI group were higher than in the COVID-19 group, differing by 1.26 times ( $p < 0.001$ ), TNFR2 – by 1.57 times ( $p < 0.001$ ), TGF $\beta$ 1 – by 1.57 times ( $p < 0.001$ ), CD206 – by 1.35 times ( $p < 0.001$ ), and TRAIL – by 1.06 times ( $p < 0.05$ ). No significant differences in CD14, HLA-DR, CD32, or CD68 expression were found between these groups ( $p > 0.05$ ).

When assessing the correlation between changes in monocyte response parameters and AMI development in COVID-19 patients, correlations were identified with CD14 ( $p = -0.43$ ,  $p < 0.05$ ), HLA-DR ( $p = -0.71$ ,  $p < 0.01$ ), CD206 ( $p = 0.74$ ,  $p < 0.01$ ), TNFR1 ( $p = 0.34$ ,  $p < 0.05$ ), TNFR2 ( $p = 0.43$ ,  $p < 0.05$ ), TRAIL ( $p = 0.75$ ,  $p < 0.01$ ), and TGF $\beta$ 1 ( $p = 0.77$ ,  $p < 0.01$ ).

In a further integrated assessment of the contribution of monocyte CD markers to AMI development in COVID-19 patients, ROC analysis was performed (Fig.). Decreased monocyte HLA-DR expression (AUC = 0.713,  $p = 0.002$ ) and increased CD206 (AUC = 0.849,  $p < 0.001$ ), TRAIL (AUC = 0.752,  $p < 0.001$ ), and TGF $\beta$ 1 (AUC = 0.836,  $p < 0.001$ ) levels are prognostically significant risk factors for AMI in COVID-19 patients (Table 2).

## DISCUSSION

The identified changes in the phenotypic composition of peripheral blood monocytes in young patients with COVID-19 and with the combination of COVID-19 and AMI reflect complex immune response processes, where phases of hyperinflammation, functional “exhaustion” of immune cells, and tissue remodeling alternate sequentially. The decreased monocyte expression of CD14 and HLA-DR observed in patients with COVID-19 and COVID-19 + AMI compared to the control group may indicate the formation of a functionally “exhausted” cell pool – CD14<sup>+</sup>HLA-DR<sup>neg/low</sup>, previously described in sepsis and cancer [13], and in later studies – in severe SARS-CoV-2 infection [14]. Moreover, in the COVID-19 + AMI group, the decrease in HLA-DR expression was more pronounced than in the COVID-19 group. Our results are consistent with those of Fraccarollo D. et al., who reported an increase in CD14<sup>+</sup>HLA-DR<sup>neg/low</sup> monocyte



**FIG.**

Roc curve illustrates the discriminatory ability of monocyte response parameters in predicting the risk of AMI in patients with COVID-19

**TABLE 2**

**ROC CURVE PARAMETERS IN THE GROUP OF PATIENTS WITH COVID-19 AND AMI**

| Parameters    | AUC   | 95% CI      | p      |
|---------------|-------|-------------|--------|
| CD14          | 0.634 | 0.567–0.701 | 0.038  |
| HLA-DR        | 0.713 | 0.624–0.802 | 0.002  |
| CD11b         | 0.406 | 0.259–0.553 | 0.051  |
| CD206         | 0.849 | 0.794–0.904 | <0.001 |
| CD32          | 0.549 | 0.469–0.630 | 0.467  |
| TNFR2         | 0.365 | 0.289–0.441 | 0.047  |
| TNFR1         | 0.505 | 0.411–0.600 | 0.038  |
| CD68          | 0.392 | 0.302–0.481 | 0.111  |
| TRAIL         | 0.752 | 0.670–0.833 | <0.001 |
| TGF $\beta$ 1 | 0.836 | 0.766–0.906 | <0.001 |

expression in the blood of patients with AMI was reported [15]. HLA-DR is one of three class II major histocompatibility complex (MHC) glycoproteins expressed on antigen-presenting cells, functioning to present antigens internalized by the cell to T-cell receptors (TCR), leading to T-cell activation. Decreased HLA-DR expression on CD14<sup>+</sup> cells may indicate a suppression of monocyte antigen-presenting capacity to T cells

[11]. *In vitro* studies have shown that culturing monocytes with cytokines such as TGFβ1 leads to decreased HLA-DR surface expression [16]. Similar changes, involving alveolar macrophages, were described by Bühling F. et al. [17]. The authors demonstrated an inverse correlation between high TGFβ1 concentration and HLA-DR expression, and a direct correlation with CD11b levels in alveolar macrophages of patients with pneumonia. CD11b is one of the α-chains of β2 integrins and is a receptor for iC3b, ICAM-1,2,4, fibrinogen, and CD14. It plays an important role in macrophage interaction with endothelial cells or extracellular matrix proteins [17]. The study suggested that microbial antigens are the primary regulators of CD11b expression. We found that CD11b levels in patients with COVID-19 and COVID-19 + AMI were elevated compared to the control group but did not differ between the groups, which further supports the influence of infection on antigen expression. Similar changes were observed in other CD markers, such as CD32 and CD68.

It should be noted that monocytes with reduced HLA-DR levels in patients with COVID-19 and COVID-19 + AMI do not exhibit pronounced immunosuppressive activity. This is confirmed by increased monocyte expression of immunoregulatory molecules and inflammatory markers, such as CD206, TNFR1, TNFR2, TRAIL, and TGFβ1, which are most pronounced in the COVID-19 + AMI group compared to the COVID-19 group. These results may support the role of monocytes with low HLA-DR levels in the mechanisms of AMI development. *In vitro* experiments have shown that macrophages derived from CD14<sup>+</sup>HLA-DR<sup>neg/low</sup> monocytes from AMI patients, upon IFN-γ stimulation, produce large amounts of inflammatory cytokines TNF-α, IL-6, and IL-1β compared to macrophages with high HLA-DR expression [15]. Increased cellular expression of TNFR1, TNFR2, and TRAIL is a marker for systemic inflammation and endothelial dysfunction [18]. The authors demonstrated that activation of the TNF-α/TNFR1 complex induces the release of extracellular vesicles from endothelial cells, which are involved in vascular injury and promote apoptosis, and inflammatory damage to endothelial cells. TNFR2 initiates the recruitment of TRAF2 and TRAF5 proteins, activating the NF-κB and MAPK signaling pathways to sustain inflammation and lead to the production of pro-inflammatory cytokines (IL-6 and IL-8) [2]. In recent years, there has been a significant increase in interest in the role of TRAIL in the pathogenesis of cardiovascular pathology. According to Goncalves I. et al., TRAIL induces the apoptosis of macrophages, vascular smooth muscle cells, and endothelial cells, and maintains the inflammatory activity of atherosclerotic plaques, leading to their instability and increasing the risk of thrombosis and AMI development [19]. Alonso-Herranz L. et al. showed that TGFβ1 hyperactivation in macrophages after ischemic myocardial injury promotes local

endothelial-to-mesenchymal transition of cells and formation of myofibroblasts involved in cardiac fibrosis and remodeling [20]. A review by Budnevsky A.V. et al. confirmed the role of post-COVID syndrome in TGFβ1 overexpression, leading to prolonged immunosuppression and fibrosis [21].

The above literature data are supported by our studies, in which ROC analysis demonstrated that changes in monocyte expression of HLA-DR, CD206, TRAIL, and TGFβ1 can serve as biomarkers for high AMI risk in patients with COVID-19.

This study has several limitations. First, the sample size was relatively small, which may reduce the statistical power of the analysis and limit the generalizability of the results to a broader population. Second, the study design was single-center, potentially introducing bias related to patient selection and treatment protocols specific to a single institution. Third, the absence of longitudinal patient follow-up precludes assessment of long-term cardiovascular outcomes and dynamics of monocyte phenotypic composition in the post-hospital period. Despite these limitations, the data obtained are valuable for understanding the early immunopathogenetic mechanisms of cardiovascular complications in patients who have recovered from COVID-19.

## CONCLUSION

COVID-19 induces changes in monocyte phenotype, leading to the formation of a subpopulation with immunosuppressive, pro-inflammatory, and pro-fibrotic characteristics, which increases the risk of AMI development.

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### Conflict of interest

The authors declare no conflict of interest.

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## CARDIOLOGY

### ASSOCIATION OF SEX AND AGE WITH THE CERAMIDE PROFILE OF ADIPOSE TISSUE IN PATIENTS WITH CORONARY ARTERY DISEASE

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#### ABSTRACT

**Background.** Coronary artery disease (CAD) is associated with complex molecular changes in adipose tissue (AT) that go beyond simple energy storage. Current studies indicate the active participation of AT in metabolic regulation and secretion of bioactive lipid mediators, including ceramides, the role of which in CAD pathogenesis remains poorly understood, particularly with regard to age and sex differences.

**Aim.** To characterize the ceramide profile in subcutaneous, epicardial and perivascular AT (SAT, EAT, PVAT) in patients with CAD, with regard to age and sex differences.

**Materials and Methods.** A lipidomic study of the ceramide profile was conducted in SAT, EAT, PVAT samples obtained from 58 patients with CAD who underwent coronary artery bypass grafting (single-center prospective clinical study). The ceramide composition was analyzed by high-performance liquid chromatography with tandem mass spectrometric detection.

**Results.** Age- and sex-related differences in the AT ceramide profile were identified. In men, the concentrations of cardioneutral ceramides (Cer d18:1/14:0, Cer d18:1/18:0, Cer d18:1/20:0, Cer d18:1/22:0) and Cer d18:1/24:1 were higher ( $p < 0.05$ ). In PVAT of men, the levels of Cer 14:0, 16:0 and 22:4 were increased ( $p < 0.001$ ). In EAT and PVAT of women, increased levels of Cer 18:0 and 23:0 were noted ( $p < 0.05$ ). In EAT and PVAT of women, the concentrations of Cer 20:0, 22:0 and 24:1 were higher ( $p < 0.05$ ). The highest levels of cardioneutral ceramides were observed in PVAT of patients aged over 75 years. The ratio of cardioneutral to cardioprotective ceramides was higher in EAT in individuals aged 60–74 years and in PVAT in those aged over 75 years.

**Conclusion.** Significant sex- and age-related differences in the AT ceramide profile of patients with CAD were identified, emphasizing the need to consider these factors when assessing cardiovascular risk. The data obtained may contribute to the development of new therapeutic strategies aimed at modulating the ceramide profile of AT to reduce cardiovascular risk.

**Keywords:** ceramides, age, sex, epicardial adipose tissue, perivascular adipose tissue, risk factors, coronary artery disease

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## АССОЦИАЦИЯ ПОЛА И ВОЗРАСТА С ЦЕРАМИДНЫМ ПРОФИЛЕМ ЖИРОВОЙ ТКАНИ ПАЦИЕНТОВ С ИШЕМИЧЕСКОЙ БОЛЕЗНЬЮ СЕРДЦА

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### РЕЗЮМЕ

**Актуальность.** Ишемическая болезнь сердца (ИБС) связана с комплексными молекулярными изменениями в жировой ткани (ЖТ), выходящими за рамки простого хранения энергии. Современные исследования указывают на активное участие ЖТ в метаболической регуляции и секреции биоактивных липидных медиаторов, включая церамиды, роль которых в патогенезе ИБС остается недостаточно изученной, особенно с учетом половозрастных характеристик.

**Цель.** Охарактеризовать церамидный профиль в подкожной, эпикардимальной и периваскулярной ЖТ (ПЖТ, ЭЖТ, ПВЖТ) у пациентов с ИБС с учетом половозрастных особенностей.

**Материалы и методы.** Проведено липидомическое исследование церамидного профиля в образцах ПЖТ, ЭЖТ, ПВЖТ, полученных от 58 пациентов с ИБС, которым выполнялось коронарное шунтирование (одноцентровое проспективное клиническое исследование). Анализ церамидного состава осуществлялся методом высокоэффективной жидкостной хроматографии с tandemным масс-спектрометрическим детектированием.

**Результаты.** Выявлены половозрастные различия в церамидном профиле ЖТ. У мужчин концентрации кардионегативных церамидов (Cer d18:1/14:0, Cer d18:1/18:0, Cer d18:1/20:0, Cer d18:1/22:0) и Cer d18:1/24:1 были выше ( $p < 0,05$ ). В ПВЖТ мужчин уровни Cer 14:0, 16:0 и 22:4 были повышены ( $p < 0,001$ ). В ЭЖТ и ПВЖТ женщин отмечалось повышение уровней Cer 18:0 и 23:0 ( $p < 0,05$ ). В ЭЖТ и ПЖТ женщин концентрации Cer 20:0, 22:0 и 24:1 были выше ( $p < 0,05$ ). Наибольшие уровни кардионегативных церамидов наблюдались в ПВЖТ пациентов старше 75 лет. Соотношение кардионегативных и кардиопротективных церамидов было выше в ЭЖТ у лиц 60–74 лет, а в ПВЖТ – старше 75 лет.

**Заключение.** Выявлены значимые половые и возрастные различия в церамидном профиле ЖТ у пациентов с ИБС, что подчеркивает необходимость учета этих факторов при оценке риска сердечно-сосудистых осложнений. Полученные данные могут способствовать разработке новых терапевтических стратегий, направленных на модуляцию церамидного профиля ЖТ для снижения сердечно-сосудистого риска.

**Ключевые слова:** церамиды, возраст, пол, эпикардимальная жировая ткань, периваскулярная жировая ткань, факторы риска, ишемическая болезнь сердца

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## INTRODUCTION

Complex molecular changes in adipose tissue (AT) play a crucial role in the pathogenesis of coronary artery disease (CAD). Adipose tissue is no longer seen as merely a passive energy store, but rather as an active participant in metabolic regulation. It has been shown to secrete bioactive lipid mediators, such as ceramides [1].

Ceramides (Cer) are a class of sphingolipids with both structural and signaling functions. They act as secondary messengers, influencing various cellular processes, including growth, proliferation, differentiation, and apoptosis [2]. Given their importance in regulating cellular functions under both normal and pathological conditions, the impact of ceramides on the cardiovascular system (CVS) and the development of cardiovascular diseases (CVD) have been actively investigated in recent decades.

Under conditions of metabolic stress, the accumulation of lipid metabolites in tissues poses a significant threat. Lipidomics studies have demonstrated that ceramides exhibit pronounced toxic effects, leading to lipotoxicity, which, in turn, results in mitochondrial dysfunction and cellular hypoxia. This process serves as a crucial mechanism in the development of various cardiometabolic disorders [3].

Although there is interest in investigating plasma ceramide levels as biomarkers of cardiovascular risk, information on the characteristics of ceramide profiles in different AT depots in patients with CAD remains limited, particularly regarding age- and sex-related differences [4].

A comprehensive analysis of the composition of ceramides in AT can help identify specific lipid biomarkers associated with different stages of disease progression and develop novel diagnostic and therapeutic approaches aimed at regulating ceramide metabolism and reducing cardiovascular risks.

## AIM

To characterize the ceramide profile in subcutaneous, epicardial and perivascular AT (SAT, EAT, PVAT) in patients with CAD, with regard to age and sex differences.

## MATERIALS AND METHODS

A lipidomic study of the ceramide profile was performed in subcutaneous, epicardial, and perivascular adipose tissues (SAT, EAT, and PVAT) from patients with coronary artery disease (CAD) who required surgical myocardial revascularization through coronary artery bypass grafting (CABG). The study was approved by the local ethics committee of the Research Institute for Complex Issues of Cardiovascular Diseases (Protocol No. 12, dated March 20, 2023) and it adhered to the principles outlined in the Declaration of Helsinki

of the World Medical Association (as amended in 2013) and the "Clinical Practice Guidelines of the Russian Federation".

The study included 58 participants, with a majority of male patients (58 %). The mean age of participants was 64.9 (47.8; 78.5) years, and the median body mass index was 26.4 (22.5; 30.2) kg/m<sup>2</sup>. A significant number of participants had comorbid conditions, including arterial hypertension (56.9 %), dyslipidemia (43.1 %), diabetes mellitus (24.1 %), and chronic kidney disease (10.3 %). A history of myocardial infarction was present in 70.7 % of patients. Among women (41.4 % of total), postmenopausal status was confirmed in 87.5 %, indicating a high proportion of postmenopausal women in this group. Patients predominantly had angina pectoris classes II (46.6 %) and III (50.0 %), as well as chronic heart failure classes I (39.7 %) and II (13.8 %). Extensive coronary artery (CA) atherosclerosis was also observed, with 81 % of patients having three or more affected coronary arteries. The median left ventricular ejection fraction was 53.6 (46.3; 58.9) %. Patients had elevated levels of total cholesterol (5.89 (4.12; 8.25) mmol/L), LDL cholesterol (3.31 (2.95; 5.65) mmol/L), and triglycerides (1.75 (1.52; 2.83) mmol/L), as well as an atherogenic index above the normal range (3.38 (2.75; 4.33)). Most patients received standard pharmacological treatment, including aspirin (93.1 %), beta-blockers (89.7 %), ACE inhibitors (77.6 %), and statins (100 %).

### Collection and Storage of Adipose Tissue Samples

During elective CABG surgery, AT samples were collected: SAT from the subcutaneous fat of the lower angle of the median sternotomy incision; EAT from the right heart chambers (atrium and ventricle); and PVAT from the region around the left main coronary artery. To preserve the lipid content, the samples were immediately placed in liquid nitrogen for freezing and then stored at -150°C.

### Lipid Extraction

A modified Folch method (1957) was employed to extract lipid components from homogenized AT samples. Approximately 100 mg of tissue was homogenized in a 2:1 (v/v) chloroform/methanol mixture. The samples were then subjected to sonication for 10 minutes to enhance extraction efficiency. Purified water was added, and the mixture was vigorously vortexed and centrifuged to separate the organic and aqueous layers. The organic layer was collected, re-extracted with a fresh aliquot of chloroform/methanol solution, and both organic phases were combined and allowed to evaporate to dryness under a nitrogen stream at room temperature. The resulting dried residue was reconstituted with a solution of isopropanol and acetonitrile (50:50, v/v) and further centrifuged to remove any insoluble impurities.

### Ceramide Profile Analysis

High-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) was used to determine ceramide composition. The methodology for ceramide profile analysis by mass spectrometry

has been thoroughly described previously [5], ensuring the reproducibility and comparability of the results obtained.

**The quantification of ceramides (ng/mL) in AT samples** was performed using the Cer d18:1/17:0 standard (17:0 Ceramide N-heptadecanoyl-D-erythro-sphingosine, >99%, Avanti). For the preparation of the Cer d18:1/17:0 stock solutions, 5 mg of the Cer d18:1/17:0 standard was placed into a 15 mL screw-cap vial, dissolved in 2.5 mL of solvent, and an additional 2.5 mL of the same solvent was added, and the mixture was vortexed. The concentration of the resulting solution was 1 mg/mL, calculated as Cer d18:1/17:0. The solution was stored at -70°C. The work was conducted using the equipment provided by the Shared Research Facility "Clinical Mass Spectrometry" of the National Medical Research Center for Obstetrics, Gynecology and Perinatology named after Academician V.I. Kulakov, Ministry of Health of the Russian Federation.

#### Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 8 software (GraphPad Software). Quantitative data are presented as the median with an interquartile range (25<sup>th</sup> and 75<sup>th</sup> percentiles). A non-parametric Kruskal – Wallis test was used to compare the three independent groups, followed by a pairwise comparison using the Mann – Whitney U test. Categorical variables were analyzed using either the  $\chi^2$  test or Fisher's exact test. Differences were considered statistically significant at  $p < 0.05$ .

## RESULTS

Ceramides (N-acylsphingosines) are a subgroup of sphingolipids that have the structural backbone of the alcohol sphingosine. Sphingosine has 18 carbon atoms and contains one trans-double bond between C4 and C5. This fragment, designated as d18:1, is bonded to a fatty acid (FA) residue through an amide bond, forming the ceramide molecule. Ceramides may contain various FAs, including saturated fatty acids such as C14:0 (myristic), C15:0 (pentadecanoic), C16:0 (palmitic), C17:0 (margaric), C18:0 (stearic), C19:0 (nonadecanoic), C20:0 (arachidic), C21:0 (heneicosanoic), C22:0 (behenic), C23:0 (tricosanoic), C24:0 (lignoceric), C25:0 (pentacosanoic), C26:0 (cerotic) and unsaturated FAs (C18:1 (oleic), C22:1 (erucic), C24:1 (nervonic), C22:2 (docosadienoic), C22:4 (docosatetraenoic), and C22:5 (docosapentaenoic).

A wide range of ceramides was identified in the PVAT, EAT, and SAT from CAD patients. All ceramides detected contained the C18 sphingoid base, also known as sphingosine (d18:1). In the AT samples analyzed, ceramides containing both saturated FAs (Cer d18:1/14:0, Cer d18:1/15:0, Cer d18:1/16:0, Cer d18:1/17:0, Cer d18:1/18:0, Cer d18:1/19:0, Cer d18:1/20:0, Cer d18:1/21:0, Cer d18:1/22:0, Cer d18:1/23:0, Cer d18:1/24:0, Cer d18:1/25:0, Cer d18:1/26:0) and unsaturated FAs (Cer d18:1/18:1, Cer

d18:1/22:1, Cer d18:1/24:1, Cer d18:1/22:2, Cer d18:1/22:4, Cer d18:1/22:5) were identified.

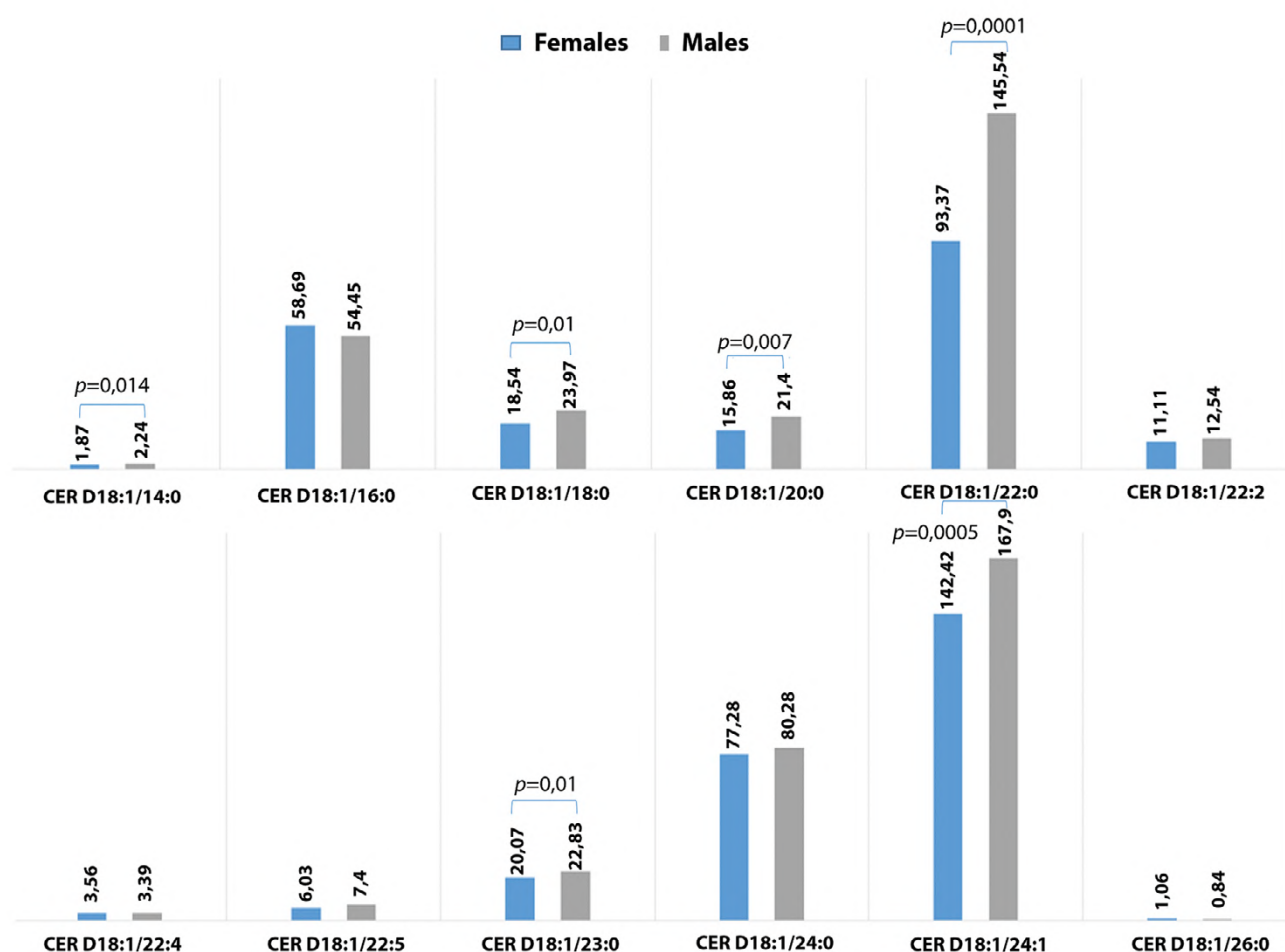
Given the known cardiovascular effects of various ceramides, the identified compounds were divided into groups associated with cardioneurogenic (Cer d18:1/14:0, Cer d18:1/16:0, Cer d18:1/18:0, Cer d18:1/20:0, Cer d18:1/24:1) and cardioprotective (Cer d18:1/23:0, Cer d18:1/24:0, Cer d18:1/25:0, Cer d18:1/26:0) properties [2].

Analysis of ceramide concentrations in AT samples, considering patient sex, revealed statistically significant differences in the levels of individual ceramide species within AT in men, regardless of location. In particular, the concentrations of Cer d18:1/14:0, Cer d18:1/18:0, Cer d18:1/20:0, and Cer d18:1/22:0, containing saturated FAs and associated with cardioneurogenic effects that were significantly higher in male AT. Furthermore, increased levels of Cer d18:1/24:1, containing a mono-unsaturated FA and exhibiting potential pathological effects on the cardiovascular system, including proatherogenic action, were observed [2]. Notably, a concurrent increase in the concentration of Cer d18:1/23:0, which has cardioprotective properties, was also noted (Fig. 1).

Considering the sex and anatomical location of the patients, it was found that PVAT of males had elevated levels of certain ceramides compared to that of females: Cer 14:0 (1.7-fold,  $p = 0.000017$ ), Cer 16:0 (1.4-fold,  $p = 0.0005$ ), and Cer 22:4 (1.4-fold,  $p = 0.00002$ ). At the same time, in EAT of females, an increase in Cer d18:1/17:2 concentration was observed (1.5-fold,  $p = 0.006$ ). In EAT and PVAT of females, levels of Cer 18:0 (1.6-fold,  $p = 0.0005$  and 1.4-fold,  $p = 0.045$ , respectively) and Cer 23:0 (1.2-fold,  $p = 0.045$  and 1.7-fold,  $p = 0.0005$ , respectively) were higher compared to that of males. In EAT and SAT of females, concentrations of certain ceramides were higher than in the male group: Cer 20:0 (1.2-fold,  $p = 0.004$  and 1.7-fold,  $p = 0.045$ , respectively), Cer 22:0 (1.8-fold,  $p = 0.007$  and 1.5-fold,  $p = 0.00001$ , respectively), and Cer 24:1 (1.7-fold,  $p = 0.006$  and 1.5-fold,  $p = 0.00001$ , respectively). In SAT of females, elevated levels of Cer d18:1/22:2 (1.6-fold,  $p = 0.0005$ ), Cer 22:4 (1.2-fold,  $p = 0.046$ ), and Cer 22:5 (1.4-fold,  $p = 0.000017$ ) were found compared to that of males.

Patients with CAD were then divided into three age groups:  $\leq 60$  years, 60–74 years, and  $\geq 75$  years. Based on this stratification, it was found that the highest levels of ceramides with adverse cardiovascular effects were seen in PVAT of patients aged 75 and over: Cer d18:1/14:0, Cer 16:0, and Cer 22:0 compared to patients under the age of 60 and those aged 60–74 years ( $p < 0.005$ ).

The level of Cer d18:1/14:0 in EAT of individuals aged 60–74 years was statistically significantly higher compared to the group under the age of 60 and showed a trend towards an increase compared to the group over the age of 75. The highest levels of Cer d18:1/18:0 in EAT and PVAT were found in the group aged over the age of 75. Cer d18:1/20:0 was higher in EAT of patients aged 60–74 years and in PVAT of the group aged over 75 years.



**FIG. 1.**  
Concentration of ceramides in adipose tissue of patients with CAD, ng/ml

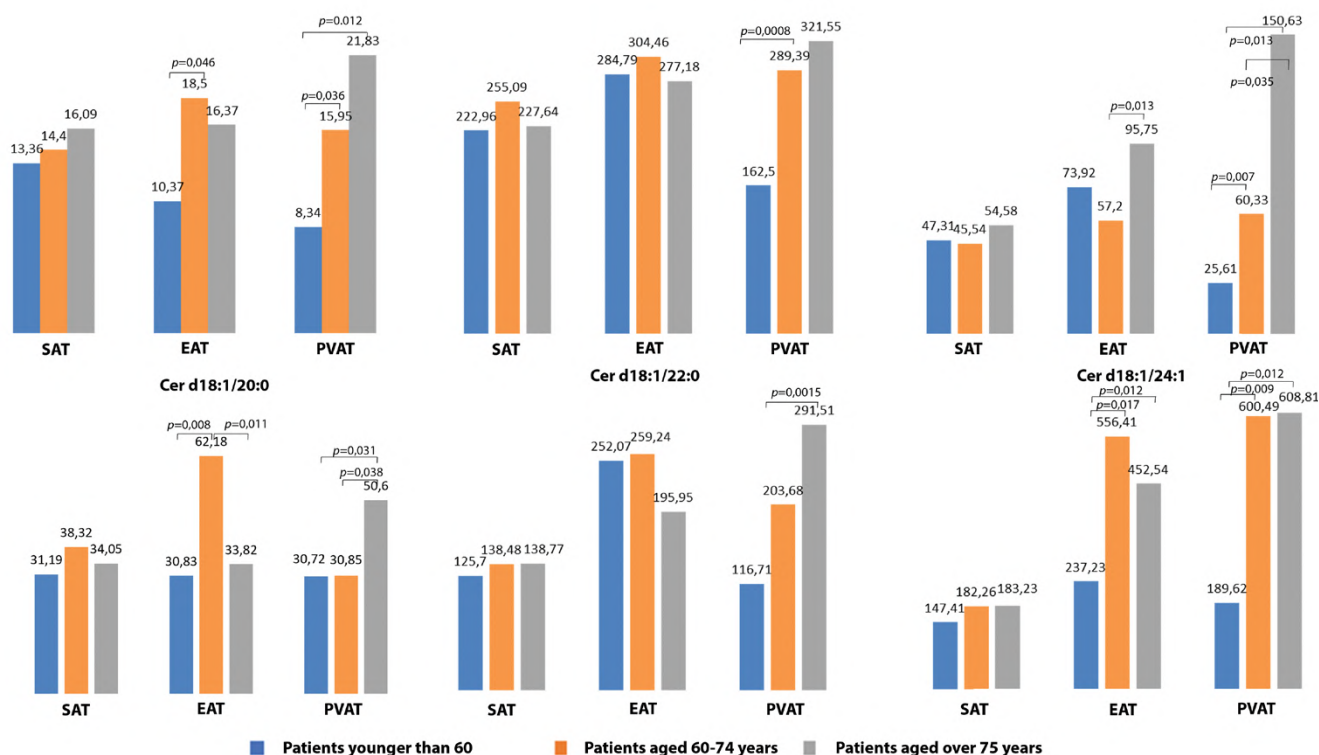
Levels of Cer d18:1/24:1 in cardiac AT of patients aged 60–74 and over the age of 75 exceeded those in patients under the age of 60 (Fig. 2).

The concentration of the cardioprotective ceramide Cer d18:1/23:0 in PVAT of patients aged 60–74 years and those older than 75 years was higher compared to the group younger under the age of 60. Levels of Cer d18:1/24:0 in EAT and PVAT in the 60–74 and ≥ 75 age groups exceeded those in patients younger than 60. The lowest concentrations of Cer d18:1/26:0 in both EAT and PVAT were observed in the 60–74 age group compared to those older than 75 and younger than 60 (Fig. 3).

The highest levels of ceramides with uncertain effects on the cardiovascular system (CVS) were observed in the following: Cer d18:1/15:0 was elevated in SAT ( $p = 0.018$ ) and PVAT ( $p = 0.024$ ) of patients over the age of 75; Cer d18:1/18:1 was elevated in SAT of patients aged 60–74 compared to those over 75

( $p = 0.031$ ); Cer d18:1/19:0 was elevated in SAT of patients over 75 ( $p = 0.015$ ) and in PVAT of patients aged 60–74 ( $p = 0.028$ ). In contrast, the concentration of Cer d18:1/17:0 in AT did not significantly differ according to age among CAD patients ( $p > 0.05$ ).

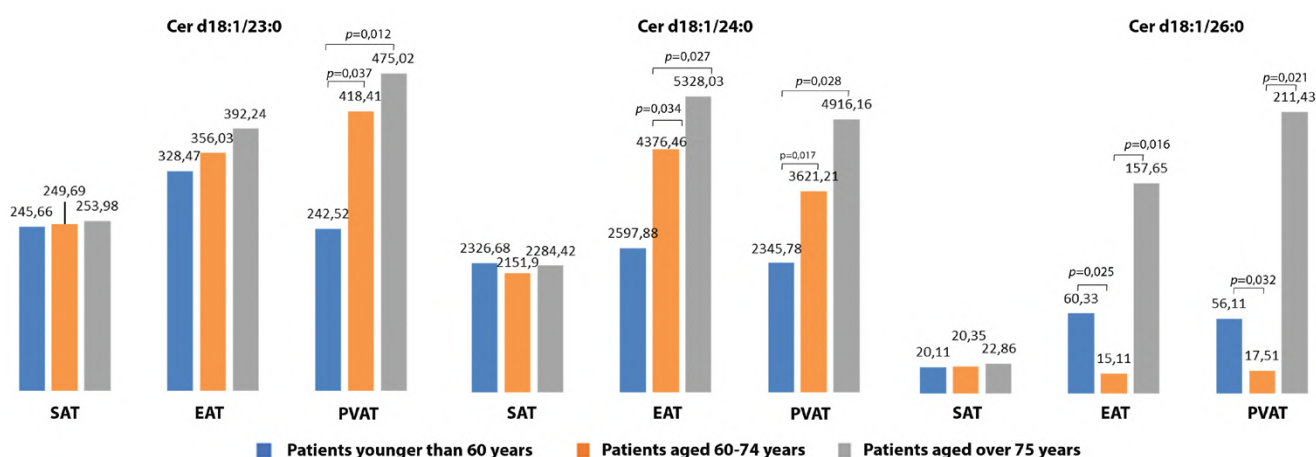
When calculating the ratios of Cer 16:0/24:0, Cer 18:0/24:0, Cer 18:1/24:0, and Cer 24:1/24:0, the Cer 16:0/24:0 ratio was significantly higher in EAT of patients under the age of 60 ( $p = 0.023$ ) and those aged 60–74 ( $p = 0.011$ ) than in patients older than 75 years, with no differences observed between the first two groups. The highest Cer 18:0/24:0 ratio was found in SAT ( $p = 0.012$ ) and PVAT ( $p = 0.017$ ) of patients aged over 75 years and in EAT of patients under the age of 60 ( $p = 0.039$ ). The Cer 18:1/24:0 ratio was highest in EAT of patients aged 60–74 years ( $p = 0.0004$ ) compared to those older than 75 years. The Cer 24:1/24:0 ratio was highest in EAT ( $p = 0.027$ ) and PVAT ( $p = 0.013$ ) among the 60–74 age group.



**Note.** PVAT – perivascular adipose tissue; SAT – subcutaneous adipose tissue; EAT – epicardial adipose tissue; Cer – ceramides.

**FIG. 2.**

Concentration of ceramides with adverse cardiovascular effects in tissue lipid extracts of local fat depots of patients with CAD depending on age, ng/ml



**Note.** PVAT – perivascular adipose tissue; SAT – subcutaneous adipose tissue; EAT – epicardial adipose tissue; Cer – ceramides.

**FIG. 3.**

Concentration of ceramides with cardioprotective properties in lipid extracts of tissues of local fat depots of patients with CAD depending on age, ng/ml

## DISCUSSION

Regardless of AT location, a consistent increase in Cer d18:1 with saturated fatty acid residues (C14:0, C18:0, C20:0, C22:0, and C23:0) and monounsaturated fatty acid (C24:1) was observed in men with CAD. These findings are consistent with the data from Blachnio–Zabielska A.U. et al., who demonstrated that men exhibit preferential accumulation of Cer (d18:1/18:0), Cer (d18:1/20:0), and Cer (d18:1/22:0) in AT. Moreover, the total ceramide content (pg/adipocyte) was also higher in men compared to women (34 % vs. 31 %) [6]. However, in our study, Cer C24:1 levels were higher in male AT, which contradicts the results of these authors, who found that the concentration of this ceramide was higher in female AT. This discrepancy may be explained by differences in the AT location used for analysis. Specifically, Blachnio–Zabielska A.U. et al. exclusively focused on SAT collected laterally to the umbilicus through needle liposuction. Our study involves the analysis of visceral depots, EAT and PVAT, allowing us to assess their contribution to the pathogenesis of cardiometabolic diseases [7]. Considering that women exhibit an increase in superficial SAT precisely in the lateral area around the umbilicus [8], Cer C24:1 concentration in their SAT might be underestimated when analyzing other depots. The concentration of Cer (d18:1/16:0) in female AT was found to be significantly higher than that in males, which confirms the results of Brusatori M. et al. [7], who identified a specific accumulation of this ceramide in the visceral AT of women.

In our study, PVAT of men was characterized by elevated levels of Cer 14:0, 16:0, and 22:4 compared to women. At the same time, EAT of women showed an increase in Cer d18:1/17:2 concentrations. Additionally, both EAT and PVAT showed an increase in Cer 18:0 and 23:0 compared to men. In EAT and SAT of women, the concentrations of the following ceramides were higher than in the male group: Cer 20:0, 22:0, and 24:1. In SAT of women, elevated levels of Cer d18:1/22:2, 22:4, and 22:5 were found compared to men.

The identified differences in AT ceramide profiles may be due to differences in dietary habits between men and women. It is known that men on average consume more saturated fats than women, which stimulates the activity of ceramide synthase (CERS), enzymes that catalyze ceramide synthesis [9]. This leads to increased concentrations of ceramides containing saturated FAs, such as C16:0 and C18:0, in the AT. Therefore, increased saturated fat intake may contribute to the accumulation of cardioneegative saturated FA ceramides in male AT and, consequently, to the development of metabolic dysfunction [10].

The influence of sex hormones on ceramide synthesis and degradation should also be considered. Androgens, such as testosterone, can inhibit ceramide synthesis enzymes, e.g., CERS6. Six different CERS (CERS1-6) have been identified in mammals, with each having a specific fatty acid specificity. CERS6 plays

a primary role in the synthesis of ceramides containing palmitic and stearic FAs, which have been associated with adverse effects on the CVS [11]. The inhibition of CERS6 by testosterone observed in mouse experiments explains the lower levels of Cer C16:0 and C18:0 in the liver of males compared to females [12]. However, data suggest that sphingolipids and their metabolites can modulate steroid hormone secretion by regulating both their release and homeostasis [13]. This creates a feedback loop in which changes in the ceramide profile may affect hormonal status and in turn influence ceramide synthesis. Therefore, the ability of sex hormones to affect ceramide synthesis enzymes in human AT requires further study. The identified sex differences in AT ceramide profiles among CAD patients may be of prognostic significance, as elevated concentrations of certain ceramides, particularly those containing saturated FAs, are associated with increased cardiovascular risk [14].

Previous studies have convincingly demonstrated the relationship between circulating long-chain ceramides and age [15]. Age has also been shown to be closely associated with the prevalence of CAD [16]. Although there is a well-established association between blood long-chain ceramide levels and age [17], data on ceramide content in AT, especially in cardiac and vascular depots, and its age-related changes are extremely limited.

In this study, we identified a wide range of ceramides in AT, including those with both saturated and unsaturated acyl chains. Our data indicate that AT, especially EAT and PVAT, which are localized close to atherosclerotic lesion sites, may serve as a source of ceramides that can have both favorable and adverse effects [18].

We determined the characteristics of ceramide content in AT of different locations, depending on age, in CAD patients. The highest levels of cardioneegative ceramides Cer d18:1/14:0, 16:0, 18:0, and 22:0 were observed in EAT of individuals aged 60–74 years, and in PVAT of patients older than 75 years. The lowest concentrations of the cardioprotective ceramide Cer d18:1/26:0 in cardiac AT were found in the 60–74 age's group, which may be an unfavorable indicator for individuals of this age group. It is possible that cardiac AT undergoes more significant remodeling with age.

Bonzón–Kulichenko E. et al. [19] showed increased levels of Cer 18:0, 20:0, 22:0, and 24:1 in brown adipose tissue (BAT) and increased levels of Cer 18:1 and 20:0 in white adipose tissue (WAT) of 15-month-old male rats when compared to 2-month-old males. Moreover, in 24-month-old male rats, there were increased levels of Cer 16:0 and 20:0, and decreased levels of Cer 24:1 in WAT when compared to 3-month-old animals [20]. These findings suggest that the age-associated changes in the AT ceramide profile may vary depending on AT type and ceramide acyl chain length.

In addition to the acyl chain length, the presence of double bonds in the FA residues of ceramides must

be considered. Unsaturated FAs in ceramides are more prone to oxidation, which may lead to the formation of reactive oxygen species (ROS) and increased oxidative stress. Notably, in older patients with CAD, increased levels of ceramides containing double bonds have been observed [20]. This finding is consistent with the data on the differential effects of saturated and unsaturated ceramides on the CVS: for example, Cer 24:0 has a protective effect, while Cer 24:1 does not [21]. These differences may be due to the dysregulation of ceramide metabolism in CVD.

We then calculated the ratio of cardioneutral to cardioprotective ceramides in AT for each age group of CAD patients. The highest EAT ceramide ratios were observed in CAD patients aged 60–74 years, which may indicate an increased risk of adverse cardiovascular events and mortality from these diseases in this age group. This is consistent with the findings of Akawi N. et al. [22], who showed that elevated Cer d18:1/16:0 levels in EAT were associated with an increased risk of heart failure among elderly patients. Age-associated changes in the EAT phenotype, such as increased secretion of pro-inflammatory adipokines, are likely to contribute to the development of cardiomyopathy and myocardial fibrosis [23].

## CONCLUSION

Significant sex- and age-related differences in the AT ceramide profile have been identified in patients with CAD. Our data suggest that there is an age-related modulation of ceramide synthesis, while sex-specific differences in the ceramide profile indicate a possible influence of hormonal factors on this process. When analyzing the AT ceramide profile and its impact on cardiovascular risk, it is important to consider sex. With age, the cardiac and vascular AT undergo significant remodeling. The observed age-related shift in lipid balance, manifested by an increased ratio of cardioneutral to cardioprotective ceramides in EAT in patients aged 60–74 years and in PVAT in patients older than 75 years, suggests a potential increase in cardiovascular risk in these age subgroups. Modifying the ceramide profile of EAT and PVAT in these age groups represents a promising therapeutic approach to reducing cardiovascular risk and requires further prospective study.

### Conflicts of interest

The authors declare no conflicts of interest.

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## CLINICAL LABORATORY DIAGNOSTICS

### COMPARATIVE EVALUATION OF THE MAGNETIC RESONANCE CONTRAST PROPERTIES OF GADOLINIUM-BASED CONTRAST AGENTS

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#### ABSTRACT

**Background.** Restrictions on the use of gadolinium-containing radiocontrast agents due to the risk of acute and long-term complications are stimulating the search for new, safer, and more effective gadolinium-based nanoparticle formulations.

**Aim.** Comparative assessment of the magnetic contrast properties of a gadolinium nanocomposite and a gadolinium-based contrast agent.

**Methods.** The dynamics of substance accumulation and visual MRI patterns were studied in 5 healthy female Wistar rats using a Siemens Avanto device with a magnetic field of 1.5 T. Anesthetized animals were placed ventrally within the head coil, after which a preliminary MRI scan was performed. A gadolinium nanocomposite solution and a reference solution (Omniscan®) were injected at equivalent gadolinium doses into the lateral tail vein or into the abdominal cavity, followed by repeat MRI scans with image acquisition every 2–3 minutes. The primary imaging areas were the abdominal cavity and retroperitoneal space, including the thoracic cavity.

**Results.** Comparative analysis showed that gadolinium nanoparticles stabilized with arabinogalactan exhibit magnetocontrast properties, as evidenced by T1-weighted image enhancement of the liver parenchyma after intravenous administration and of the abdominal cavity after intraperitoneal administration. The magnetic contrast image of the kidneys and bladder was higher after Omniscan® administration than after gadolinium nanocomposite administration. However, the maximum contrast coefficient of the gadolinium nanocomposite in the liver parenchyma increased by 20 % compared to baseline. Omniscan® administration resulted in a 13 % increase in T1-weighted image intensity of the liver parenchyma.

**Keywords:** gadolinium, nanocomposite, nanoparticles, magnetic contrast properties, white rats

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## СРАВНИТЕЛЬНАЯ ХАРАКТЕРИСТИКА МАГНИТОКОНТРАСТНЫХ СВОЙСТВ ГАДОЛИНИЙСОДЕРЖАЩИХ ПРЕПАРАТОВ ДЛЯ МРТ-ДИАГНОСТИКИ

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### РЕЗЮМЕ

**Обоснование.** Ограничения применения гадолинийсодержащих рентгеноконтрастных веществ, обусловленные развитием острых и отдаленных осложнений, стимулируют поиск новых, более безопасных и эффективных форм на основе наночастиц гадолиния.

**Цель работы.** Сравнительная оценка магнитоконтрастных свойств нанокompозита гадолиния и содержащего гадолиний диагностического препарата.

**Методы.** Динамика органного накопления и визуальной МРТ-картины изучены на 5-ти здоровых белых крысах-самках линии Wistar на аппарате Siemens Avanto с магнитным полем 1,5 Тл. Для проведения МРТ анестезированных животных укладывали в вентральном положении в катушку, предназначенную для головы, проводили предварительное исследование на МРТ-томографе, затем в латеральную хвостовую вену или в брюшную полость с помощью катетера вводили раствор нанокompозита гадолиния или препарата сравнения (Омнискан®) в эквивалентных по гадолинию дозах и проводили повторное МРТ-исследование, делая снимки через каждые 2–3 минуты. Центром области исследования были брюшная полость и забрюшинное пространство с захватом в поле зрения грудной клетки.

**Результаты.** Выполненный сравнительный анализ показал, что наночастицы гадолиния, стабилизированные арабиногалактаном, обладают магнитоконтрастными свойствами, доказательством чему явилось усиление T1-взвешенного изображения в паренхиме печени при внутривенном введении и брюшной полости при внутрибрюшинном введении. Магнитоконтрастное изображение почек, мочевого пузыря при введении препарата Омнискан® выше, чем при введении нанокompозита гадолиния. Однако максимальная величина коэффициента контрастирования нанокompозита гадолиния в паренхиме печени возрастала по сравнению с исходным состоянием на 20 %, при введении Омнискана® наблюдалось усиление интенсивности T1-взвешенного изображения паренхимы печени на 13 %.

**Заключение.** Использование нанокompозита гадолиния является перспективным для МРТ-диагностики. На данном этапе пока преждевременно говорить о возможностях применения исследуемого нанокompозита гадолиния в клинической практике, требуется более детальное рассмотрение его влияния на процессы релаксации как в нормальных, так и в патологически измененных тканях.

**Ключевые слова:** гадолиний, нанокompозит, наночастицы, магнитоконтрастные свойства, белые крысы

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## BACKGROUND

Extensive data on the development of acute and long-term consequences of gadolinium-based contrast agents used in MRI diagnostics have been collected in clinical practice. The most severe of these is nephrogenic systemic fibrosis (NSF). It has been established that free gadolinium  $Gd^{3+}$  is highly toxic to tissues and accumulates in the liver, brain, and bones after administration [1–4]. At the same time, clinical manifestations of this accumulation may not manifest immediately or in every case. Given that the ionic radius of  $Gd^{3+}$  is almost similar to that of  $Ca^{2+}$ , gadolinium functions as an inorganic blocker of voltage-gated calcium channels, suppressing physiological processes dependent on the inward  $Ca^{2+}$  current (in particular, contraction of smooth, striated muscle and myocardium) and the activity of certain enzymes (in particular, a number of dehydrogenases and kinases) [5–7]. Furthermore, calcium-sensing receptors on hepatocytes, renal cells, fibroblasts, and certain other cells may be activated by gadolinium. The most significant acute toxic effects of free gadolinium occur in the liver, causing hepatocyte necrosis [8]. Most of the gadolinium is excreted by the kidneys, but traces of the substance may remain in the body over an extended period. The risk of gadolinium retention is increased in pregnant women, children, and patients with impaired renal excretory function. Among the risk factors for adverse effects associated with contrast agents, the primary one is the dose of administered contrast agent. In MRI diagnostics, administration of up to 2–3 doses (0.2–0.3 mmol/kg body weight) of gadodiamide is permitted. Although NSF may develop even after administration of a single dose, the risk of this disease is increased with a single administration of a higher dose, as well as after repeated injections [9]. Since historical data on the contrast agents used and their doses are often unavailable, information on the possible cumulative effect of repeated contrast agent administration is very limited. The stability of the chelate complex of gadolinium-based agents is also of great importance for the development of adverse effects. The greatest caution is required when using agents with low stability (Omniscan® or Optimark®), which include non-ionic linear substances. After administration of the latter, there is always a high probability of gadolinium deposition in the patient's body. At the same time, in the opinion of clinicians, the benefits of the studies continue to outweigh the risks.

In light of the above, there has been an increasing focus in recent years on the development of safe gadolinium nanoparticles. This work has emphasized the need to minimize drug toxicity and the generation of artifacts in resulting images. Recently, nanoparticles based on cerium, gadolinium, and gadolinium orthoferrites have been developed for magnetic resonance contrast enhancement. The creation of contrast agents in nanoform can significantly improve diagnostic

accuracy and, most importantly, reduce the risk of adverse consequences for patients. Nanoparticles offer a number of advantages: increased relaxivity, the possibility of targeted delivery [10, 11], the ability to be eliminated more rapidly from the body, and, importantly, a fundamentally different route of excretion, often not associated with renal filtration. There is a second pathway of  $Gd^{3+}$  excretion, which is through the liver. The presence of this pathway significantly reduces the risk of adverse processes associated with gadolinium accumulation. Overall, gadolinium nanoparticles may become the basis for a new generation of MRI contrast agents. This would make diagnostic procedures more informative and safe.

In addition to the lack of toxicity and high magnetic contrast properties, increasing the bioavailability of gadolinium nanoparticles is essential. For effective delivery of nanoparticles to body structures, a natural polysaccharide derived from Siberian larch (arabinogalactan) is used. In our studies, an innovative nanocomposite consisting of gadolinium nanoparticles encapsulated in a matrix of the natural polymer arabinogalactan, which allows the creation of stable solutions suitable for use in biological media, was used for the first time. The properties of arabinogalactan (water solubility, the presence of functional groups for modification, low immunogenicity) make it an ideal basis for the creation of nanoconjugates with  $Gd^{3+}$  ions [12, 13]. The use of gadolinium nanoparticles encapsulated in an arabinogalactan matrix for magnetic resonance contrast diagnostics, in our opinion, will allow a reduction in the administered dose of the contrast agent while maintaining effectiveness, which will significantly reduce the toxic load on the body.

## AIM

Comparative assessment of the magnetic contrast properties of a gadolinium nanocomposite and a gadolinium-based contrast agent.

## MATERIALS AND METHODS

As the test material for MRI, we used a preparation containing gadolinium nanoparticles stabilized with arabinogalactan (Gd-AG), which was produced at the V.V. Voevodsky Institute of Chemical Kinetics and Combustion of the Siberian Branch of the Russian Academy of Sciences. Arabinogalactan is a natural nanostabilizing polysaccharide, which is a water-soluble white or cream powder, tasteless and odorless, consisting of two monosaccharides: galactose and arabinose. The nanocomposite consists of chelate complexes of bioflavonoids and  $Gd^{3+}$  gadolinium nanoparticles encapsulated in water-soluble arabinogalactan macromolecules. The gadolinium content (4.8 %) was determined using a TM 3000 scanning microscope (Hitachi,

Japan) with an X-ray energy-dispersive microanalysis attachment. Electron microscopy for particle size distribution was performed on a Leo 906 E transmission electron microscope (Zeiss, Germany); the sizes of the metal-complex nanoparticles ranged from 1 to 72 nm, with an average size of 12–15 nm. The shape of the nanoparticles and the nanocomposite macromolecules was spherical. As a reference preparation for MRI studies, we used the commercially available preparation Gadodiamide (brand name – Omniscan®), a paramagnetic contrast agent based on a monomolecular gadolinium chelate complex. It is used for intravenous administration in MRI, with a concentration of 0.5 mmol/mL. The active ingredient in Gadodiamide is gadodiamide, and the solution contains 0.5 mmol (287 mg) of gadodiamide per milliliter of solution. Gadodiamide is a positive MRI contrast agent and a clinically safe gadolinium-chelate complex. It is administered as a parenteral solution of gadolinium, and its chemical structure is represented by the formula [5,8-bis(carboxymethyl)-11-[2-(methylamino)-2-oxoethyl]-3-oxo-2,5,8,11-tetraazatridecan-13-oato(3-)] gadolinium ( $C_{16}H_{26}GdN_5O_8$ ).

The study of the magnetic resonance contrast properties of the gadolinium nanocomposite and the reference preparation (Omniscan®), administered intravenously to Wistar rats, was conducted using an MRI system at the Best Veterinary Clinic in Novosibirsk, Russia. The MRI system used in the study was a Siemens Avanto (SIEMENS) scanner with a 1.5-T magnetic field. A T1-weighted imaging protocol was employed, which included fat suppression (T1vibe\_fs): TR (repetition time) – 5.5 ms; TE = 2.7 ms, FOV = 160, slice thickness – 2.5 mm. Images were obtained using a 256 x 256 pixel matrix.

At the first stage, a pilot study was conducted to investigate the intensity of T1-weighted images of phantoms that contained a solution of the gadolinium nanocomposite and the reference preparation (Omniscan®) with equivalent concentrations of gadolinium. The gadolinium nanocomposite solution was prepared by adjusting the amount of gadolinium to match the therapeutic dose of Omniscan® (0.1 mmol/kg). Both phantom studies and *in vivo* experiments used this gadolinium solution. Equal volumes (1.5 mL) of the prepared solutions contained 5.5 mg/mL of gadolinium in ionic form and in nanoform. A control tube contained only 1.5 mL physiological saline.

At the second stage, the effect of the gadolinium nanocomposite on the intensity and quality of MRI images upon administration of gadolinium-containing agents to healthy female white rats was evaluated. To assess the contrast properties of the gadolinium nanocomposite, 5 rats underwent MRI before and after receiving a gadolinium-containing agent (intravenous injection,  $n = 2$ ; intraperitoneal injection,  $n = 1$ ) and Omniscan®

(intravenously,  $n = 2$ ). The MRI procedure was performed under anesthesia using ketamine injections through a peripheral 29G catheter according to the following scheme: an initial anesthetic dose of 100 mg/kg ketamine for anesthesia induction, followed by two subsequent injections of 50 mg/kg. Anesthetized animals were placed on a feedback-controlled heating pad at 37°C to maintain body temperature. Adequate anesthesia was confirmed by the absence of the righting reflex and response to tail pinch. The next dose (50 mg/mL) was administered when the animals regained response to tail pressure stimuli. The average anesthetic duration was 2.5 hours. Anesthetized animals received intravenous injections of the following doses of test agents: Omniscan® – 0.2 mL/kg (0.025 mmol/kg equivalent to 14.3 mg/kg gadolinium) and gadolinium nanocomposite – 0.346 mg/kg in 1.5 mL physiological saline (equivalent to 3.5–4.13 mg gadolinium depending on body weight). For MRI, the animals were positioned in a ventral position within a head coil. A preliminary MRI scan was performed, followed by the administration of either the gadolinium nanocomposite or the reference preparation via a catheter inserted into the lateral tail vein. A second MRI scan was then performed, with images acquired every 2–3 minutes.

The center of the scanning area was the abdominal cavity and the retroperitoneal space, with the chest being included in the field of view. During the image processing using the region-of-interest tools on the T1-weighted MRI images of the white rats, various regions were selected, including muscles, the heart, urinary bladder, renal cortex and medulla, as well as liver parenchyma.

The contrast effect of the nanocomposite and the reference preparation was assessed using the contrast coefficient (CC). This was calculated as the ratio of the mean intensity value obtained from five measurements of internal organs (liver parenchyma, myocardium, renal cortex, renal medulla, and urinary bladder) in rats, to the mean intensity value from five measurements in the lower limb muscle region of the experimental animals.

Image processing was performed using the Vidar Dicom Viewer software package (version 3.4.2)<sup>1</sup>.

Statistical analysis of the study results was conducted using Statistica 6.1 (StatSoft). The Shapiro – Wilk W-test was employed to determine the distribution type. For comparing groups (before and after contrast agent administration), Student's *t*-test for paired samples was used. Null hypotheses regarding no differences between the groups were rejected if the significance level was  $p \leq 0.05$ .

Experimental studies were conducted at the vivarium of the East-Siberian Institute of Medical and Ecological Research (ESIMER) using sexually mature white Wistar rats weighing 180–230 g. The animals were

<sup>1</sup> <https://povidar.ru/posts/2025/03/31/vidar-dicom-viewer-3-4-2.html>

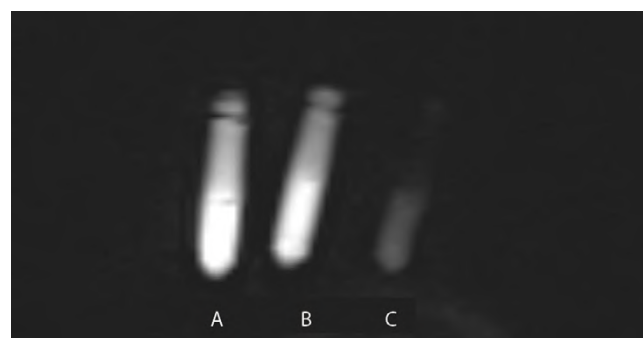
kept in a controlled environment with a 12-hour light/dark cycle and regulated temperature ( $22 \pm 3^\circ\text{C}$ ). The humidity was maintained at 60 %, with free access to clean drinking and food that contained all necessary vitamins and trace elements. The vivarium of the ESIMER has received a Certificate from the Angarsk Animal Disease Control Station, (April 21, 2023), confirming that it meets all veterinary and sanitary requirements for housing, handling, and conducting experiments on laboratory animals, as well as ensuring the safety of laboratory animals in terms of veterinary and sanitary standards. The work was conducted in compliance with the principles of humane treatment of animals, as outlined in the Helsinki Declaration on the Humane Treatment of Animals adopted by the World Medical Association (revised in October 2008) and the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes, signed in Strasbourg on March 18, 1986, as well as the “Rules of Laboratory Practice”, issued by the Ministry of Health and Social Development on August 23, 2010 (Order No. 708n). Prior to initiating the study, approval was obtained from the local ethics committee of the ESIMER, which confirmed that the procedures for handling animals were in accordance with ethical standards (Protocol No. 5, November 27, 2024).

## RESULTS

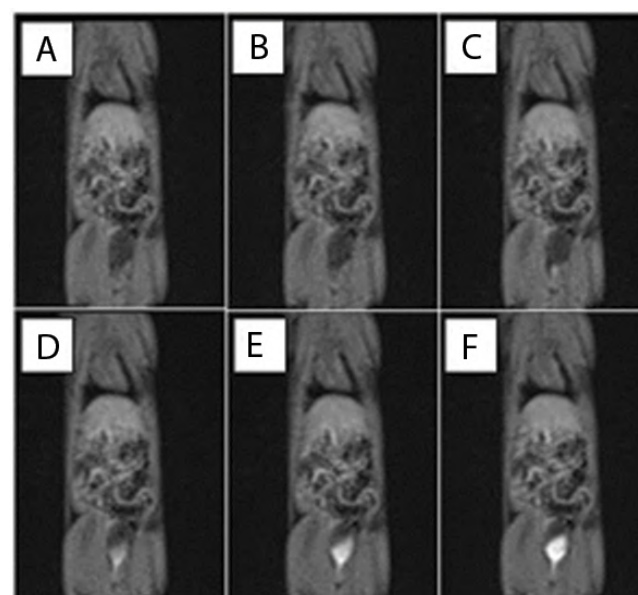
The study of the intensity of T1-weighted images of phantoms containing a solution of a gadolinium nanocomposite and a reference preparation (Omniscan®) at equivalent concentrations (in terms of gadolinium) showed that both agents significantly enhanced T1-weighted image contrast at this concentration. Equal volumes (1.5 mL of physiological saline) of prepared solutions containing 5.5 mg/mL of gadolinium in ionic form and in nanoform, were used. The control tube contained only 1.5 mL of physiological saline. It was found that the MR relaxation parameters for Omniscan® and Gd-AG in aqueous solution were  $507 \pm 40$  arbitrary units and  $356 \pm 27$  arbitrary units, respectively, which exceeded the signal from physiological saline by 7–8 times (Fig. 1).

Therefore, the results of the study on phantoms demonstrated that the gadolinium nanocomposite exhibits imaging properties that make it a potential diagnostic magnetic contrast agent.

Figures 2 and 3 show T1-weighted images of a white rat's urinary bladder and kidneys at the levels of maximum long-axis projection and maximum cross-sectional area, respectively, illustrating the dynamics of Omniscan® accumulation over 20 minutes after administration. In the kidneys of white rats, gadodiamide was visualized as early as 3 minutes ( $p = 0.01$ ) after intravenous administration (Fig. 3), while in the urinary bladder, it was seen from 5 minutes (from 7 minutes,



**FIG. 1.**  
T1-weighted image of phantoms containing Omniscan® (A), Gd-AG (B), and saline (C) (TR = 4.6 ms; TE = 2.1 ms)



**FIG. 2.**  
T1-weighted image showing the dynamics of Omniscan® accumulation in the rat urinary bladder before (A) and at 3 (B), 5 (C), 10 (D), 15 (E), and 20 (F) minutes after intravenous administration

$p = 0.0001$ , Fig. 2). The contrast coefficient increased 1.4–2.2-fold in the kidneys and 6.5-fold in the urinary bladder. The intensity of T1-weighted images of the rat myocardium did not change over time after Omniscan® administration.

Figure 4 presents T1-weighted images of the body of a white rat before and after the administration of the gadolinium nanocomposite solution. After the intravenous injection of the gadolinium nanocomposite, there was no noticeable increase in the intensity of the images in any of the organs or tissues, including the kidneys, heart, muscle, subcutaneous fat, and intestinal structures, over the course of 30 minutes.

The absence of a contrast effect of the gadolinium nanocomposite in the kidneys may be due to the branched structure and high molecular weight (approximately 20 kDa) of the arabinogalactan macromolecule that serves as a matrix for stabilizing the gadolinium nanoparticles. It is worth noting that the comparative characteristics of the renal barrier in different animal species demonstrate significant differences in glomerular membrane permeability. In humans, the barrier is impermeable to molecules larger than 15,000 Daltons, while in rats it is impermeable to molecules exceeding 2,000 Daltons [14]. Given the larger molecular weight exceeding the glomerular filtration threshold and the negative charge of the studied gadolinium nanocompound, it is likely that these factors hinder their penetration into the filtrate.

At the same time, after administration of the gadolinium nanocomposite, there was an increase in the T1-weighted signal in the liver parenchyma of white rats, which reached statistical significance ( $p = 0.015$ ) at 26 minutes compared to the pre-administration level (Fig. 5). In the liver parenchyma, the maximum contrast coefficient CC, which is the ratio of image intensities of internal organs to that of the muscles in the rat's hind limb, increased by 13 % compared to baseline values (Table). Following the administration of Omniscan®, a significant ( $p = 0.032$ ) increase in the intensity of the T1-weighted image of the liver parenchyma was observed only once, at 2.5 minutes after administration (Fig. 5).

The obtained results indicate that the studied gadolinium nanocomposite accumulated in the liver parenchyma and enhanced its image contrast.

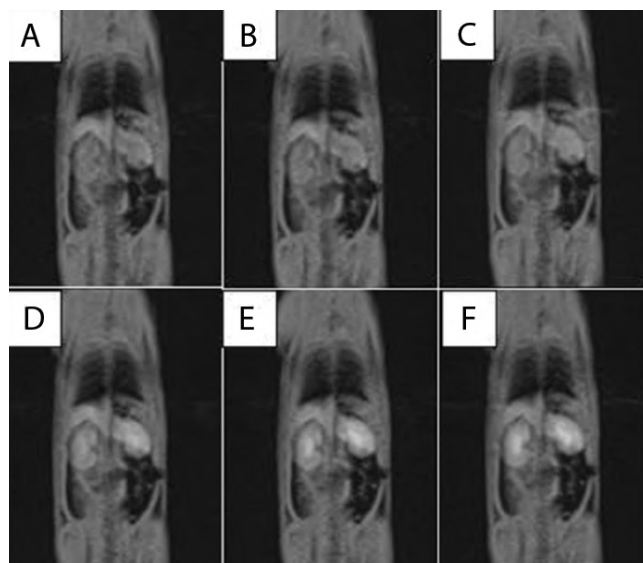
Additionally, the imaging characteristics were evaluated following intraperitoneal administration of the gadolinium nanocomposite into white rats at doses similar to those used for intravenous administration. Figure 6 shows frontal T1-weighted MRI sections of the body of a white rat after intraperitoneal infusion of the gadolinium nanocomposite solution.

As can be seen from Figure 6, when Gd-AG was administered intraperitoneally to rats, it was clearly visible in the abdominal cavity, confirming its potential for enhancing contrast.

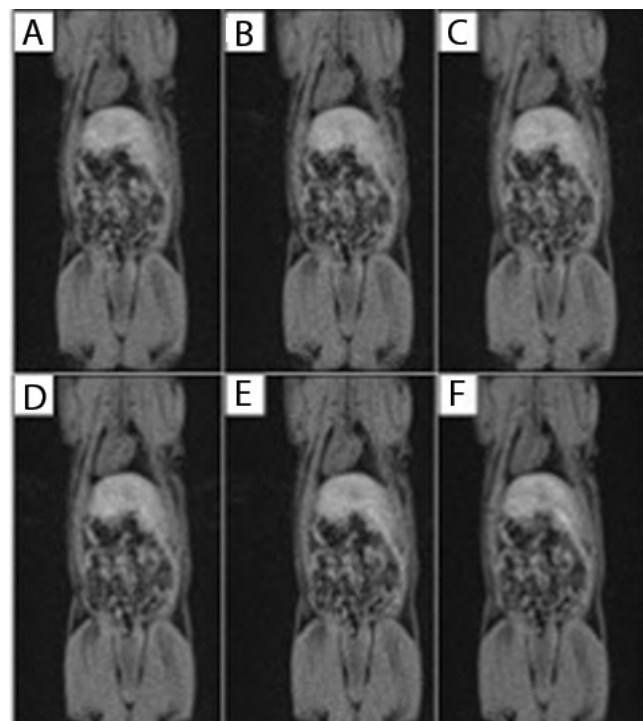
## DISCUSSION

As a result of the study, the contrast properties of the gadolinium nanocomposite were evaluated in laboratory animals. The unequivocal presence of contrast properties in the gadolinium nanocomposite was confirmed through phantom studies, which demonstrated its imaging capabilities, as evidenced by significant enhancement of T1-weighted images similar to those produced by the reference agent Omniscan®. Additionally, potential magnetic contrast properties were also established upon intraperitoneal administration of the gadolinium nanocomposite in rats,

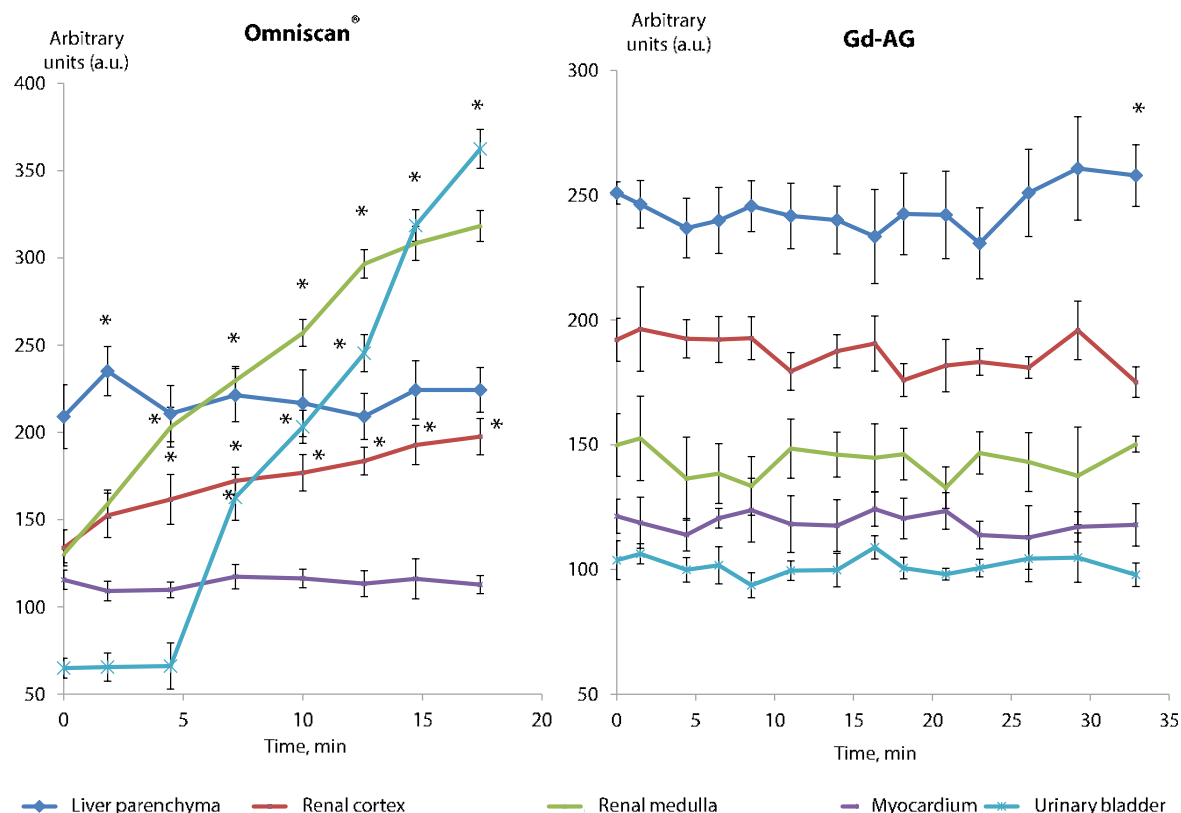
with clear visualization in the abdominal cavity using MRI. In this case, the studied nanocomposite remained in the abdominal cavity in an almost undiluted state,



**FIG. 3.** T1-weighted images showing the dynamics of Omniscan® accumulation in the rat kidneys before (A) and at 3 (B), 5 (C), 10 (D), 15 (E), and 20 (F) minutes after intravenous administration



**FIG. 4.** T1-weighted images showing the dynamics of gadolinium nanocomposite accumulation in the rat heart and liver parenchyma before (A) and at 8 (B), 16 (C), 22 (D), 26 (E), and 30 (F) minutes after intravenous administration



Note. \* – statistically significant differences between pre- and post-contrast values ( $p < 0.05$ ).

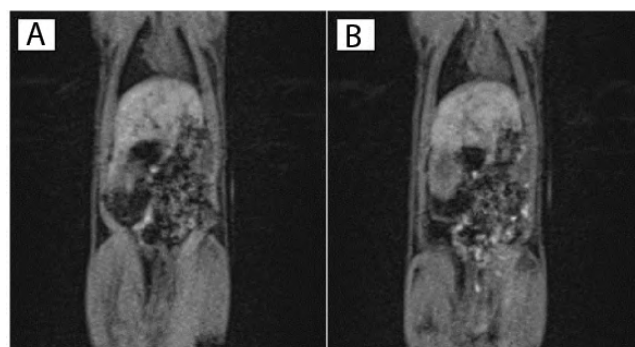
**FIG. 5.**

Changes in T1-weighted image intensity in rat internal organs after intravenous administration of the gadolinium nanocomposite and Omniscan®

as there is typically a small amount of serous fluid present between abdominal organs. Since the circulating blood volume in white rats averages 50–70 mL/kg [15], upon intravenous administration, the studied contrast agents were diluted in 9–16.8 mL of blood. At the same time, the concentration levels of these agents were 9–11 times lower than those at which image intensity enhancement was recorded in phantom studies. It can be assumed that the concentration of the nanocomposite after intravenous administration was insufficient for its contrast properties to manifest, and further studies are required to evaluate the effective dose of this agent.

However, the pharmacodynamics properties of gadolinium nanoparticles encapsulated within arabinogalactan in terms of their localization, organ accumulation, and the magnitude and duration of magnetic resonance contrast effects, differed from the gadolinium chelate contained in the commercial preparation Omniscan®. The studied compounds had significant differences in the degree of renal and urinary bladder enhancement. Contrast enhancement of the kidneys

and urinary bladder with Omniscan® was pronounced and observed as early as the second minute after administration. Upon administration of the gadolinium



**FIG. 6.**

T1-weighted images showing Gd-AG accumulation in the rat abdominal cavity before (A) and at 20 (B) minutes after intraperitoneal administration

TABLE

**MAXIMUM CONTRAST COEFFICIENTS ON T1-WEIGHTED IMAGES IN RAT ORGANS AFTER ADMINISTRATION OF THE GADOLINIUM NANOCOMPOSITE AND OMNISCAN®**

| Agent     | Liver parenchyma | Myocardium | Renal cortex | Renal medulla | Urinary bladder |
|-----------|------------------|------------|--------------|---------------|-----------------|
| Gd-AG     | 1.77             | 0.76       | 1.16         | 0.97          | 0.65            |
| Omniscan® | 1.57             | 0.87       | 1.37         | 2.24          | 2.63            |

nanocomposite at a dose equivalent to one diagnostic dose of Omniscan® (in terms of gadolinium), gadolinium was not visualized in the kidneys or urinary bladder for 40–50 minutes after intravenous administration. In our opinion, this is due to the large molecular weight and the negative charge of the molecule of the studied gadolinium nanocomposite, which impede its penetration into the renal filtrate.

At the same time, the maximum contrast coefficient of the gadolinium nanocomposite within the liver parenchyma was higher than with Omniscan® administration. Gadolinium nanoparticles encapsulated in arabinogalactan accumulated in the liver 26 minutes after intravenous administration, with the maximum contrast coefficient of the gadolinium nanocomposite increasing by 20 % compared to baseline. Whereas with Omniscan® administration, an increase in the intensity of the T1-weighted image of the liver parenchyma was observed only by 13 %. It is known that arabinogalactan has a high affinity for asialoglycoprotein receptors, the greatest number of which are found on hepatocytes, and belongs to polysaccharides that enter the cell via receptor-mediated endocytosis [16]. Available literature data on the pharmacokinetics and biodistribution of arabinogalactan indicate that upon intravenous administration, arabinogalactan is distributed mainly in the liver, accounting for up to 52 % of the administered dose [17]. The substance also accumulates in the liver for an extended period, lasting more than 5 days.

It should be noted that the studies conducted were performed on healthy experimental animals with no pathology or lesions. Pathological tissues metabolically differ from healthy tissues, and therefore capture contrast agents more effectively, leading to more contrast-rich MRI images. In this context, the evaluation of the contrast properties of the studied gadolinium nanocomposite under conditions of experimental modeling of liver pathology, including both oncological and non-oncological diseases, becomes particularly important. A more in-depth examination of its effects on relaxation processes in both normal and pathological tissues is necessary.

**Study limitations**

The studies were limited to the analysis of the MRI contrast properties of gadolinium nanoparticles in healthy white rats, without any pathological

processes in the tissues that could potentially affect the final imaging contrast enhancement outcomes.

**CONCLUSION**

Overall, the use of gadolinium nanocomposite for MRI diagnostics is promising. However, at present, it is premature to discuss the clinical application of the studied gadolinium nanocomposite. Further research is needed to fully understand their effects on relaxation processes in normal and abnormal tissues. At the same time, the use of arabinogalactan molecules as a stabilizing ligand in the nanocomposite, which has a hepatocellular targeting effect, expands the potential uses of this nanocomposite for receptor-mediated delivery of gadolinium nanoparticles to parenchymal liver cells when visualizing pathological changes in liver tissue. Safety and efficacy of gadolinium nanoparticle-arabinogalactan conjugates will be confirmed through further animal studies and clinical trials.

**Conflicts of interest**

The authors declare no conflicts of interest.

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## MICROBIOLOGY AND VIROLOGY

### ECOLOGICAL ASPECTS OF PARAMYXOVIRUS CIRCULATION IN WILD BIRD POPULATIONS ON THE TERRITORY OF CONTINENTAL EURASIA

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#### ABSTRACT

**Background.** Avian paramyxoviruses (subfamily Avulavirinae) are widespread avian pathogens that constantly circulate in wild and domestic animal populations. The subfamily Avulavirinae is divided into 3 genera, comprising 22 species. Epizootic surveillance of avulaviruses is conducted worldwide and is of critical importance due to their rapid spread via bird migrations. Avian viruses can cause significant economic damage to the poultry industry both in developing countries and in regions with sustainable economic development. Their infectious potential is of great interest and remains poorly understood.

The majority of intercontinental flyways of migratory birds pass through the territory of continental Eurasia, where large numbers of migratory birds congregate in confined areas during stopovers, nesting and wintering. This creates favorable conditions for virus transmission and the emergence of new genetic variants.

**Aim.** To analyze the species distribution of paramyxoviruses in wild birds across continental Eurasia.

**Materials and Methods.** As a result of long-term studies of viruses in wild bird populations across continental Eurasia, avian avulavirus isolates were obtained.

**Results.** Avian avulavirus isolates of APMV -1, -3, -4, -6, -8, -9, -11, -12, -13, -16, -20, and -21 were obtained. APMV-1 and APMV-4 were detected throughout Eurasia, most frequently in Anseriformes. APMV-3, -6, -9, -11, and -12 were recorded in the European part of Eurasia, APMV-8, -13, and -20 in its central part, and APMV-6, -16, and -21 in the Asian part, in Anseriformes, Passeriformes, Charadriiformes, Ciconiiformes, and Psittaciformes.

**Keywords:** bird paramyxoviruses, avulaviruses, metaavulaviruses, orthoavulaviruses, paraavulaviruses, continental Eurasia, Newcastle disease virus, wild birds

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## ЭКОЛОГИЧЕСКИЕ АСПЕКТЫ ЦИРКУЛЯЦИИ ПАРАМИКСОВИРУСОВ В ПОПУЛЯЦИЯХ ДИКИХ ПТИЦ НА ТЕРРИТОРИИ КОНТИНЕНТАЛЬНОЙ ЕВРАЗИИ

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### РЕЗЮМЕ

**Цель настоящей работы** заключается в анализе видового распространения парамиксовирусов диких птиц на территории континентальной Евразии. Парамиксовирусы птиц (подсемейство Авулавирусы) — широко распространенные патогены птиц, постоянно циркулирующие в популяциях диких и домашних животных. Подсемейство Авулавирусы разделено на 3 рода, включающих 22 вида. Эпизотоологический надзор за авулавирусами ведется по всему миру и очень важен из-за их быстрого распространения благодаря миграциям птиц. Вирусы птиц способны нанести значительный экономический ущерб отрасли птицеводства. Их инфекционный потенциал представляет собой большой интерес и является малоизученным.

Через территорию континентальной Евразии проходит основная часть межконтинентальных перелетных путей мигрирующих птиц, где на ограниченной местности концентрируется огромное количество перелетных птиц во время стоянок, гнездования и зимовок, что создает благоприятные условия для распространения вирусов и возникновения новых генетических вариантов.

В результате многолетних исследований вирусов в популяциях диких птиц на территории континентальной Евразии были выделены образцы авулавирусов птиц АPMV-1, -3, -4, -6, -8, -9, -11, -12, -13, -16, -20, -21. По всей территории Евразии были выявлены АPMV-1 и АPMV-4, наиболее часто — у гусеобразных. Виды АPMV-3, -6, -9, -11, -12 были отмечены в европейской части Евразии, виды АPMV-8, -13, -20 в ее центральной части, виды АPMV-6, -16, -21 в азиатской части у гусеобразных, воробьинообразных, ржанкообразных, аистообразных, попугаевых.

**Ключевые слова:** парамиксовирусы птиц, авулавирусы, метаавулавирусы, ортоавулавирусы, параавулавирусы, континентальная Евразия, вирус болезни Ньюкасла, дикие птицы

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## INTRODUCTION

Viral pathogens are widespread and continuously circulate in human, animal, and bird populations, posing a major threat to health. Viruses of the family *Paramyxoviridae* have a broad host range and are isolated from fish, reptiles, birds, animals, and humans worldwide [1]. Avian viruses (subfamily *Avulavirinae*) – avulaviruses, particularly the Newcastle disease virus (NDV), receive special attention due to their ability to spread rapidly across the globe through bird migrations. Avulaviruses can also cause significant economic damage to the poultry industry, both in developing countries and in regions with sustainable economic development. Many of the known avian paramyxovirus species have been identified through international epidemiological surveillance programs for influenza viruses.

To analyze the species distribution of avulaviruses across continental Eurasia, information was retrieved from the eLibrary.ru, CyberLeninka, ScienceJournals, Scopus, Web of Science, ScienceDirect, SpringerLink, and PubMed databases for the past 10 years.

Avulaviruses cause viral infections in various species of domestic and wild birds [2]. Numerous cases of infection in synanthropic bird species have also been reported [3], which constitute a distinct reservoir where viruses can concentrate, persist, and undergo genetic changes. Synanthropic birds, having been infected with avulaviruses from wild birds, can transmit the infection to domestic poultry. For instance, transmission of Newcastle disease virus from pigeons to chickens has been described [4].

Newcastle disease virus in poultry, which belongs to the genus *Avian orthoavulavirus*, is highly pathogenic and capable of causing massive epizootics among wild and domestic birds. This fact is particularly significant as it poses a major economic threat to poultry industries worldwide. For example, the Advisory Committee of the Eurasian Economic Commission on Dangerous Pathogens has classified NDV as a biosafety level 2 pathogen in the United Kingdom [5].

The infectious potential of other avian paramyxovirus species (hereinafter APMV) remains poorly understood. However, in recent years, these viruses have been increasingly isolated from wild and domestic birds worldwide and represent a potential health threat, as infection of birds with certain virus species (avian paramyxoviruses -2, -3) is associated with respiratory tract lesions, decreased egg production, and neurological symptoms [5]. There is also a possibility of infection in non-avian species. It has been shown that avian paramyxoviruses 1-9, except avian paramyxovirus -5, can replicate in mice and hamsters upon intranasal infection, with avian paramyxoviruses -1, -2, -6, -7, -9 causing broncho-interstitial pneumonia of varying severity and significant weight loss [6].

### Classification

Avian paramyxoviruses belong to the subfamily *Avulavirinae*, which is part of the family *Paramyxoviridae*.

Species division is based on hemagglutination inhibition and neuraminidase inhibition assays. According to the latest taxonomic data, the order *Mononegavirales*, subfamily *Avulavirinae* comprises three genera [7]:

Genus *Metaavulavirus* (14 species, including *Avian metaavulavirus* -2, -6; *Avian paramyxovirus* -2, -5-8, -10, -11, -14, -15, -20, -22);

Genus *Orthoavulavirus* (11 species, including *Orthoavulavirus* sp. *Virus*, *Antarctic penguin virus* A, B, C, *APMV* -1, -9, -12, -13, -16, -21, *AOAV* -12);

Genus *Paraavulavirus* (including species *Paraavulavirus* -3, -4).

Viruses APMV -1, -9, APMV -2, -5-8, and species APV -3, -4 were isolated and characterized before 1980, while APMV -10, -11, APMV -12, -13 were identified between 2005 and 2013. APMV -14, isolated from a duck in Japan in 2011 [8], AMAV-15, isolated from a sandpiper (Bonaparte's sandpiper, family *Scolopacidae*) in Brazil in 2012 [9], and APMV -16, isolated from a wild duck in South Korea in 2014 [10], were included in the subfamily *Avulavirinae* in 2018 [11]. Antarctic penguin viruses A, B, C were isolated from Antarctic penguins (Chinstrap penguin, Adelie penguin) in 2017 [12]. Also, in 2013 and 2014, AMAV-20 (GenBank: MH844489.1) was isolated from gulls in Kazakhstan [13]. APMV-21 was isolated from wild birds in South Korea in 2017 [14]. APMV-22 was isolated from samples collected from red turtle doves in Taiwan [15].

### Ecological Aspects of Avulavirus Circulation in Wild Bird Populations of Continental Eurasia

Continental Eurasia is a vast territory through which most intercontinental migratory flyways of birds pass: the East Asian-Australasian, Central Asian, West Asian-East African, Mediterranean-Black Sea, and Atlantic flyways [16], where large numbers of migratory birds concentrate in confined areas during stopovers, nesting, and wintering. Currently, there are about 250 species of wild birds that are natural carriers of NDV and other avulaviruses. Thus, favorable conditions are created for the further spread of various avulavirus types among birds and infection of healthy individuals. In this regard, monitoring of viral diseases in birds and characterization of obtained virus isolates are of great importance. Surveillance of avulaviruses in natural environments, as well as in high-risk areas for virus transmission between domestic and wild birds, will provide information on the ecology, epizootiology, and genetic relationships of viruses. These data can offer insight into currently circulating virus types and contribute to risk assessment of epizootics in domestic and wild bird populations.

### Ecological Aspects of Avian Avulavirus-1 Circulation in Wild Bird Populations

Avian Avulavirus-1 is currently the most studied, as it includes all strains of NDV, which is economically

significant for poultry production due to the high contagiousness of the disease it causes in commercial and domestic poultry. Infection with viruses of this species can vary in severity – from asymptomatic or mild carriage to severe disease, accompanied by fever, respiratory tract lesions, pneumonia, multiple hemorrhagic lesions of internal organs, CNS involvement, encephalitis, leading to death [5]. Based on pathogenicity, these strains are classified as velogenic, mesogenic, and lentogenic types. The varying levels of pathogenicity are determined by the amino acid sequence composition of the protease cleavage site of the  $F_0$  [17]. NDV strains cause viral diseases in wild and domestic birds. A separate group includes avulavirus-1 strains infecting wild and domestic pigeons, which are also contagious to other bird species, posing a primary threat to domestic and commercial poultry populations [17].

NDV is endemic to developing countries in Africa, Asia (India, Pakistan, Iran), and Central America with predominantly agricultural economies, where poultry production plays a significant role. Despite vaccination programs, periodic outbreaks are also recorded in developed countries such as the USA, Australia, and the EU. NDV was first characterized in 1926 in Indonesia and the United Kingdom, then in 1928 in India, and in the 1920s-1950s caused the first panzootic wave of Newcastle disease. The virus subsequently spread to eastern regions of Eurasia, causing a second wave of epizootics in Middle Eastern and European countries in the 1960s. Phylogenetic analysis has shown relatedness between isolates collected in Denmark, Sweden, Switzerland, and Austria and Middle Eastern isolates from the 1960s, whereas isolates from Germany, Belgium, the Netherlands, Italy, and Spain had a more recent origin and were novel to the European region, being genetically similar to isolates from the Far East [18]. In the 1980s, no major NDV outbreaks were observed in European countries, but since the early 1990s, the number of registered NDV cases has been steadily increasing. In 1996, NDV caused outbreaks in Scandinavian countries, and a genetically related NDV strain was isolated from birds in Germany in 1993–1996, then in Finland and Denmark in 2001 [19], as well as in Sweden and Denmark in 2002–2004. In the period 2000–2009 and in 2016, pigeon NDV infections were regularly reported in Europe. In Switzerland in 1996, antibodies to APMV-1 were found in 10 % of wild birds during serological surveys. Individual NDV epizootic outbreaks were recorded in Italy from 1990 onward, and then almost annually from 2000 to 2009 in wild mallard populations. In 2005, PPMV-1 strains were isolated from various bird species: mallards (order Anseriformes) and robins (order Passeriformes); similar isolates were also identified in northern Italy from pigeons. NDV cases were reported in Austria in 2004 and in France in 2006 in Columbidae birds [19]. Virological screening of samples from wild waterfowl in Finland in 2006–2010 detected 39 NDV strains. Positive NDV samples were most frequently found

in Eurasian teal and mallards. The highest NDV prevalence among wild birds was recorded in 2010 at 11.8 %. Phylogenetic analysis of these samples revealed their close relatedness to strains from wild and domestic birds in China [20]. In 2010, a study of NDV prevalence among wild mallard populations captured in Sweden during the autumn migration found that 1 % of birds were infected with NDV. Phylogenetic analysis showed close relatedness to strains from central and northern Europe, as well as to a small group of strains isolated from birds in southeastern China [20]. NDV strains detected in 2006–2008 in wild waterfowl populations in Luxembourg were genetically close to viruses found in birds in Finland. These data suggest the potential spread of NDV by migratory birds [21]. In Serbia, NDV was detected in wild birds (mallard, Eurasian sparrowhawk, collared dove, feral pigeon) in 2007, phylogenetically close to strains from China. However, it was noted that the strain isolated in Serbia was genetically similar to samples most commonly isolated in Europe at that time from domestic poultry, suggesting that domestic birds could have been the source of infection for wild birds [19]. From 2010 to 2016, 35 fatal cases among wild birds caused by NDV were detected in Catalonia (northern Spain), with the number of infections increasing over this period. Most cases were recorded from September to February, which the authors attribute to favorable conditions for virus persistence in the environment (in water) during this period, as NDV is transmitted via the fecal-oral route [22]. During viral infection surveillance in Romania from 2010 to 2016, antibodies to NDV were detected in 5.4 % of the wild bird population, *the first* such detection in this territory [23]. These data indicate active circulation of NDV strains among wild waterfowl and Columbidae, as well as further spread of NDV across continental Europe [24]. In Russia, in 2001, NDV isolates were obtained from wild birds in the Astrakhan region (Volga River basin): little tern, great cormorant, coot, whiskered tern, and common redshank. In 2001, an NDV strain was isolated during virological monitoring of wild bird populations in Primorye. In 2004, an NDV strain was isolated from teal, which exhibited greater pathogenic properties compared to the 2001 isolate [25]. NDV isolates were also obtained from wild ducks in Adygea in 2008 and from a pigeon in Altai in 2010 [26]. In 2011, NDV was isolated from a wild mallard in Yakutia, phylogenetically close to strains from China, Japan, and South Korea [27]. From 1998 to 2005, periodic NDV outbreaks were observed in domestic poultry in Kazakhstan and Kyrgyzstan [28]. During virological surveillance in 2011 and 2014, APMV-1 viruses were detected in wild birds from 17 families across nine regions of Kazakhstan [28]. Nine NDV isolates were obtained from virological analysis of wild birds in South Korea during 2007–2010, and their similarity to isolates from live bird markets in Korea and China was noted, confirming the role of wild migratory birds in NDV spread [29]. Regular NDV outbreaks have been reported in China

since 1996. Genetic analysis of NDV isolates has shown that NDV can actively mutate, generating unique variants with novel properties and pathogenic potential capable of causing a panzootic in the Asian region in the future [30]. In India in 2012, cases of NDV infection in wild peafowl were reported, with strains genetically related to those from Western Europe, Israel, Indonesia, Taiwan, and India [31]. A large number of NDV outbreaks were recorded in Pakistan during 2010–2012, including among captive wild birds [17]. The presented data show that NDV is persistently present and continuously circulates in the European and Asian parts of Eurasia, periodically causing epizootics among wild and domestic birds. A case of mixed infection of a wild mallard with NDV and Avian Avulavirus-4 was noted in Israel in 1997. The fact of mixed infection of a single individual with different paramyxovirus species is of great importance for epizootiology, as it may alter the clinical presentation of diseases, enhancing or attenuating their severity [32].

According to reports on avian paramyxovirus detection in the European Union in 2016, 12 countries reported no paramyxovirus isolates on their territory (Croatia, Estonia, Finland, Hungary, Northern Ireland, Latvia, Lithuania, Luxembourg, Norway, Poland, Greece), while 19 countries reported detection of avian paramyxoviruses. Among these, 11 EU countries recorded avulavirus cases in wild birds: Austria – Avian Avulavirus-1 in feral pigeons; Belgium – Avian Avulavirus-1 in pigeons, Avian Avulavirus-3 in captive Psittaciformes, Avian Avulavirus-6 in mallards. In Cyprus, positive samples of Avian Avulavirus-1 were found in urban pigeons; in the Czech Republic – Avian Avulavirus-1 in collared doves; in Denmark – Avian Avulavirus-1, -4 in wild mallards and Avian Avulavirus-9 in farmed mallards. In France – Avian Avulavirus-1 in doves and pigeons; in Italy – in doves and sparrowhawks; in the Netherlands – in wild and urban ducks; in Portugal – in common and collared doves; in Slovakia – in pigeons and common doves; in Spain – in doves, Eurasian tawny owls, and pigeons. The final report on avian paramyxovirus detection noted that in 2016, three NDV epizootics were recorded in EU countries compared to two in 2015, indicating a persistent and increasing threat of new epizootics. The report also noted a large number of infections in pigeons and Columbidae, which may contribute to virus spread in commercial and domestic poultry populations. It also emphasized the particular importance of monitoring not only Avian Avulavirus-1 but also other avian avulavirus species [33]. Surveillance of other Avian Avulavirus species has shown their constant presence in wild bird populations of continental Eurasia. Thus, serological surveys of 18 wild bird species in Andalusia (southern Spain) in 1990–1992 detected antibodies to all Avian Avulavirus-1–9 species except Avian Avulavirus-5. Among waterfowl, Avian Avulavirus-8 (43 %) and Avian Avulavirus-6 (21 %) predominated, mainly in Anseriformes, while among non-aquatic

bird species, Avian Avulavirus-2 (60 %) in sparrows constituted the largest proportion. It was also noted that the highest antibody levels to avulaviruses were recorded from May to September, during the period of maximum juvenile density in bird flocks [3].

### **Ecological Aspects of Avian Avulavirus-3 Circulation in Wild Bird Populations**

Isolation of an Avian Avulavirus-3 strain was reported only once – from Psittaciformes in Belgium in 2016. Avian Avulavirus-3 infection caused encephalitis in birds of this family. Avian Avulavirus-3 can also infect chickens and turkeys, causing respiratory tract lesions of varying severity, pancreatitis, and CNS involvement in passerine birds [5, 33].

### **Ecological Aspects of Avian Avulavirus-4 Circulation in Wild Bird Populations**

Avian Avulavirus-4 has become widespread across continental Eurasia. It is typically isolated from wild waterfowl. It was first isolated from a duck in Hong Kong in 1975, and subsequently from mallards, grey ducks, teals, and geese during surveillance programs in the USA and China. In Eurasia, Avian Avulavirus-4 was isolated in Italy from mallards and ducks, and in Belgium from mallards in 2006 and 2007, respectively [34]. In 2016, it was isolated from wild mallards in Denmark [33]. In Ukraine, Avian Avulavirus-4 isolation cases were reported in 2010 from mallards, teals, and starlings; in 2011–2013 from mallards, and in 2014 from teals [35]. In Russia, Avian Avulavirus-4 was isolated from teals in 2017 and 2018, and from a mallard in 2019 [25]. In Kazakhstan – from wild Anseriformes on Lake Alakol and from a curlew on the Caspian Sea coast in 2003 and 2009 [36]. In Mongolia – from mallards in 2012 [37]. In China, Avian Avulavirus-4 isolates were obtained during 2012–2016 from wild waterfowl (mallard, swan, greater white-fronted goose, and grey duck) [38]. In South Korea, Avian Avulavirus-4 samples were isolated in 2006, 2007, 2009–2014, 2016, and 2017 from wild waterfowl (mallard, grey duck) [39]. The pathogenic potential of Avian Avulavirus-4 is poorly understood. Experimental infection of chickens caused moderate pneumonia and catarrhal tracheitis.

### **Ecological Aspects of Avian Avulavirus-6 Circulation in Wild Bird Populations**

Avian Avulavirus-6 is also most frequently isolated from wild waterfowl. This serotype was first isolated in 1977 in Hong Kong from a duck. In 2007, an isolate from a teal in Italy was characterized as Avian Avulavirus-6 [5]. It was also isolated in 2007 and 2016 in Belgium [33]. In 2003, it was isolated in Russia in Primorye from grey herons [3], and in 2009 from a teal [40]. In Kazakhstan – from a red-crested pochard in 2013

[41]. In China, Avian Avulavirus-6 samples were isolated from wild mallards in 2001, 2011, and 2015 [41]. In South Korea – from wild ducks in 2010, 2011, 2013, and 2014 [42]. The infectious properties of Avian Avulavirus-6 are poorly understood. It is known that Avian Avulavirus-6 is avirulent for chickens, but causes moderate respiratory disease and reduced egg production in turkeys.

#### **Ecological Aspects of Avian Avulavirus-8 Circulation in Wild Bird Populations**

Avian Avulavirus-8 was first isolated from a Canada goose in 1976 among waterfowl of the Atlantic flyway, and then from a wild pintail duck in 1978 in Japan. In Eurasia, Avian Avulavirus-8 isolates were obtained in Mongolia from wild waterfowl in 2012 [37] and in Kazakhstan from wild waterfowl (waders, whooper swans, greater white-fronted geese) in 2013 [36]. Avian Avulavirus-8 isolates are most frequently found in waterfowl, but a case of antibodies to Avian Avulavirus-8 in chickens has been noted, as well as its ability to replicate in mammals (hamsters, mice, rhesus macaques).

#### **Ecological Aspects of Avian Avulavirus-9 Circulation in Wild Bird Populations**

Avian Avulavirus-9 isolation cases were reported in Italy in 2004, 2007, and 2008 in wild waterfowl of the Anatidae family (pintail, mallard, wigeon) [5]. In Denmark in 2016, an Avian Avulavirus-9 infection case was noted in farmed mallards. Avian Avulavirus-9 can infect other animal species (mice, hamsters, macaques) with clinical signs of disease.

#### **Ecological Aspects of Avian Avulavirus-11 Circulation in Wild Bird Populations**

In 2010, an Avian Avulavirus-11 isolate was obtained from a snipe in France. The habitat of these birds includes muddy shores and wetlands. Phylogenetically, its closest relative is Avian Avulavirus-2. The Avian Avulavirus-11 sample was obtained from a bird without clinical signs. Its pathogenic potential and virulence properties have not yet been studied [5].

#### **Ecological Aspects of Avian Avulavirus-12 Circulation in Wild Bird Populations**

In Italy in 2005, as part of an influenza virus surveillance program, an Avian Avulavirus isolate was obtained from cloacal swabs of wigeons (order Anseriformes) and was characterized as a new species, Avian Avulavirus-12. Serological testing with Avian Avulavirus-1-9 antisera showed genetic relatedness of the Avian Avulavirus-12 isolate to Avian Avulavirus-1, and to a lesser extent to Avian Avulavirus-9. Virulence tests on day-old chicks demonstrated low

virulence of Avian Avulavirus-12 for chickens (ICPI 0.45) [5].

#### **Ecological Aspects of Avian Avulavirus-13 Circulation in Wild Bird Populations**

Avian Avulavirus-13 was isolated during annual viral surveillance of wild bird populations in Kazakhstan in 2013 from a greater white-fronted goose. Phylogenetically, Avian Avulavirus-13 is closest to Avian Avulavirus-12, as well as to Avian Avulavirus-1 and Avian Avulavirus-9 [43].

#### **Ecological Aspects of Avian Avulavirus-16 Circulation in Wild Bird Populations**

In 2014, as part of an avian influenza surveillance program in southeastern South Korea, a novel paramyxovirus sample was isolated from the feces of wild birds inhabiting wetlands. Morphologically, viral particles exhibited pleomorphic shapes typical of paramyxoviruses, with characteristic surface glycoprotein arrangements. Serological tests identified this paramyxovirus sample as a novel species, Avian Avulavirus-16. Phylogenetically, it is most closely related to Avian Avulavirus-1, followed by Avian Avulavirus-9, -12, and to a lesser extent -13. Serological testing of samples from various wild bird species showed that Avian Avulavirus-16 was found only in Anseriformes (Eurasian teal, Eurasian wigeon, mallard, and spot-billed duck) [10].

#### **Ecological Aspects of Avian Avulavirus-20 Circulation in Wild Bird Populations**

In Kazakhstan in 2013 and 2014, a novel avian paramyxovirus was isolated from nasopharyngeal and cloacal swabs of wild black-headed gulls (order Charadriiformes) and characterized as Avian Avulavirus-20. Morphological examination showed that virions had the structure typical of paramyxoviruses. Virological studies demonstrated the absence of pathogenic properties of Avian Avulavirus-20 in infected chickens. It is important to note that Avian Avulavirus-20 was isolated only from Laridae, whereas most avulavirus species are characteristic of Anatidae. It can be hypothesized that Charadriiformes may have greater susceptibility to Avian Avulavirus-20 [13].

#### **Ecological Aspects of Avian Avulavirus-21 Circulation in Wild Bird Populations**

In 2015, a novel avian paramyxovirus, Avian Avulavirus-21, was isolated from the feces of wild migratory birds in South Korea (Cheonsu Bay, western region of South Korea). Among the wild birds found in this area are numerous waterfowl such as the greater white-fronted goose, bean goose, black crane, ruddy shelduck, white-tailed sandpiper, common sandpiper,

wood sandpiper, yellow-billed egret, Eurasian spoon-bill, as well as the Japanese buzzard, Eurasian hobby, grey-headed bunting, and Japanese shrike [14].

### Vaccination Challenges

Currently, low-pathogenicity strains such as LaSota are used for NDV vaccination of domestic and commercial poultry. However, their ability to provide sufficiently effective immune responses against new wild strains phylogenetically distant from vaccine strains remains questionable [19, 44]. Another current issue in poultry vaccination is the interspecies transmission of vaccine virus strains from domestic poultry to wild birds. Various outbreaks caused by vaccine strains in wild birds have been identified in Mexico, Japan, and Finland [44, 45]. In light of this situation, the strategy and criteria for vaccine strain selection need to be reconsidered. Continuous monitoring of wild and synanthropic bird species for viral infections is also essential.

### Practical Applications of Avulaviruses

An oncolytic effect has been discovered in some paramyxoviruses (NDV), allowing their use in the development of antitumor therapies [46].

The replication characteristics and conserved genomic properties make them suitable and safe for the development of vector vaccines. Currently, there is an urgent need for effective vaccines against highly virulent NDV strains. Naturally occurring low-virulence NDV strains are currently used as live attenuated vaccines against NDV. However, as a result of routine vaccination, domestic poultry in some cases becomes less sensitive to currently used vaccines. Avulavirus species that do not cause clinical signs of disease could serve as material for the development of recombinant NDV vaccines [47].

## CONCLUSION

From the data presented, it is evident that the primary carriers of avulaviruses among wild birds are waterfowl. The most widespread and best studied is Avian Avulavirus-1, known as Newcastle disease virus. Its reservoir and vectors in the wild are most frequently birds of the orders Anseriformes and Columbiformes. Cases of Avian Avulavirus-1 detection have also been recorded in birds of the orders Passeriformes, Galliformes, Gaviiformes, Strigiformes, and Accipitriformes, indicating a wide diversity of potential hosts and vectors for this virus. The particular attention to this avulavirus variant is explained by the fact that it causes massive epizootics among domestic poultry, primarily chickens.

Avian Avulavirus-3 has been isolated only once (Belgium) from captive Psittaciformes in the European part of continental Eurasia.

Avian Avulavirus-4 has most frequently been recorded in the Asian part of Eurasia (China, South Korea). Avian Avulavirus-4 has been isolated from waterfowl of the order Anseriformes, but also from Passeriformes and Charadriiformes.

Avian Avulavirus-6 has been recorded across the entire territory of Eurasia in birds of the orders Anseriformes and Ciconiiformes.

Avian Avulavirus-8 has been isolated in central Eurasia (Kazakhstan and Mongolia) from birds of the orders Anseriformes and Passeriformes.

Avian Avulavirus-9 has been recorded only in the European part of Eurasia, in Anseriformes.

Avian Avulavirus-11 has been isolated only once – in France from a snipe (order Charadriiformes).

Avian Avulavirus-12 was isolated from Anatidae in the European part of Eurasia (Italy). Avian Avulavirus-13 (from Anseriformes) and Avian Avulavirus-20 (from Charadriiformes) were isolated in central Eurasia (Kazakhstan). Avian Avulavirus-16 was isolated from Anatidae in South Korea (Asian part of continental Eurasia). Avian Avulavirus-21 was isolated from wild migratory birds in South Korea.

The analysis of available literature data indicates that avulaviruses are widely distributed in wild bird populations across the entire territory of Eurasia. The epidemic and epizootic potential of these viruses, with the exception of Avian Avulavirus-1 (Newcastle disease virus), remains poorly understood, and these viruses may pose a potential threat to humans and, particularly, to poultry production, underscoring the need for their surveillance in the wild.

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# MORPHOLOGY, PHYSIOLOGY AND PATHOPHYSIOLOGY

## COMPARATIVE ANALYSIS OF THE RESULTS OF PREDICTING DIAMETERS OF DAUGHTER SEGMENTS OF CORONARY ARTERY BIFURCATIONS UNDER NORMAL CONDITIONS, OBTAINED USING GRAPH NEURAL NETWORKS AND KNOWN NUMERICAL MODELING METHODS

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### ABSTRACT

**Background.** The accurate determination of human coronary artery (HCA) diameters plays a pivotal role in the diagnosis, risk assessment, and treatment planning of ischemic heart disease. Given that vascular networks possess a fractal structure and consist of arterial bifurcations (AB), a critical task is the prediction of the diameters of daughter (distal) arterial segments (AS) based on the known diameter of the mother (proximal) AS. Existing numerical modeling methods (ENMM) for predicting arterial diameters rely on approximate analytical dependencies derived empirically.

**Aim.** To conduct a comparative analysis of the results of predicting diameters of daughter segments (bifurcation components) obtained using Graph Neural Networks and known numerical modeling methods based on morphometric data of real healthy human coronary arteries.

**Methods.** To achieve this objective, various GNN architectures were implemented and tested: GCN, GraphSAGE, GAT, and TransformerConv. Additionally, ENMM and a Multilayer Perceptron (MLP) were evaluated. A comparative statistical analysis of the results was conducted. The study utilized previously obtained morphometric data on the internal diameters of AS comprising 30 corrosion cast graphs of healthy HCAs.

**Results.** This study proposes the use of GNNs for this specific task for the first time. Comparative analysis demonstrated that all GNN models surpassed both ENMM and the Multilayer Perceptron in accuracy when predicting the diameters of smaller-caliber daughter AS. The TransformerConv architecture demonstrated the best performance, achieving a coefficient of determination  $R^2 = 0.96$  and RMSE = 0.199 for predicting the larger daughter segment diameter, and  $R^2 = 0.76$  and RMSE = 0.221 for the smaller segment.

**Conclusion.** The developed models may serve as the foundation for creating clinical decision support systems, enabling more precise assessments of the structural status of human coronary arteries.

**Keywords:** human heart, coronary arteries, graph neural networks, diameter prediction, arterial bifurcation, medical diagnostics

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# СРАВНИТЕЛЬНЫЙ АНАЛИЗ РЕЗУЛЬТАТОВ ПРОГНОЗИРОВАНИЯ ДИАМЕТРОВ ДОЧЕРНИХ СЕГМЕНТОВ БИФУРКАЦИЙ ВЕНЕЧНЫХ АРТЕРИЙ В НОРМЕ, ПОЛУЧЕННЫХ С ИСПОЛЬЗОВАНИЕМ ГРАФОВЫХ НЕЙРОННЫХ СЕТЕЙ И ИЗВЕСТНЫХ МЕТОДОВ ЧИСЛЕННОГО МОДЕЛИРОВАНИЯ

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## РЕЗЮМЕ

**Обоснование.** Точное определение диаметров венечных артерий сердца человека (ВАСЧ) играет ключевую роль в диагностике, оценке рисков и планировании лечения ишемической болезни сердца. Поскольку сосудистые сети имеют фрактальную структуру и состоят из артериальных бифуркаций (АБ), важной задачей является прогнозирование диаметров дочерних (дистальных) артериальных сегментов (АС) на основе известной величины диаметра материнского (проксимального) АС. Известные методы численного моделирования – прогнозирования диаметров артерий используют приближенные аналитические зависимости, полученные эмпирически.

**Цель.** Провести сравнительный анализ результатов прогнозирования диаметров дочерних сегментов (составных частей бифуркации), полученных с использованием графовых нейронных сетей и известных методов численного моделирования (ИМЧМ), основанных на данных морфометрии реальных венечных артерий сердца человека в норме.

**Методы.** Для достижения поставленной цели были реализованы и протестированы различные архитектуры GNN: GCN, GraphSAGE, GAT и TransformerConv. А также ИМЧМ и многослойный перцептрон. Проведен сравнительный статистический анализ результатов. Использовали ранее полученные данные морфометрии величин внутренних диаметров АС, составляющих 30 графов коррозионных препаратов ВАСЧ в норме.

**Результаты.** Впервые предложено использовать GNN для решения данной задачи. Сравнительный анализ результатов показал, что все GNN-модели превосходили по точности ИМЧМ и многослойный перцептрон, в прогнозировании величин диаметров дочерних АС меньшего калибра. Наилучшие результаты продемонстрировала TransformerConv, достигнув коэффициента детерминации  $R^2 = 0,96$  и  $RMSE = 0,199$  для прогноза большего дочернего диаметра и  $R^2 = 0,76$  и  $RMSE = 0,221$  — для меньшего.

**Заключение.** Разработанные модели могут стать основой для создания клинических систем поддержки принятия решений, позволяющих более точно оценивать состояние структуры ВАСЧ.

**Ключевые слова:** сердце человека, венечные артерии, графовые нейронные сети, прогнозирование диаметров, артериальная бифуркация, медицинская диагностика

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## BACKGROUND

The importance of predicting diameters of human coronary arteries (HCA) in modern cardiology is immense. The accurate determination of these parameters is crucial for the diagnosis, prevention, and treatment of ischemic heart disease. This includes the detection of stenosis, assessment of hemodynamic risk, and planning for revascularization procedures.

From a morphological perspective, intraorgan arterial networks can be considered as fractal systems [1, 2]. Their primary structural component is the arterial bifurcation (AB), which consists of a parent (proximal) arterial segment (AS) with diameter  $D$  and two daughter (distal) ASs with diameters  $d_{max}$  and  $d_{min}$ . These daughter segments differ in their internal diameter. Therefore, it is of practical interest to predict the diameter of these daughter segments based on the diameter of the parent segment. To address this challenge, existing numerical modelling methods have been proposed [3, 4]. However, modern machine learning techniques, such as neural networks, have not yet been applied in this field.

The vascular network structure can be represented as a graph, specifically a binary tree. A specialized class of algorithms, Graph Neural Networks (GNNs), has been developed to process data with this structure [5]. Typical GNN applications include classification and link prediction, but this study proposes using GNNs for regression, specifically predicting the diameter of daughter ASs based on the parent AS diameter.

## AIM

To conduct a comparative analysis of the results of predicting diameters of daughter segments (bifurcation components) obtained using graph neural networks and known numerical modeling methods based on morphometric data from real healthy human coronary arteries.

## METHODS

**Study Design.** The study was based on the results of a morphometric analysis of 30 human coronary arteries, obtained through corrosion casting [6]. The initial data were provided in Excel format. From the entire set of features, the following variables were selected for model training and validation: parent AS diameter ( $D$ , mm); sex (1 – male, 2 – female); age group (1 – second period of mature age (males 36–60 years, females 36–55 years); 2 – elderly age (males 61–75 years, females 56–75 years) (Classification of the Academy of Medical Sciences of the USSR, 1965); and branching level – the newly formed AB series, ranging from 1 to 20. The target variables to be predicted were the diameters of the two daughter ASs:  $d_{max}$  (mm) – diameter of the larger AS, and  $d_{min}$  (mm) – diameter of the smaller AS. The sample

size included 30 specimens, each containing more than 100 measured ASs. The data were structured as 30 graphs. Branching level and all diameter values were considered as numerical variables, whereas sex and age were considered categorical. A preliminary statistical analysis using the Pandas and NumPy libraries revealed that the distribution of AS diameters deviated from normal and contained outliers.

**Data Preprocessing and Graph Construction.** The PyTorch Geometric (PyG) framework was used to implement GNNs [7]. Tabular data from Excel were preprocessed using the Pandas library and converted to PyTorch tensors. Anatomical connections (for constructing the graph data structure) between ASs were determined using the NetworkX library [8]. To avoid data leakage, the split into training and test sets was performed at the level of entire HCA trees (graphs). The training set consisted of 22 HCA trees (graphs) (73 %), and the test set contained 8 (27 %). This approach ensures that the model does not use information about the structure of trees (graphs) from the test set during training. Training data were normalized using RobustScaler from the scikit-learn library [9], which is robust to outliers. Normalization parameters were saved for subsequent application to test data and during inference.

**GNN Architectures.** Four key GNN architectures were implemented: GCN (Graph Convolutional Network), GraphSAGE (Sample and Aggregate), GAT (Graph Attention Network), and TransformerConv (Graph Transformer). To compare the effectiveness of the GNN approach, a Multilayer Perceptron (MLP) trained on the same input features but without considering the graph structure, as well as the results of ENMM, were also used.

**Hardware and Training Process.** All experiments were conducted using a PC with Windows 11 and WSL2 subsystem and Ubuntu 24.04 distribution. Hardware configuration: Intel Core i7-14700KF processor, 64 GB RAM, Nvidia GPU with 32 GB video memory. Library versions used: PyTorch 2.7.0+cu128 and PyTorch Geometric 2.6.1. The AdamW optimizer [10] with L2 regularization (weight decay) was used to train the models to prevent overfitting; the Huber loss function [11] was employed, which combines the advantages of MSE (for small errors) and MAE (for large errors), making it robust to outliers. Dropout technique and early stopping (with no improvement in loss for 50 epochs) were used for regularization. Automated hyperparameter search was performed using the Optuna library [12] over 200 trials. All experiments were logged in the MLflow environment.

**ENMM – Baseline Models for Comparison.** To verify the results of neural network predictions, ENMM were used [3, 4]. The model based on [3] utilizes the relationship between the parent AS diameter ( $D$ ), the larger and smaller daughter AS diameters ( $d_{max}$  and  $d_{min}$ ), and the asymmetry ratio (*ratio*):

$$D^{\frac{7}{3}} = d_{max}^{\frac{7}{3}} + d_{min}^{\frac{7}{3}} \quad (1)$$

where  $D$  is the parent AS diameter,  $d_{max}$  is the diameter of the larger daughter AS, and  $d_{min}$  is the diameter of the smaller daughter AS. The authors also express branching asymmetry as the ratio of daughter diameters:

$$ratio = \frac{d_{min}}{d_{max}}. \quad (2)$$

From (1) and (2), the diameters of the daughter ASs can be calculated:

$$d_{max} = D \cdot \left( \frac{1}{(1+ratio^{\frac{7}{3}})} \right)^{\frac{3}{7}}, \quad (3)$$

$$d_{min} = ratio \cdot d_{max}. \quad (4)$$

where  $ratio=0.625$

The model based on [4] relates the diameters of the daughter ASs ( $d_{max}$  and  $d_{min}$ ) to the parent AS diameter ( $D$ ) and the asymmetry parameters ( $\alpha, \beta$ ):

$$d_{max} = \alpha D, \quad d_{min} = \beta D. \quad (5)$$

where  $\alpha \approx 0.9, \beta \approx 0.6$ .

*Comparative analysis of the modeling results.* Two metrics were used to assess prediction accuracy:  $R^2$  (coefficient of determination) and RMSE (root mean square error). Prediction quality was evaluated on the test set using the coefficient of determination ( $R^2$ ). This metric determines what proportion of the variance in the dependent variable is explained by the regression model. The coefficient of determination ( $R^2$ ) was calculated using the formula presented below:

$$R^2 = 1 - \frac{\sum_{i=1}^n (y_i - \hat{y}_i)^2}{\sum_{i=1}^n (y_i - \bar{y})^2} \quad (6),$$

where  $y_i$  is the actual value,  $\hat{y}_i$  is the model prediction,  $\bar{y} = \frac{1}{n} \sum_{i=1}^n y_i$  is the mean value, and  $n$  is the number of samples in the test set. The closer  $R^2$  is to 1, the better the model performance. A negative value indicates that the model performs worse than simply predicting the mean value. The Root Mean Squared Error (RMSE) was also used as a metric – a standard error measure in regression tasks. It preserves the same units as the original data (mm), making it convenient for interpretation:

$$RMSE = \sqrt{\frac{1}{n} \sum_{i=1}^n (y_i - \hat{y}_i)^2} \quad (7).$$

The closer the RMSE is to 0, the better. Five state-of-the-art GNN architectures were compared, along with two classical analytical models from the scientific literature.

## RESULTS

Preliminary analysis of the results (Table 1) shows that predicting  $d_{min}$  is significantly more challenging than predicting  $d_{max}$ . The calculated  $R^2$  and RMSE values for various MLP and GNN architectures, as well as for the numerical modeling methods described in the literature [3, 4], indicate that for all models except TransformerConv, the  $R^2$  value for the smaller daughter AS is substantially lower, while the RMSE is higher. This is logical, as the diameter of the smaller daughter AS may be more strongly influenced by hemodynamic and topological factors.

The results of predicting two key parameters of HCA ASs are presented:  $d_{max}$  is the diameter of the larger daughter AS of the AB, and  $d_{min}$  is the diameter of the smaller daughter AS of the AB. The Multilayer Perceptron (MLP) demonstrates satisfactory results, particularly for predicting  $d_{max}$ . This performance is notably better than that of GCN and GraphSAGE, suggesting that even without explicitly accounting for the graph structure, the network is able to capture the underlying patterns.

However, its inferiority to the best GNN models confirms the importance of considering structural connections. The results obtained using GCN and GraphSAGE showed moderate, rather modest results for  $d_{max}$ . Their simple mechanisms for aggregating information from neighboring nodes seem insufficient for accurately modeling the HCA structure. It should be noted that GraphSAGE performs slightly better at predicting  $d_{min}$  than MLP and GCN architectures.

GAT (Graph Attention Network) demonstrates selectivity. For  $d_{max}$  it ranks second in accuracy after TransformerConv ( $R^2 = 0.71$ ), while for  $d_{min}$  it shows the highest RMSE among the networks studied. It is possible that the attention mechanism of GAT helps to identify key “parent” features for the larger daughter AS ( $d_{max}$ ), but proves less effective for the smaller AS ( $d_{min}$ ).

TransformerConv demonstrates exceptional accuracy for both parameters:  $R^2$  values close to ideal (0.95 and 0.76), and RMSE values minimal (0.199 mm and 0.221 mm). This indicates that the attention mechanism underlying this GNN architecture is ideally suited for capturing complex, non-local dependencies in HCA structure. All GNN architectures showed positive and significant  $R^2$  values. The numerical modeling methods [3, 4], based on physical principles, showed unsatisfactory or even extremely poor results, particularly for ( $R^2 = -0.79$  and  $-0.51$ ). This indicates that real HCA bifurcations are more complex than

TABLE 1

**R<sup>2</sup> AND RMSE VALUES FOR VARIOUS MLP AND GNN ARCHITECTURES, AS WELL AS ENMM, DESCRIBED IN THE LITERATURE**

| Model                             | Prediction results |           |                |           |
|-----------------------------------|--------------------|-----------|----------------|-----------|
|                                   | $d_{max}$          |           | $d_{min}$      |           |
|                                   | R <sup>2</sup>     | RMSE (mm) | R <sup>2</sup> | RMSE (mm) |
| MLP                               | 0.59               | 0.566     | 0.37           | 0.359     |
| GCN                               | 0.51               | 0.625     | 0.37           | 0.359     |
| GraphSAGE                         | 0.53               | 0.612     | 0.43           | 0.341     |
| GAT                               | 0.71               | 0.479     | 0.28           | 0.385     |
| TransformerConv                   | 0.95               | 0.199     | 0.76           | 0.221     |
| Model by Olufsen M.S. et al. [12] | 0.42               | 0.675     | -0.79          | 0.609     |
| Model by Huo Y. & Kassab G. [3]   | 0.46               | 0.653     | -0.51          | 0.559     |

**Note.**  $d_{max}$  – diameter of the larger daughter arterial segment (AS);  $d_{min}$  – diameter of the smaller daughter AS; R<sup>2</sup> – coefficient of determination; RMSE – root mean square error; MLP – Multilayer Perceptron; GCN (Graph Convolutional Network) – graph convolutional network; GraphSAGE (Sample and Aggregate) – graph sampling and aggregation network; GAT (Graph Attention Network) – graph attention network; TransformerConv (Graph Transformer) – graph transformer.

described by the analytical laws traditionally used for this purpose.

## DISCUSSION

Unlike traditional neural networks designed for processing isolated objects such as tables or images, GNNs operate on structured systems composed of interconnected graph nodes. These networks extract information not only from the individual components (nodes), but also from the topology (connections) between them (edges). The operating principle of GNNs is based on the message-passing mechanism. Each node collects features from its nearest neighbors. The collected messages are then aggregated using a permutation-invariant function (e.g., sum, mean, max). The aggregated information is then combined with the node's current features to update its state. This multi-layer architecture allows nodes to incorporate information from nodes that are increasingly distant from it.

In this study, key GNN architectures were implemented. GCN (Graph Convolutional Network) [5] aggregates the features of neighboring nodes, taking into account the degree (number of connections). This architecture is highly efficient, but it does not differentiate between the importance of each neighbor. GraphSAGE (Sample and Aggregate) [13] uses fixed-size sampling method instead of considering all neighbors, making it scalable for large graphs. GAT (Graph Attention Network) [14] assigns a weighting coefficient to each neighboring node, determining its relevance to the central node using an attention mechanism. TransformerConv (Graph Transformer) [15] employs an advanced attention mechanism inspired by the transformer architecture [16], allowing it to capture more complex global dependencies in the graph. Its superiority is explained by global attention. Unlike GAT, which

only looks at nearest neighbors, the transformer can evaluate the importance of any node in the graph. This allows the model to take into account the complex influence of remote regions of the vascular network on the local AB geometry, resulting in high accuracy.

A comparison of model performance has established a clear correlation between modeling outcomes and AS diameter. Numerical modeling techniques [3, 4] exhibit limited accuracy in forecasting the parameter for ASs with greater diameters. This suggests that the models establish an explicit link between the daughter AS diameter and the parent AS diameter. At the same time, all neural network architectures show clear superiority over numerical modeling methods [3, 4].

For smaller-diameter daughter ASs, this trend continues: all investigated neural networks outperform numerical modeling methods. This advantage is explained by the ability of neural networks to identify complex, non-obvious connections between the values of studied parameters, while numerical modeling methods [3, 4], based on simplified mathematical formulations, does not account for the individual phenotypic characteristics of HCAs.

For smaller-diameter daughter ASs, the situation is similar: all investigated neural networks outperform numerical modeling methods. This advantage is explained by the ability of neural networks to identify complex and non-obvious relationships between the values of the studied parameters, whereas numerical modeling methods [3, 4], based on simplified mathematical representations, do not take into account the individual phenotypic characteristics of HCAs.

Interestingly, despite the natural structure of blood flow, only one graph network, the transformer (TransformerConv), was able to extract sufficient information from this structure to exceed the 70 % R<sup>2</sup> threshold for predicting the diameter of the smaller daughter AS.

This achievement is due to the attention mechanism, which takes into account both local and global connections within the HCA network. Analysis of the prediction results of the numerical modeling methods [3, 4] shows that they perform poorly in predicting  $d_{min}$ , with a negative  $R^2$  indicating that their predictions are not only inaccurate, but also systematically biased. This raises questions about the universality of underlying physical principles, such as minimum energy or shear stress laws. Their relatively better performance (though still poor) in predicting  $d_{max}$  may indicate that the larger daughter AS diameter may follow some generally accepted empirical rules.

The significant superiority of neural network models, especially graph neural networks, compared to numerical modeling methods in predicting the diameters of smaller daughter ASs confirms their potential for addressing theoretical and practical (clinical) biomedical challenges. The high  $R^2$  values for all models studied in the context of the larger daughter AS indicates a strong correlation between the diameters of this daughter branch and the parent AS, which is also confirmed by the high accuracy of hydrodynamic models.

## CONCLUSION

Neural network models have shown promising results in predicting the diameters of daughter arterial segments within coronary artery bifurcations. This indicates the potential of the proposed approach. GNNs, especially TransformerConv, are a powerful tool for predicting bifurcation geometry and significantly outperform conventional numerical modeling methods. However, these models should not be applied to predict the diameter of the smaller daughter AS ( $d_{min}$ ) in the studied dataset as their use may lead to significant errors. The prediction of  $d_{min}$  is a more challenging task that requires the use of advanced architectures such as TransformerConv or the development of novel specialized approaches.

The primary limitation of this research is the relatively small size of the training dataset, which has a negative impact on prediction accuracy. In order to enhance model performance, it would be beneficial to enlarge and refine the training data set, minimizing the occurrence of outliers.

For practical applications, such as medical modeling or surgical planning, graph transformers (TransformerConv) are recommended, as they demonstrate maximum accuracy and reliability.

Researchers in the field of hemodynamics are advised to reconsider or significantly supplement the existing branching laws based on data collected through machine learning methods. Further research in this area should focus on expanding model functionality by simultaneously predicting not only the diameters but also the lengths of daughter ASs and bifurcation angles. This would significantly improve the accuracy of HCA models and greatly enhance their potential for widespread use in theoretical and practical medical applications.

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## Conflicts of interest

The authors declare no conflicts of interest.

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## CHARACTERISTICS OF MICROCIRCULATORY PARAMETERS OF BOYS AND GIRLS ACCORDING TO LASER DOPPLER FLOWMETRY (LDF)

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### ABSTRACT

*The method of laser doppler flowmetry is one of the relevant and informative ways to study the state of the microcirculation.*

**The aim** of the work was to conduct a comparative analysis of the microcirculatory system of boys and girls according to laser Doppler flowmetry (LDF).

**Materials and methods.** The study involved 128 second-year students of the Medical Institute of the North-Eastern Federal University named after M.K. Ammosov. All individuals were of Yakut ethnicity. The boys' group consisted of 44 people, the girls' group consisted of 84 people. The state of microcirculation and the functioning of its regulatory mechanisms were assessed by laser Doppler flowmetry (LDF) using two identical single-channel portable LASMA PF analyzers (SPE "LAZMA", Moscow). The measurement was carried out on the lower third of the right and left forearms for 5 minutes.

**Results.** Significant intergroup differences in microcirculatory parameters between the examined boys and girls were established. The results showed significant sex differences in the state of the microcirculatory system: the boys recorded higher values of shunt blood flow ( $1.71 \pm 0.13$ ) and a large amplitude of fluctuations due to vascular endothelial activity ( $0.04 \pm 0.01$ ). The girls had significantly lower shunt blood flow ( $1.34 \pm 0.07$ ) and endothelial activity ( $0.01 \pm 0.01$ ). At the same time, the levels of nutritional blood flow, as well as the activity of neurogenic and myogenic regulatory mechanisms, were similar in both sexes. A group of people with a low level of respiratory fluctuations in blood flow prevailed among the subjects (68.2 % of boys and 73.8 % of girls). And the indicators of cardiac fluctuations in blood flow were at a normal or reduced level and also did not depend on gender.

**Conclusion.** The study highlights the need to take into account the age and gender characteristics of the microcirculatory system in the diagnosis and monitoring of diseases of the cardiovascular system, especially in young people living in the extreme climatic conditions of Yakutia.

**Keywords:** microcirculation, LDF, boys, girls, Yakutia

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## ХАРАКТЕРИСТИКА ПОКАЗАТЕЛЕЙ МИКРОЦИРКУЛЯТОРНОГО РУСЛА ЮНОШЕЙ И ДЕВУШЕК ПО ДАННЫМ ЛАЗЕРНОЙ ДОППЛЕРОВСКОЙ ФЛОУМЕТРИИ (ЛДФ)

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### РЕЗЮМЕ

Метод лазерной доплеровской флоуметрии является одним из актуальных и информативных способов изучения состояния микроциркуляторного русла.

**Целью** работы явилось проведение сравнительной характеристики показателей микроциркуляторного русла юношей и девушек по данным лазерной доплеровской флоуметрии (ЛДФ).

**Материалы и методы исследования:** В исследовании приняли участие 128 студентов второго курса Медицинского института Северо-Восточного федерального университета им. М.К. Аммосова. Все обследованные были якутской национальности. Группа юношей состояла из 44 человек, группа девушек из 84 человек. Состояние микроциркуляции и функционирование ее регуляторных механизмов оценивали методом лазерной доплеровской флоуметрии (ЛДФ) с помощью двух одинаковых одноканальных портативных анализаторов «ЛАЗМА ПФ» (ООО НПП «ЛАЗМА», Москва). Измерение проведено на нижней трети правого и левого предплечий, запись велась в течение 5 минут.

**Результаты.** Установлены достоверные межгрупповые различия в показателях микроциркуляторного русла между обследованными юношами и девушками. Полученные результаты показали значимые половые различия в состоянии микроциркуляторного русла: у юношей зафиксированы более высокие значения шунтового кровотока ( $1,71 \pm 0,13$ ) и большая амплитуда колебаний, обусловленных активностью эндотелия сосудов ( $0,04 \pm 0,01$ ). У девушек же отмечались достоверно меньшие показатели шунтового кровотока ( $1,34 \pm 0,07$ ) и активности эндотелия ( $0,01 \pm 0,01$ ). При этом уровни нутритивного кровотока, а также активность нейрогенных и миогенных механизмов регуляции были сходны у представителей обоих полов. Среди обследуемых преобладала группа лиц с низким уровнем дыхательных колебаний кровотока (68,2 % юношей и 73,8 % девушек). А показатели сердечных колебаний кровотока находились в нормальном или сниженном уровне и также не зависели от пола.

**Заключение.** Исследование подчеркивает необходимость учитывать возрастные и половые особенности показателей микроциркуляторного русла при диагностике и мониторинге заболеваний сердечно-сосудистой системы, особенно у молодых людей, проживающих в экстремальных климатических условиях Якутии.

**Ключевые слова:** микроциркуляция, ЛДФ, юноши, девушки, Якутия

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## INTRODUCTION

One of the key aspects of cardiovascular system function is microcirculation. Disturbances at the level of the microcirculatory bed play a crucial role in the pathogenesis of a wide range of diseases, from arterial hypertension and diabetes mellitus to chronic heart failure and autoimmune vasculitides.

One of the most informative and modern approaches for assessing microcirculation is laser Doppler flowmetry (LDF) [1–3]. This method is based on recording the intensity of light scattering reflected by moving erythrocytes, which allows determination of blood flow velocity and volume in the smallest blood vessels [4]. In studies utilizing LDF various parameters are recorded that characterize blood circulation at the microcirculatory level, such as the microcirculation index, reactivity indices, flow velocity, and signal variability [5, 6]. By obtaining objective quantitative data, a comprehensive analysis of the functional status of microcirculation can be conducted, including assessment of its response to external stimuli and identification of potential abnormalities.

Interpretation of microcirculatory parameters requires consideration of various individual and population factors, primarily sex and age, as well as climatic and geographic conditions of residence [7–9]. Extreme continental climate of Yakutia, characterized by prolonged periods of low temperatures, sharp fluctuations in atmospheric pressure, and specific light conditions, creates specific requirements on cardiovascular function, particularly the microcirculation. Establishing differences in sex-specific microcirculatory parameters can lead to the personalization of strategies for early diagnosis, prevention, and management of cardiovascular disease. To date, no studies on the microvascular bed using LDF in young individuals have been conducted in the Republic of Sakha (Yakutia). This highlights the significance and originality of this research.

## AIM

To conduct a comparative analysis of microcirculatory parameters in young males and females using laser Doppler flowmetry (LDF).

## MATERIALS AND METHODS

The study was conducted at the Medical Institute of M.K. Ammosov North-Eastern Federal University (NEFU) in April and May 2025. A total of 128 second-year students, who were of Yakut ethnic origin, participated in the study, including 44 males and 84 females. All participants met the following inclusion criteria: absence of chronic cardiovascular and autoimmune diseases, as well as acute pathological conditions at the time of the examination. Participation in the study was voluntary, and it was carried out only after obtaining written informed consent

from each participant. The study received approval from the local ethics committee of the Medical Institute of M.K. Ammosov (Protocol No. 5, dated February 14, 2024).

The status of microcirculation and the functioning of its regulatory mechanisms were evaluated using laser Doppler flowmetry (LDF), employing two identical single-channel portable LASMA PF analyzers (SPE LAZMA, Moscow), in accordance with the requirements of Roszdravnadzor (Resolution No. 660/2018). To assess microcirculatory parameters, sensors were attached to the lower third of the right and left forearms of the subjects. During the recording of blood perfusion, the subjects were in a state of physical and psychological rest. The arms were placed at heart level, and the study was conducted at least 2 hours following meals and physical activity. Each measurement lasted 5 minutes, and the temperature in the testing environment was maintained at  $23 \pm 2^\circ\text{C}$ .

The processing of the acquired LDF signals was carried out using specialized software. Based on the acquired data, quantitative parameters were calculated. The following parameters were recorded: the integrated perfusion index (M, perfusion units, p.u.) and the coefficient of variation (CV, %) that characterize microcirculatory variability. The component level of microcirculation was determined by the parameters of nutritional (Mnutr) and shunt (Mshunt) blood flow. Wavelet analysis was used to evaluate the amplitude-frequency spectrum of microcirculation oscillations and calculate the amplitude characteristics of the main rhythms of microvascular blood flow regulation: endothelial (Ae), neurogenic (An), myogenic (Am), respiratory (Ar), and cardiac (Ac). After measurement completion, the software of the instrument automatically generated a report indicating perfusion, variability, and regulatory parameters. In the forearm area, the following parameters were considered within the normal range: M – 3.5 to 9.5 p.u., Mshunt – 2.0 to 6.0 p.u., Mnutr – 1.5 to 3.5 p.u., KV – 6.0 to 11.0 %, Ae – 0.1 to 0.2 p.u., An – 0.12 to 0.35 p.u., Am – 0.2 to 0.35 p.u., Ar – 0.15 to 0.25 p.u., Ac – 0.15 to 0.35 p.u.

During the study, the LASMA PF device simultaneously recorded local skin temperature in the forearm area. This allowed the thermal component to be taken into account when interpreting microcirculatory parameters [10]. The data obtained provided a comprehensive assessment of the baseline state of the microvascular bed and the functional activity of the neurohumoral and metabolic regulatory systems.

Statistical analysis was conducted using SPSS-22 software. Mean (M), standard deviation (SD), median (Me), and interquartile range [ $Q_{25}$ ;  $Q_{75}$ ] were calculated. The normality of the distribution was assessed using the Kolmogorov-Smirnov test. Depending on the distribution type, parametric (Student's *t*-test) and non-parametric (Mann-Whitney U-test) methods were employed for intergroup comparison. The significance of differences between relative parameters was determined using Pearson's chi-square ( $\chi^2$ ) test. Differences were considered statistically significant at  $p < 0.05$ .

## RESULTS

The results of microcirculatory parameters in young males and females obtained using LDF were analyzed. The parameters recorded by the LASMA-PF device are presented in Table 1.

A significant intergroup difference was observed in shunt blood flow values. The mean shunt blood flow was significantly higher in males ( $p = 0.034$ ). The amplitude of blood flow oscillations, determined by the endothelial mechanism of vascular tone regulation, was also significantly higher in males ( $p = 0.039$ ).

Analysis of microcirculation level distribution showed that in males, normal microcirculation was recorded in 52.3 % of cases, while reduced microcirculation was observed in 47.7 %, although this difference was not statistically significant. Similar distribution patterns were observed in females, with normal microcirculation occurring in 42.9 % and reduced level in 57.1 % of cases. The mean local skin temperature of the forearm was  $32.22 \pm 1.65^\circ\text{C}$  in males and  $32.09 \pm 1.56^\circ\text{C}$  in females.

Levels of nutritional and shunt blood flow are presented in Table 2.

Table 3 presents data on the amplitude of blood flow oscillations, as determined by endothelial, neurogenic, and myogenic mechanisms of vascular tone regulation, in young males and females.

During the study, differences in the amplitude of blood flow oscillations that are determined by endothelial, neurogenic, and myogenic mechanisms of vascular tone regulation were recorded in young males and females of Yakut ethnicity. The levels of respiratory and cardiac blood flow oscillation amplitudes for both groups are presented in Table 4.

## DISCUSSION

The level of the microcirculatory bed showed no sex differences. A comparison of our data with the results of Parshakova V.E. et al. (2024) [11] revealed that the absolute index of microcirculation (M) was lower in both

**TABLE 1**  
**INDICATORS OF MICROCIRCULATION OF THE FOREARM OF BOYS AND GIRLS OF YAKUT ETHNICITY**

| Parameter    | Males (n=44) |                                        | Females (n=84) |                                        | p       |
|--------------|--------------|----------------------------------------|----------------|----------------------------------------|---------|
|              | M±SD         | Me [Q <sub>25</sub> ;Q <sub>75</sub> ] | M±SD           | Me [Q <sub>25</sub> ;Q <sub>75</sub> ] |         |
| M, p.u.      | 3.67±1.23    | 3.37 [2.86; 4.16]                      | 3.73±2.24      | 3.41 [2.79; 4.15]                      | p=0.630 |
| Mnutr, p.u.  | 2.15±1.12    | 1.80 [1.33; 2.50]                      | 2.37±2.91      | 1.87 [1.32; 2.68]                      | p=0.972 |
| Mshunt, p.u. | 1.68±0.85    | 1.50 [1.02; 2.0]                       | 1.41±1.27      | 1.39 [0.91; 1.87]                      | p=0.034 |
| Sigma        | 0.55±0.39    | 0.41 [0.29; 0.69]                      | 0.48±0.33      | 0.38 [0.28; 0.55]                      | p=0.298 |
| CV, %        | 14.72±9.05   | 11.85[9.51; 17.66]                     | 12.92±5.77     | 11.13 [9.19; 15.09]                    | p=0.278 |
| Ae, p.u.     | 0.04±0.07    | 0.02 [0.01; 0.36]                      | 0.002±0.04     | 0.001 [0.0; 0.30]                      | p=0.039 |
| An, p.u.     | 0.17±0.16    | 0.12 [0.07; 0.22]                      | 0.17±0.15      | 0.13 [0.08; 0.20]                      | p=0.819 |
| Am, p.u.     | 0.23±0.21    | 0.16 [0.11; 0.27]                      | 0.21±0.15      | 0.17 [0.11; 0.29]                      | p=0.843 |
| Ar, p.u.     | 0.17±0.18    | 0.11 [0.08; 0.18]                      | 0.13±0.11      | 0.09 [0.07; 0.15]                      | p=0.090 |
| Ac, p.u.     | 0.20±0.12    | 0.18 [0.12; 0.21]                      | 0.18±0.11      | 0.15 [0.11; 0.21]                      | p=0.390 |
| T, C°        | 31.85±1.65   | 32.14[30.31; 33.23]                    | 31.96±1.56     | 31.86 [30.95; 33.10]                   | p=0.580 |

**TABLE 2**  
**DISTRIBUTION OF BOYS AND GIRLS BY LEVELS OF NUTRITIONAL AND SHUNT BLOOD FLOW**

| Parameter              | Level     | Males (n=44) | Females (n=84) |
|------------------------|-----------|--------------|----------------|
| Nutritional blood flow | Normal    | 23 (52.3)    | 48 (57.1)      |
|                        | Increased | 8 (18.2)     | 8 (9.6)        |
|                        | Reduced   | 13 (29.5)    | 28 (33.3)      |
| Shunt blood flow       | Normal    | 13 (29.5)    | 15 (17.9)      |
|                        | Reduced   | 31 (70.5)    | 69 (82.1)      |

groups groups ( $3.67 \pm 1.23$  and  $3.73 \pm 2.24$  p.u. vs.  $5.2 \pm 1.0$  p.u.). Neither study found any significant difference in this parameter between males and females.

Analysis of microcirculatory components revealed that the mean shunt blood flow was significantly higher in males ( $p = 0.034$ ) compared to females. Furthermore, the amplitude of endothelium-dependent blood flow oscillations was also significantly higher in males ( $p = 0.039$ ). These findings can be attributed to a combination of structural and physiological features: males typically have higher anthropometric measurements (height, weight, muscle mass, etc.), a faster metabolic rate, and a more extensive network of arteriovenous anastomoses. These features lead to increased shunt blood flow. It is known that decreased testosterone levels are associated with impaired vascular endothelial function [12].

Skin temperature was measured using an integrated thermosensor within the flowmeter measuring head and recorded simultaneously with the LDF signal. The local skin temperature on the forearm showed no significant difference between males ( $32.22 \pm 1.65^\circ\text{C}$ ) and females ( $32.09 \pm 1.56^\circ\text{C}$ ). Simultaneous temperature recording

improves the reliability of the data, as local skin temperature directly affects microvascular tone and consequently perfusion levels. Minor temperature fluctuations can influence vasomotor activity and potentially affect the accuracy of regulatory mechanism assessments.

A heterogeneous pattern was observed in terms of nutritional blood flow levels. A significant proportion of both males and females (52.3 % and 57.1 %, respectively) exhibited normal nutritional blood flow ( $\chi^2 = 5.454$ ,  $p = 0.020$ ;  $\chi^2 = 22.134$ ,  $p < 0.001$ ). However, individuals with reduced and increased nutritional blood flow values were also present. A significantly larger proportion of males ( $\chi^2 = 5.002$ ,  $p = 0.026$ ) and females ( $\chi^2 = 24.257$ ,  $p = 0.001$ ) had reduced shunt blood flow (70.5 % and 82.1 %, respectively). This can be explained by the structural characteristics of forearm skin, where arteriolo-venular anastomoses are rare in this area and located in deeper skin layers inaccessible to laser probe analysis. Consequently, forearm skin perfusion primarily reflects the nutritional aspect of microcirculation [13–15].

Both males and females exhibited a predominance of reduced blood flow oscillation amplitudes associated

**TABLE 3**

**AMPLITUDE LEVELS OF BLOOD FLOW FLUCTUATIONS CAUSED BY DIFFERENT MECHANISMS OF VASCULAR TONE REGULATION IN BOYS AND GIRLS**

| Blood flow oscillation amplitude | Level     | Males (n=44) | Females (n=84) | Pearson's $\chi^2$ test; p     |
|----------------------------------|-----------|--------------|----------------|--------------------------------|
| Endothelial mechanism            | Normal    | -            | 1 (1.2)        | $\chi^2 = 0.522$ ; $p = 0.471$ |
|                                  | Increased | 6 (13.6)     | 1 (1.2)        | $\chi^2 = 7.502$ ; $p = 0.007$ |
|                                  | Reduced   | 38 (86.4)    | 82 (97.6)      | $\chi^2 = 0.205$ ; $p = 0.651$ |
| Neurogenic mechanism             | Normal    | 19 (43.2)    | 35 (41.7)      | $\chi^2 = 0.011$ ; $p = 0.917$ |
|                                  | Increased | 7 (15.9)     | 12 (14.3)      | $\chi^2 = 0.044$ ; $p = 0.834$ |
|                                  | Reduced   | 18 (40.9)    | 37 (44.0)      | $\chi^2 = 0.047$ ; $p = 0.830$ |
| Myogenic mechanism               | Normal    | 9 (20.5)     | 23 (27.4)      | $\chi^2 = 0.451$ ; $p = 0.502$ |
|                                  | Increased | 9 (20.5)     | 14 (16.7)      | $\chi^2 = 0.194$ ; $p = 0.660$ |
|                                  | Reduced   | 26 (59.1)    | 47 (56.0)      | $\chi^2 = 0.032$ ; $p = 0.859$ |

**TABLE 4**

**AMPLITUDE LEVELS OF RESPIRATORY AND CARDIAC FLUCTUATIONS IN BLOOD FLOW IN BOYS AND GIRLS**

| Blood flow oscillation amplitude | Level     | Males (n=44) | Females (n=84) | Pearson's $\chi^2$ test; p     |
|----------------------------------|-----------|--------------|----------------|--------------------------------|
| Respiratory                      | Normal    | 8 (18.2%)    | 11 (13.1%)     | $\chi^2 = 0.432$ ; $p = 0.511$ |
|                                  | Increased | 6 (13.6%)    | 11 (13.1%)     | $\chi^2 = 0.006$ ; $p = 0.941$ |
|                                  | Reduced   | 30 (68.2%)   | 62 (73.8%)     | $\chi^2 = 0.075$ ; $p = 0.785$ |
| Cardiac                          | Normal    | 23 (52.3%)   | 38 (45.2%)     | $\chi^2 = 0.200$ ; $p = 0.655$ |
|                                  | Increased | 7 (15.9%)    | 6 (7.1%)       | $\chi^2 = 1.938$ ; $p = 0.164$ |
|                                  | Reduced   | 14 (31.8%)   | 40 (47.6%)     | $\chi^2 = 1.249$ ; $p = 0.264$ |

with the endothelium (86.4 % and 97.6 %, respectively). A higher amplitude of blood flow oscillations mediated by the endothelial mechanism of vascular tone regulation was more common in males ( $\chi^2 = 7.502$ ;  $p = 0.007$ ). We suggest that the increased amplitude of endothelium-dependent blood flow oscillations in males may be due to a higher baseline vascular tone under the influence of androgens, requiring a stronger reactive release of nitric oxide for vasodilation in response to stimuli [16].

Assessment of blood flow oscillation amplitude, mediated by the neurogenic mechanism of vascular tone regulation, in Yakut males showed an approximately equal distribution between normal (43.2 %) and reduced (40.9 %) levels. A similar distribution was observed in the female group (41.7 % and 44.0 %, respectively). An increased level was seen in a significantly lower proportion of both males ( $\chi^2 = 4.323$ ;  $p = 0.038$ ) and females ( $\chi^2 = 8.897$ ;  $p = 0.003$ ). There were no statistically significant differences in the distribution of neurogenic regulation levels between the studied groups. This indicates a low level of sympathetic nervous system involvement in microcirculation regulation for both males and females. The myogenic mechanism also showed no significant sex differences, with reduced activity seen in the majority of males and females. Our study found that different regulatory mechanisms were equally involved, indicating a balanced and adaptable organization of vasomotor control in the group of young participants. Each mechanism made a comparable and balanced contribution to the regulation of microcirculation.

In the examined groups of males and females, a predominance of reduced respiratory blood flow oscillation amplitude was observed (68.2 % and 73.8 %, respectively). The amplitude of respiratory blood flow oscillations is an important indicator of venous outflow and overall autonomic regulation of the cardiovascular system. Cardiac blood flow oscillations reflect the transmission of the pulse wave from the large arteries to the microcirculatory bed and depend on several factors, including blood pressure, arterial wall stiffness, peripheral vascular resistance, and the state of capillary beds and tissue drainage [17]. Analysis of cardiac blood flow oscillation amplitude revealed that, in the majority of subjects, it was within normal or reduced ranges and did not statistically differ between males and females ( $p > 0.05$ ). Increased levels were only observed in a small percentage of participants. This finding indicates that, despite known physiological differences between males and females (e.g., lower blood pressure and lower muscle mass in females), the transmission of pulse waves to the microcirculation is comparable between the two sexes. On the one hand, this could be seen as an indication of preserved arterial wall elasticity and appropriate peripheral resistance. However, on the other hand, the decreased amplitude, particularly in almost half of the female participants (47.6 %), could suggest more effective pulse wave attenuation in peripheral vessels. This is often associated with the vasodilative effect of estrogens. To verify this hypothesis, a study involving direct measurement of hormone levels would be required.

Numerous studies have demonstrated significant sex differences in microvascular perfusion parameters. Women generally exhibit higher perfusion indices and larger amplitudes of neurogenic oscillations than men, which can be attributed to the effects of estrogen on vasodilation, endothelial-dependent relaxation, and overall vascular reactivity. These findings are supported by studies in the literature [18, 19]. At the same time, studies have shown that men often exhibit signs of reduced microvascular reserve even at a younger age, which may explain their earlier manifestation of atherosclerotic and hypertensive complications compared to women.

## CONCLUSION

Therefore, significant intergroup differences in microcirculatory parameters were observed between the examined males and females. Males exhibited higher mean shunt blood flow values and a greater amplitude of blood flow oscillations associated with vascular endothelial activity. Nevertheless, the distribution of nutritional blood flow levels and the levels of neurogenic and myogenic mechanisms for vascular tone regulation did not show significant differences between the sexes. Both males and females exhibited a predominance of decreased respiratory component parameters. Cardiac flow oscillation parameters fell within the normal or decreased range and were not dependent on sex.

These results highlight the importance of considering age and sex characteristics when assessing the state of the cardiovascular system and the microcirculatory bed, particularly in young people living in the extreme climatic conditions of Yakutia.

## Conflicts of interest

The authors declare no conflicts of interest.

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## NEUROLOGY AND NEUROSURGERY

### PSYCHOPHYSIOLOGICAL CHARACTERISTICS OF COGNITIVE FUNCTIONS IN MIDDLE-AGED AND OLDER ADULTS AND THE EFFECT OF PHYTOCONSTITUENTS WITH NOOTROPIC PROPERTIES ON THEM

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#### ABSTRACT

**Background.** Maintaining cognitive health and preventing dementia are priorities in modern medicine and science. Nootropics play an important role in neuroprotection. Multicomponent herbal remedies containing medicinal plant extracts hold promise as phytopharmacological regulatory systems for maintaining and restoring cognitive health.

**Aim.** To analyze the psychophysiological characteristics of cognitive functions in middle-aged and older adults and to assess the effect of a phytoconstituent with nootropic properties on these characteristics.

**Materials and methods.** Before and after a course of treatment with the phytoconstituent, participants underwent a comprehensive examination that included electrocardiography with heart rate variability assessment; evaluation of short-term and long-term memory using the "10-Word Memorization" test by A.R. Luria; and assessment of logical thinking using the "Complex Analogies" test.

**Results.** The study identified two groups of participants: those with moderate and those with high levels of working memory and logical thinking. Group 1 was characterized by low parasympathetic nervous system markers and reduced heart rate variability. Group 2 demonstrated higher heart rate variability and elevated parasympathetic markers. After administration of the phytoconstituent, parasympathetic activity markers (RMSSD, HFmc<sup>2</sup>, HF%) increased in Cluster 1 and decreased in Cluster 2. Following the course of the phytoconstituent, Group 1 exhibited a more pronounced improvement in memory indices and logical reasoning abilities.

**Conclusion.** An association was found between high levels of working memory and logical thinking and a predominance of parasympathetic influences on heart rhythm. Administration of the herbal preparation with nootropic properties resulted in improved cognitive function and a balanced autonomic nervous system.

**Key words:** cognitive functions, nootropic agents, heart rate variability, autonomic nervous system

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## ПСИХОФИЗИОЛОГИЧЕСКИЕ ОСОБЕННОСТИ КОГНИТИВНЫХ ФУНКЦИЙ У ПРЕДСТАВИТЕЛЕЙ ЗРЕЛОГО И ПОЖИЛОГО ВОЗРАСТА И ВЛИЯНИЕ НА НИХ ФИТОКОМПОЗИЦИИ С НООТРОПНЫМИ СВОЙСТВАМИ

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### РЕЗЮМЕ

**Актуальность.** Сохранение когнитивного здоровья и профилактика деменции – приоритетные задачи современной медицины и науки. Ноотропы занимают важное место в нейропротекции. Многокомпонентные фитосредства с экстрактами лекарственных растений перспективны как фитотерапевтические регуляторные системы для поддержания и восстановления когнитивного здоровья.

**Цель исследования.** Провести анализ психофизиологических особенностей когнитивных функций у представителей зрелого и пожилого возраста и оценить влияние на них фитокомпозиции (ФК) с ноотропными свойствами.

**Материалы и методы.** До и после курсового приема фитокомпозиции испытуемым проводили комплексное обследование, включающее электрокардиографию с оценкой вариабельности ритма сердца, оценку функции кратковременной и долговременной памяти с помощью теста «Заучивание 10 слов» А.Р. Лурия, логическое мышление оценивали с помощью теста «Сложные аналогии».

**Результаты.** Исследование выявило две группы испытуемых: со средним и высоким уровнем развития рабочей памяти и логического мышления. Первая группа характеризовалась низкими значениями маркеров парасимпатической нервной системы и низкой вариабельностью ритма сердца. Вторая группа отличалась высоким уровнем вариабельности сердечного ритма, высокими значениями маркеров парасимпатической нервной системы. После приема ФК величины маркеров парасимпатической активности RMSSD, HFms<sup>2</sup> и HF% возрастали у членов первого кластера и снижались у членов второго кластера. После приема ФК в первой группе в большей степени улучшались показатели памяти и способности к логическому мышлению.

**Заключение.** Показана связь между высокими показателями рабочей памяти и логического мышления и преобладанием парасимпатических влияний на ритм сердца. Прием фитокомпозиции с ноотропными свойствами вызывал улучшение когнитивных функций и выравнивание баланса вегетативной нервной системы.

**Ключевые слова:** когнитивные функции, ноотропные средства, вариабельность ритма сердца, автономная нервная система

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## INTRODUCTION

A review of the literature revealed a limited number of studies comprehensively investigating the relationships between cognitive functions and autonomic regulatory mechanisms of cardiac activity, particularly in middle-aged and older adults [1].

Maintaining cognitive health and preventing dementia are priorities in modern medicine and science [2]. Nootropics play an important role in neuroprotection and can improve memory, performance, and adaptive capabilities of the brain in older adults [3, 4]. Epidemiological data indicate the need for the development of affordable domestic nootropics. Multicomponent herbal preparations containing medicinal plant extracts have the potential to be used as phytopharmacological regulatory systems for maintaining and restoring cognitive health [5, 6].

In 2023, a research team from the Institute of Biomedicine at Altai State University, as part of the project "Development of functional products for restoring body functions during age-related changes and evaluation of their effectiveness" under the Priority-2030 program, developed a phytoconstituents (PC) with nootropic properties. Identifying the mechanisms by which this PC influences human cognitive functions is of current relevance.

## AIM

To analyze the psychophysiological characteristics of cognitive functions in middle-aged and older adults and to assess the effect of a phytoconstituents with nootropic properties on these characteristics.

## MATERIALS AND METHODS

### *Study Population*

The group receiving the PC ( $n = 35$ ) consisted of conditionally healthy volunteers over 45 years of age, who were recruited from various occupations. A total of 35 women aged 45–65 years (mean age  $52.3 \pm 0.94$  years) were examined. The sample size was determined based on the small sample criterion (sample size may not exceed 30 units plus 25 % to account for potential outliers).

All participants provided informed consent prior to participating in the study. Inclusion criteria: voluntary informed consent. Exclusion criteria: chronic diseases, hypertension, blood clotting disorders, alcohol abuse, regular smoking, hypersensitivity to PC components, recurrent gastrointestinal problems, and blood pressure above 130/80 mmHg.

### *Psychophysiological Research Methods*

Before and after the course of PC administration, participants underwent a comprehensive examination that included:

- electrocardiography with the assessment of heart rate variability (HRV) using a Poly-Spectr-8/EX device (Ivanovo, Russian Federation);
- assessment of short-term and long-term memory using the "10-Word Memorization" test developed by A.R. Luria;
- assessment of logical thinking through the "Complex Analogies" test developed by E.A. Korobkova, which evaluates logical reasoning based on the ability to find logical connections between 20 word pairs (classification, antonyms, cause-effect, synonyms, structural connections, group membership), using an online testing service [7].

### *Pharmacological Substances*

The PC included components with significant nootropic, adaptogenic, antioxidant, and antihypoxic properties: astragalus membranaceus extract (20 mg), finely ground boswellia serrata resin (20 mg), Hericium erinaceus fruiting bodies (159 mg) – to stimulate cognitive functions, Ginkgo biloba extract (20 mg) and polyphenols (5 mg) – to improve blood supply, as well as a vitamin complex (vitamins A, C, D, E, K, group B, and vitamin D3). Volunteers were administered one PC capsule twice daily for a period of two weeks.

### *Ethical Approval*

The study was approved by the local ethics committee of Altai State University (Protocol No. 9 dated April 5, 2024).

### *Statistical Analysis*

Statistical data processing was performed using the SPSS 21.0 software package. To check for the normality of the distribution, the Shapiro – Wilk test was employed (significance level  $p > 0.05$ ). For quantitative data that did not follow a normal distribution, the median (Me) and interquartile range ( $Q_{25} - Q_{75}$ ) were used. The non-parametric Kruskal – Wallis test was used for intergroup comparisons. For normally distributed data, quantitative variables were presented as mean ( $M$ )  $\pm$  standard error (SE). For intergroup comparisons in cases of normally distributed data, the parametric Student's  $t$ -test for independent samples was employed. The Wilcoxon test for paired samples was used to evaluate dynamic changes. Differences were considered statistically significant at  $p < 0.05$ . Cluster analysis was used to form groups with similar characteristics.

## RESULTS

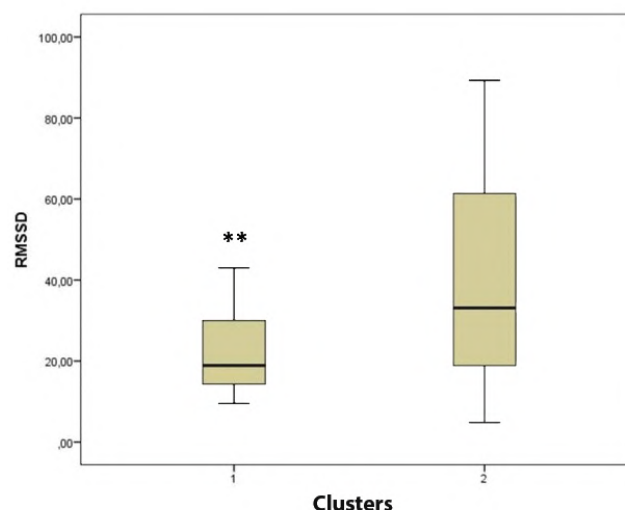
Cluster analysis identified two groups with shared characteristics (Cluster 1,  $n = 21$ ; Cluster 2,  $n = 14$ ). There was no significant difference in age between the clusters (Cluster 1:  $51.8 \pm 1.25$  years; Cluster 2:  $52.9 \pm 1.47$  years;  $p = 0.550$ ). A comparative analysis revealed statistically significant differences in several psychophysiological parameters between the study groups. Using psychophysiological measures, statistically significant intergroup differences were observed in first recall (Cluster 1:  $6.3 \pm 0.09$ ; Cluster 2:  $8.8 \pm 0.17$ ;  $p < 0.001$ ), second recall

(Cluster 1:  $8.5 \pm 0.13$ ; Cluster 2:  $9.1 \pm 0.12$ ;  $p < 0.001$ ), delayed recall (Cluster 1:  $8.8 \pm 0.16$ ; Cluster 2:  $10.0 \pm 0.03$ ;  $p < 0.001$ ), and recall after 24 hours (Cluster 1:  $8.8 \pm 0.16$ ; Cluster 2:  $9.6 \pm 0.08$ ;  $p < 0.001$ ). Analysis of the results from the “Complex Analogies” test revealed that participants in Cluster 2 ( $9.2 \pm 0.52$ ,  $p = 0.046$ ) demonstrated a significantly greater capacity for establishing logical connections between pairs of words compared to those in Cluster 1 ( $7.1 \pm 0.61$ ), i.e., subjects in Cluster 2 exhibited a higher level of cognitive functioning.

Statistically significant differences were also observed between the study groups in terms of heart rate variability parameters. To assess parasympathetic regulation, the following HRV markers were used [8]: the root mean square of successive RR interval differences (RMSSD) (Fig. 1), as well as the absolute and relative high-frequency power (HF) (Fig. 2). These parameters were significantly lower in subjects of Cluster 1, indicating reduced activity of the parasympathetic division of the autonomic nervous system.

After administration of the PC, parasympathetic activity markers increased in Cluster 1 and decreased approximately 2-fold in Cluster 2. Specifically: In Cluster 1, RMSSD increased by 50 % [before PC: Me 20.0, Q25–75 11.0–43.0; after PC: Me 30.0, Q25–75 16.0–64.0;  $p = 0.008$ ]; HF ( $\text{ms}^2$ ) increased 3-fold [before PC: Me 125.0, Q25–75 25.0–450.0; after PC: Me 375.0, Q25–75 75.0–1350.0;  $p = 0.008$ ]; HF% increased by 50 % [before PC: Me 16.0, Q25–75 8.0–46.0; after PC: Me 24.0, Q25–75 8.0–69.0;  $p = 0.008$ ]. In Cluster 2, RMSSD decreased approximately 2-fold [before PC: Me 42.0, Q25–75

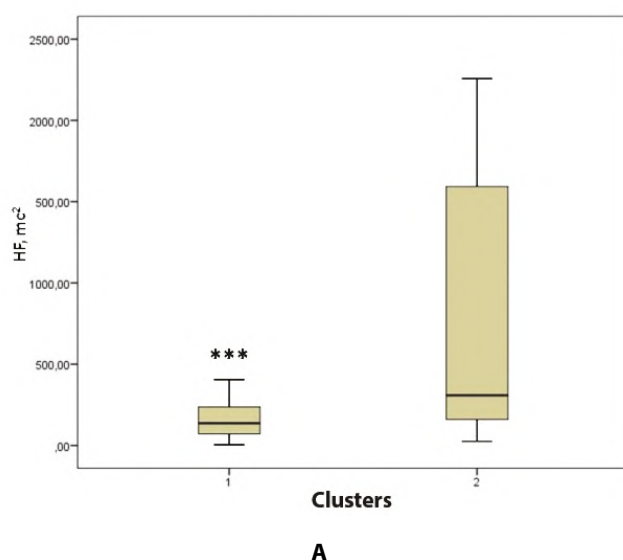
11.0–89.0; after PC: Me 21.0, Q25–75 8.0–45.0;  $p = 0.008$ ]; HF ( $\text{ms}^2$ ) decreased approximately 2-fold [before PC: Me 375.0, Q25–75 30.0–2250.0; after PC: Me 187.0, Q25–75 15.0–1125.0;  $p = 0.008$ ]; HF% decreased by 50 % [before



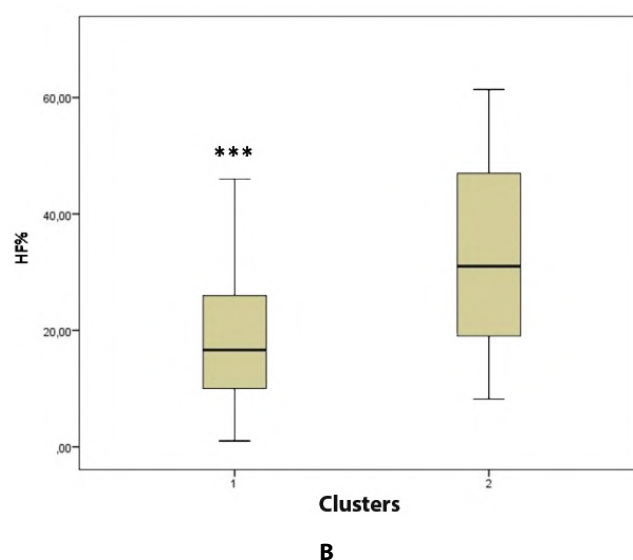
**Note.** Cluster 1 – individuals with a moderate level of working memory and logical thinking; Cluster 2 – individuals with a high level of working memory and logical thinking; Significant differences between groups: \*\* –  $p < 0.01$ .

**FIG. 1.**

Time-domain heart rate variability parameters (RMSSD) (data are presented as medians and interquartile ranges (Me,  $Q_{25}$ – $Q_{75}$ ))



**A**

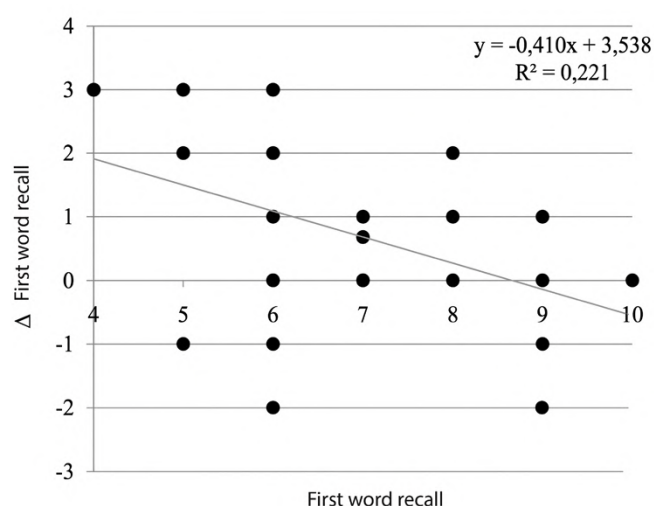


**B**

**Note.** Clusters are the same as in Fig. 1. Significant differences between groups: \*\*\* –  $p < 0.001$ .

**FIG. 2.**

High-frequency spectral parameters of heart rate variability: A – HF ( $\text{ms}^2$ ) and B – HF (%) (data are presented as medians and interquartile ranges (Me,  $Q_{25}$ – $Q_{75}$ ))



**FIG. 3.** Correlation between the first recall delta and the number of words recalled. The figure shows the regression equation

PC: Me 31.0, Q25–75 8.0–61.0; after PC: Me 15.5, Q25–75 5.0–30.0;  $p = 0.008$ ].

Administration of the PC led to a statistically significant increase in memory performance. First recall in Cluster 1 increased by 8 % (from  $6.3 \pm 0.09$  to  $6.8 \pm 0.17$ ,  $p < 0.001$ ), while in Cluster 2 it increased by 1 % (from  $8.8 \pm 0.17$  to  $8.9 \pm 0.17$ ,  $p = 0.317$ ). Delayed recall in Cluster 1 increased by 7 % (from  $8.8 \pm 0.16$  to  $9.4 \pm 0.17$ ,  $p < 0.001$ ) and in Cluster 2 by 2 % (from  $10.0 \pm 0.03$  to  $10.0 \pm 0.03$ ,  $p = 1.00$ ). Recall after 24 hours increased by 6 % in Cluster 1 (from  $8.8 \pm 0.16$  to  $9.3 \pm 0.17$ ,  $p < 0.001$ ) and by 1 % in Cluster 2 (from  $9.6 \pm 0.08$  to  $9.7 \pm 0.08$ ,  $p = 0.157$ ).

Following PC administration, there was a statistically significant increase in scores on the “Complex Analogies” test, reflecting an improvement in logical-analytical abilities. Specifically, a statistically significant improvement was observed compared to baseline, predominantly in subjects from Cluster 1 (by 39 %) and Cluster 2 (by 23 %). For Cluster 1, the mean score increased from  $7.1 \pm 0.85$  to  $9.9 \pm 0.61$ ,  $p = 0.004$ . For Cluster 2, the mean score increased from  $9.2 \pm 0.52$  to  $11.3 \pm 0.64$ ,  $p = 0.002$ .

The effectiveness of the PC is likely to be largely determined by the baseline level of brain function. The most pronounced effect of the preparation was observed among individuals with lower baseline memory performance. Specifically, the delta (difference between pre- and post-PC values) in first word recall was negatively dependent on the baseline number of words recalled (Fig. 3). Correlation analysis revealed a moderate negative correlation between these variables ( $r = -0.470$ ,  $p = 0.005$ ).

Similar results were obtained for the delta values of second recall, delayed recall, and 24-hour recall. This

indicates that the effect of the PC is more pronounced in individuals with more substantially reduced cognitive function at baseline.

## DISCUSSION

Comparison of our results with data from other researchers also showed that high heart rate variability is associated with better cognitive characteristics and effective adaptive abilities [1, 9, 10]. The results obtained in the current study confirm data previously reported in a study conducted with officers of the Royal Norwegian Navy [9].

It is known that reduced heart rate variability may be associated with decreased cognitive efficiency, particularly reduced working memory and attention function, as well as prolonged sensorimotor reaction time [11]. Moreover, low HRV values are considered a potential predictor of an increased risk of cardiovascular diseases, cognitive dysfunction, and neurodegenerative processes, especially in older adults [12]. Our results indicate the importance of parasympathetic regulation in ensuring cognitive performance efficiency.

A comparison of the results from the phytoconstituents study with data on Ginkgo biloba extract [13] revealed a similarity in the findings: in older women and individuals with initially reduced cognitive abilities, the impact on cognitive processes was more significant.

Based on the study conducted, it can be concluded that the phytoconstituents have a beneficial effect on cognitive functions, including memory and thought processes. Given the increasing interest in plant-based agents with neuroprotective properties, the development of novel phytopreparations is becoming highly relevant. The creation and implementation of the phytoconstituents may contribute to expanding the domestic range of agents with proven efficacy in the field of cognitive neuroprotection.

## CONCLUSION

The study identified two groups of participants: those with moderate and those with high levels of working memory and logical thinking. The first group had reduced parameters of the parasympathetic nervous system activity. The second group, in contrast, demonstrated increased values of the parameters reflecting the function of the parasympathetic system. After administration of the phytoconstituents, the participants in the first group exhibited a more significant improvement in cognitive functions, accompanied by a shift in autonomic nervous system balance.

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# Conflicts of interest

The authors declare no conflicts of interest.

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## RESTLESS LEGS SYNDROME: A LITERATURE REVIEW

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### ABSTRACT

*Restless legs syndrome (RLS) is a common sensorimotor neurological disorder that significantly disrupts sleep and is associated with an increased risk of chronic noncommunicable diseases. RLS is considered a common movement disorder, occurring approximately 50 % more frequently in women and in individuals of European descent. The etiology of the disease remains unknown. The pathophysiology of RLS is also not fully understood. Dopaminergic dysfunction is a key feature of RLS. Since its discovery, the role of iron deficiency has been emphasized, not iron levels in the peripheral blood, but rather its concentration in the central nervous system. The hyperdopaminergic state in RLS can lead to decreased endogenous opioid levels, which may contribute to impaired sensory processing. Overexpression of the estrogen signaling pathway has been confirmed in patients with RLS. RLS is characterized by an uncontrollable and distressing urge to move the legs and other body parts, often accompanied by unpleasant and irritating sensations. Symptoms often appear or worsen in the evening and at night and are relieved by voluntary movement. Currently, the diagnosis of RLS is based on the clinical picture, and therefore criteria have been developed for a more objective diagnosis. Non-pharmacological treatments include sleep hygiene, moderate-intensity exercise, yoga, leg massage, progressive compression devices for the lower extremity muscles, and avoiding factors that aggravate RLS. The development of new medications for the treatment of RLS has been somewhat delayed due to a limited understanding of its pathophysiology. Dopaminergic agonists,  $\alpha 2\delta$  calcium channel ligands, and opioids are used in the treatment of RLS. The disorder is under-recognized by physicians and often remains undiagnosed. RLS is a treatable disease, but the risk of augmentation during drug therapy makes it necessary to develop effective non-drug treatment methods.*

**Keywords:** restless legs syndrome, dopamine,  $\alpha 2\delta$  calcium channel ligands, iron

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## СИНДРОМ БЕСПОКОЙНЫХ НОГ: ОБЗОР ЛИТЕРАТУРЫ

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### РЕЗЮМЕ

Синдром беспокойных ног (СБН) — распространенное сенсомоторное неврологическое расстройство, которое значительно нарушает сон и связан с повышенным риском хронических неинфекционных заболеваний. СБН считается распространенным двигательным расстройством, встречается примерно на 50 % чаще у женщин, а также у лиц европейского происхождения. Этиология заболевания до сих пор не установлена. Патофизиология СБН также до конца не изучена. Дофаминергическая дисфункция является ключевым признаком СБН. С самого открытия заболевания особо выделяли роль дефицита железа, но не его содержание в периферической крови, а именно концентрация в ЦНС. При СБН могут снижаться уровни эндогенных опиоидов, что может способствовать нарушению обработки сенсорных стимулов. У пациентов с СБН подтвердили избыточную экспрессию сигнального пути эстрогена. СБН характеризуется неконтролируемым и мучительным желанием двигать ногами и другими частями тела, и оно часто сопровождается неприятными и раздражающими ощущениями. Симптомы чаще появляются либо усиливаются в вечернее и ночное время, и уменьшаются при произвольных движениях. В настоящее время диагностика СБН основывается на клинической картине, в связи чем были разработаны критерии для более объективной постановки диагноза. К немедикаментозным методам лечения относятся: физические упражнения умеренной интенсивности, йога, массаж мышц ног, устройства для последовательной компрессии мышц нижних конечностей, избегание причин, усугубляющих СБН, гигиена сна. Разработка новых лекарственных препаратов для лечения СБН невозможна из-за ограниченного понимания его патофизиологии. В лечении СБН используются дофаминергические агонисты, лиганды  $\alpha 2\delta$  кальциевых каналов и опиоиды. Врачи недостаточно осведомлены о данном заболевании, оно часто остается не диагностируемым. СБН является курабельным заболеванием, но риск развития аугментации на фоне лекарственной терапии делает необходимой разработку эффективных немедикаментозных методов лечения.

**Ключевые слова:** синдром беспокойных ног, дофамин, лиганды  $\alpha 2\delta$  кальциевых каналов, железо, генетика

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## INTRODUCTION

Restless legs syndrome (RLS) is a common sensorimotor neurological disorder characterized by discomfort in the lower extremities, occurring especially at night. People with RLS often experience an uncontrollable and distressing urge to move their legs in order to relieve the unpleasant sensation [1, 2].

It is a well-known fact that sleep disturbances are linked to an increased risk of cardiovascular diseases, diabetes mellitus, acute cerebrovascular accident, depression, and anxiety. The experience from the Irkutsk Sleep Medicine Center and the Laboratory of Somnology and Neurophysiology at the Scientific Center for Family Health and Human Reproduction Problems has convincingly shown that various sleep disorders significantly reduce quality of life and can lead to premature aging [3–5]. However, among all sleep disorders, RLS remains the least studied pathology, requiring close attention from sleep specialists, neurologists, and geriatricians.

Therefore, RLS constitutes a significant clinical concern and a public health challenge that necessitates enhanced awareness, improved diagnostic approaches, and more effective and accessible treatment modalities to mitigate its widespread impact on patients and the healthcare system.

## AIM

To systematize the current data on the pathophysiology and treatment of RLS published over the past 5 years.

We conducted a review of contemporary Russian and international literature on restless legs syndrome. The following databases were used: eLibrary.ru, Scopus, PubMed, and Google Scholar. A search was performed using the keywords: restless legs syndrome, sleep, ferritin, and dopamine. Selection criteria included publication dates (2021–2026) and relevance to the subject matter of the literature review. Following a thorough verification of source credibility, 33 articles were chosen for citation in this study. Two additional articles from 2015 and 2018 were incorporated due to their practical significance in terms of treatment methods described therein.

This syndrome was first described by English physician Thomas Willis in 1685, but it was not until 1945 that Karl Ekbom defined the clinical characteristics of RLS and established it as a distinct nosological entity [6].

### Epidemiology

RLS is considered the most common movement disorder, affecting up to 10 % of older adults (60–74 years old according to the World Health Organization's classification) and 20 % of those aged 80 years and over. It can also occur in children, although diagnosis in this age group can be challenging and reliable prevalence estimates cannot be reliably estimated. In adults, RLS

occurs approximately 50 % more frequently in women and in individuals of European descent [7].

### Etiology

The etiology of the disease remains unknown [8]. Traditionally, RLS has been divided into primary (idiopathic) and secondary (comorbid) forms [9]. However, recent genetic data have challenged the concept of these two distinct conditions, as RLS may be independent or associated with other medical conditions. RLS may occur independently, most often at a young age, or in combination with comorbid conditions such as cardiovascular diseases, diabetes, or arterial hypertension, making accurate diagnosis challenging [10].

Genetic factors play a significant role in RLS. Twin studies and family aggregation analyses have estimated the heritability of RLS to range between 54 % and 69.4 %. Genome-wide association studies have identified risk alleles for RLS in five specific genomic regions, including MEIS1, BTBD9, PTPRD, MAP2K/SKOR1, and TOX3/BC034767, as well as in an intergenic region on chromosome 2 (rs6747972) [11]. The non-coding region of MEIS1 represents one of the strongest genetic associations, and it is part of a regulatory network involved in iron metabolism. The precise function of this protein in the biological pathway of RLS is yet to be established. This gene is highly expressed in dopaminergic neurons of the substantia nigra, which suggests a role in movement. However, its exact function in dopaminergic cells remains unknown [1].

The most common comorbid conditions that are associated with RLS include uremia, anxiety-depressive disorders, iron deficiency anemia, vitamin B12 and folic acid deficiencies, Parkinson's disease, polyneuropathy, thyroid disorders, rheumatoid arthritis, fibromyalgia, chronic venous insufficiency, and multiple sclerosis [9]. A high prevalence of RLS has been reported in patients with migraines. Pregnancy, particularly during the third trimester, is a physiological condition that is distinctly associated with the development of RLS [11]. A number of antidepressant and antipsychotic medications may potentially induce or exacerbate RLS symptoms.

### Pathogenesis

The pathophysiology of RLS is not fully understood. However, several theories have been proposed. Dopaminergic dysfunction is a key feature of RLS, but it is not simply a lack of dopaminergic substances in the central nervous system (CNS) [12]. Dopamine levels in the cerebrospinal fluid (CSF) of RLS patients do not differ from those of a control group. However, a decrease in the activity of tyrosine hydroxylase, which is involved in dopamine synthesis, and its metabolites orthomethyldopamine and homovanillic acid has been observed.

Dysfunction of the locus coeruleus is associated with RLS, where hyperactivity of the noradrenergic system may contribute to motor restlessness. Stress-induced increased activity of the locus coeruleus may contribute to sleep disturbances. Furthermore, increased noradrenergic activity originating from the locus

coeruleus may exert a regulatory effect on the functional activity of dopaminergic neurons.

Single nucleotide polymorphisms in the gene that encodes dopamine- $\beta$ -hydroxylase may contribute to the development of RLS by altering enzyme activity. This enzyme limits dopamine production by converting it into norepinephrine. It is expressed in noradrenergic cells in the locus coeruleus, postganglionic sympathetic fibers, chromaffin cells in the adrenal medulla, and epinephrine neurons. Dopamine- $\beta$ -hydroxylase is a critical enzyme in the biosynthesis of catecholamines, which are important for neurotransmission in the adrenergic sympathetic nervous system. As a result of single nucleotide polymorphisms in the dopamine- $\beta$ -hydroxylase gene, enzyme activity is altered, leading to reduced dopamine concentrations in the brain [13].

Since the discovery of the disease, particular emphasis has been placed on the significance of iron deficiency, not only in peripheral blood, but also in the CNS. It is known that iron is a co-factor for enzymes involved in the synthesis of dopamine and glutamate, and plays a role in regulating their receptors. Impaired iron transport to critical neurons can lead to subsequent local hypoxia and myelin loss [9]. Brain iron deficiency has been identified in neuropathological samples and on magnetic resonance imaging (MRI), primarily affecting the substantia nigra and to a lesser extent the putamen, caudate nucleus, and thalamus. Patients with normal serum ferritin levels have been found to have decreased ferritin levels in CSF [11]. In the cerebral vessels of RLS patients, elevated levels of hypoxia-inducible factor-1 $\alpha$  in neurons of the substantia nigra have been observed, as well as increased levels of hypoxia-inducible factor-1 $\alpha$  and vascular endothelial growth factor [12]. It is believed that the hypoxic pathway caused by iron deficiency in the brain disrupts dopamine metabolism activating an enzymatic cascade, with possible involvement of nitric oxide and adenosine. This, in turn, induces transcriptional processes that lead to increased dopamine synthesis [11].

Patients with RLS exhibit a reduction in the overall volume of the thalamus and specific thalamic nuclei, such as the inferior pulvinar, ventral posterolateral, and medial ventral nuclei. There have also been changes in the intrinsic network of the thalamus. These thalamic alterations were observed in patients who had not been treated with medication. In patients who were receiving medication, the overall thalamic volume, thalamic nuclei volumes, and intrinsic thalamic network did not differ from healthy individuals. These changes could be related to the pathophysiology of RLS, but a direct causal relationship has not yet been established.

Serum vitamin D levels were significantly lower in RLS patients compared to the control group, both in primary and secondary RLS, and were positively correlated with disease severity. However, vitamin D administration did not improve the condition of RLS patients. Expression of the vitamin D3 receptor protein has been demonstrated in midbrain dopamine neurons and their

target striatal neurons. Vitamin D enhances the expression of tyrosine hydroxylase, a rate-limiting enzyme for dopamine synthesis in brain tissue. This enzyme converts tyrosine into dopamine. The involvement of increased expression of this enzyme in RLS pathogenesis has been previously noted [14].

Although opioids have demonstrated efficacy in treating RLS, the role of the endogenous opioid system in RLS pathophysiology remains understudied [15]. Reduced levels of the endogenous opioid  $\beta$ -endorphin in post-mortem brain tissue from RLS patients, as well as in recent CSF studies, may provide support for the rationale behind using opioids in the treatment of RLS [16].

Excessive estrogen levels also affect dopamine and glutamate signaling. Elevated expression of the estrogen signaling pathway has been observed in patients with RLS. During the third trimester of pregnancy, estrogen levels reach their peak in the plasma, which may contribute to RLS, though the precise underlying mechanisms remain unclear [11].

Since RLS symptoms are most prominent in the evening and nighttime, the relationship between RLS and circadian rhythms has been extensively researched. Melatonin levels have been linked to worsening RLS symptoms [12]. The onset of melatonin secretion coincides with the nocturnal worsening of symptoms in RLS patients, and salivary melatonin levels peak approximately 2 hours prior to the peak intensity of sensory or motor symptoms. Furthermore, melatonin facilitates peripheral vasodilation, leading to a decrease in body temperature, which also correlates with the onset of RLS. However, the administration of exogenous melatonin significantly worsens motor symptoms in RLS patients [17]. Shift work is considered a risk factor for RLS due to forced disruption of natural circadian rhythms [15].

Reduced activity of circadian genes may lead to excessive expression of ferroptosis, causing intracellular dysregulation of iron metabolism, neuronal damage, and dopaminergic dysfunction.

These mechanisms are not independent of each other and may act synergistically: iron deficiency leads to altered dopamine synthesis and oxidative stress, which is further exacerbated by excitatory neurotransmission and immune system activation.

### Clinical Features

RLS is characterized by an uncontrollable and distressing urge to move the legs and other body parts, often accompanied by uncomfortable and irritating sensations. These symptoms typically occur or worsen during the evening and night, and are relieved by voluntary movement [18]. Patients typically describe these sensations as itchiness, tightness, tension, twitches, burning, spasms, pulling, or pulsing, and less frequently as pain [8].

Although RLS primarily affects the lower extremities, it has also been reported in other body regions, such as the mouth, neck, upper limbs, face, abdomen, and genitals [12].

Patients with RLS exhibit shortened sleep, increased sleep fragmentation, and more pronounced respiratory disturbances and limb movements during sleep, compared to healthy individuals.

Pain and sleep arousal symptoms are more prevalent in women, while motor symptoms (both self-reported and detected as periodic limb movements on polysomnography) are more common among men. In contrast, women with RLS tend to experience more severe sleep disturbances and depressive moods, while men with RLS are at a higher risk for comorbid conditions such as hypertension and cardiovascular disease [19].

RLS is more prevalent in patients with other conditions, such as irritable bowel syndrome, chronic fatigue, and chronic pain in the muscles and back. Patients with RLS exhibit increased sensitivity to physical sensations and often experience high levels of anger and anxiety. Individuals with moderate to severe RLS may also experience suicidal thoughts and attempts, which is a factor that should be considered when determining the treatment plan for RLS. These factors, influencing the course of the disease, are often overlooked [20].

Sleep disturbances associated with RLS can lead to daytime consequences, such as low mood, irritability, forgetfulness, difficulty concentrating and also increase the risk of chronic non-communicable diseases [18].

The frequency and severity of RLS symptoms have direct clinical significance for treatment selection. Based on these factors, RLS can be classified into: *intermittent RLS* – symptoms that are bothersome enough to warrant treatment, but occur less than twice a week on average; *chronic RLS* – moderately or severely bothersome symptoms that occur at least twice a week and require daily treatment; *refractory RLS* – symptoms that do not respond to first-line monotherapy due to incomplete or reduced efficacy, intolerable adverse effects, or development of augmentation [12]. Augmentation refers to an iatrogenic worsening of RLS symptoms, characterized by earlier onset in the afternoon compared to before treatment, spread to the upper limbs, and shorter latency to symptom onset during rest periods.

### Diagnosis

Diagnosis of RLS is typically made in primary care settings, but the variability of symptoms and their severity, including at the time of treatment initiation, make RLS a challenging condition to diagnose and treat. This can lead to under- or over-diagnosis, as some of the symptoms are nonspecific and may be indicative of other conditions.

Currently, the diagnosis of RLS is based on the clinical presentation. Therefore, in 1995, diagnostic criteria were developed to provide a more objective basis for diagnosis. These criteria were revised in 2003 and again in 2012 to improve diagnostic specificity. The most recent update, from 2012, includes the following diagnostic criteria for RLS established by the International RLS Study Group (IRLSSG) [21]:

- An urge to move the legs, which is usually accompanied by unpleasant sensations, although this is not always the case.

- Symptoms that begin or become worse during periods of rest or inactivity, such as when lying down or sitting.

- Symptoms that are partially or totally relieved by movement, such as walking or stretching, at least as long as the activity continues.

- Symptoms that occur or worsen only in the evening or at night compared to daytime.

The presence of these symptoms cannot be explained solely by other medical or behavioral conditions (e.g., myalgia, venous stasis, leg edema, arthritis, leg cramps, discomfort in certain positions, habitual foot tapping).

The diagnosis of RLS can be challenging in certain age groups, particularly children and the elderly. In children, it is essential to rely on the child's own description, as they often use expressions such as "want to move" or "need to kick" to describe their urge to move their legs [11].

Imaging techniques are essential tools for diagnosing and comprehending RLS. Brain MRI has revealed a reduction in white matter volume within the corpus callosum, anterior cingulate gyrus, and precentral gyrus, suggesting structural changes such as myelin sheath degradation. MRI data also allow for the quantification of non-heme iron accumulation in the brain using sequences that are sensitive to tissue magnetic susceptibility. These data indicate decreased iron levels in the midbrain, which correlate with RLS severity [9].

Functional MRI has revealed abnormal activation in various areas of the sensorimotor and limbic systems, suggesting that these regions may play a role in the pathogenesis of RLS [22].

Polysomnography (PSG) is the gold standard for the objective assessment of sleep quantity and quality. It provides quantitative sleep parameters that allow clinicians to differentiate between sleep phases and stages. It is not mandatory for RLS diagnosis but can be useful, since detection of increased periodic limb movements during sleep is a supportive criterion for RLS. PSG can also identify significant objective sleep abnormalities, such as prolonged sleep onset latency and an increased arousal index [23]. In women with RLS, there is significantly less N1 sleep and more N3 sleep compared to men. Men are thought to have higher rates of periodic limb movements during sleep, and subsequent microarousals, followed by N1 sleep, while women have more N3 sleep. However, although women had more N3 sleep, they were less satisfied with their sleep than men, indicating that women perceive RLS symptoms as more distressing than men [24].

Laboratory tests to confirm the diagnosis of RLS are even less specific and provide insufficient information. Extensive research is ongoing to identify effective biomarkers for RLS. Twenty-four potential N-glycan biomarkers have been identified, which show significant

differences from a control group [21]. RLS and N-glycans are closely related to immunology and inflammation, but currently there is not enough evidence to support the use of N-glycans for clinical diagnosis of RLS.

## Treatment

### Pharmacological Methods

The development of novel drugs for RLS treatment has been slightly delayed due to a lack of complete understanding of its pathophysiology. Nevertheless, several groups of drugs with distinct modes of action have shown efficacy [16].

RLS treatment should be initiated daily when symptoms become significant, i.e., when they significantly affect quality of life. This is particularly important in chronic cases. For intermittent RLS, treatment may be given episodically [11].

Dopamine agonists have long been considered the first-line treatment for RLS, but their use is limited by adverse effects.

One of the most significant challenges in managing RLS with dopamine agonist therapy is the occurrence of a paradoxical worsening of symptoms. Patients initially respond well to dopamine agonists, but as treatment progresses, symptoms may worsen. This phenomenon, known as augmentation, was first reported by R. Allen and C. Earley in 1996 among patients treated with levodopa. Augmentation manifests itself as an increase in the severity of clinical symptoms, an earlier onset of symptoms (either during the day or in the early evening), involvement of additional body parts, and a reduction in the duration of effect of dopamine agonist medications. Augmentation may also occur with drugs from other classes, although it is less common [9]. The pathophysiology of augmentation is unclear and complex. However, it is believed that overstimulation of dopamine receptors leading to neuroplastic changes is a major contributing factor. Risk factors for augmentation therapy include long duration of disease, a history of smoking, current smoking, high levels of smoking, alexithymia, higher scores on the RLS severity scale, symptoms of impulse control disorders, and higher doses of dopamine agonist medications [25]. Despite the development of augmentation, dopamine agonist medication has been shown to be effective in managing the increases in heart rate and blood pressure that are associated with periodic limb movement disorder during sleep, a condition that can lead to cardiovascular disease. Additionally, women may experience a more acute response to dopamine agonist therapy than men, possibly due to differences in the expression of dopamine receptor genes in the CNS.

However, in order to prevent the development of this undesirable effect (augmentation), it has recently been recommended that pharmacological treatment should begin with the use of  $\alpha 2\delta$  calcium channel ligands, such as pregabalin, gabapentin, and gabapentin enacarbil. These agents are considered to be as effective as dopamine agonists, but they have a significantly lower risk of causing augmentation [6]. The mechanism

of their action is believed to involve reducing glutamate release.  $\alpha 2\delta$  calcium channel ligands have shown promising results in reducing hyperexcitability, hyperactivity, and improving deep sleep in RLS patients. There is evidence suggesting that the rate of visits to emergency departments or hospitalizations due to mental health issues was higher among patients who started treatment with gabapentinoids compared to those who began treatment with dopaminergic medications; however, there was no statistically significant increase in risk associated with the use of gabapentinoids [26].

Opioid analgesics can be effective in treating RLS, but they should be used with caution due to their potential risk of dependency. It is recommended to start with opioid drugs with lower potency and then gradually increase the dosage if necessary [23].

In the case of refractory RLS, a sequential monotherapy approach using different drug classes (e.g.,  $\alpha 2\delta$  calcium channel ligands, dopaminergic agents, opioids, etc.) should be considered. In cases where augmentation is required, it may be beneficial to reduce the dose and/or completely withdraw dopaminergic medications, although this may be difficult to achieve without the addition of "rescue" or transitional therapies using drugs from other classes, such as  $\alpha 2\delta$  calcium channel ligands or opioids.

### Non-Pharmacological Methods

Non-pharmacological treatment methods include: moderate-intensity physical activity, yoga, lower limb muscle massage, sequential compression devices for the lower limb muscles, peripheral peroneal nerve stimulation, transcranial magnetic stimulation, and transcranial direct current stimulation. Additionally, it is recommended to avoid factors that may exacerbate RLS, such as alcohol, caffeine, nicotine, and certain antidepressants. Sleep hygiene should also be maintained [27–30].

Physical exercise is a promising non-pharmacological treatment for RLS, but high-intensity exercise may in some cases worsen the condition. There is variability in the individual response to exercise, with some people experiencing symptom improvement and others experiencing worsening after exercise. In studies conducted, participants noted that morning exercise generally alleviated symptoms, whereas evening physical activity tended to exacerbate them. In mild cases of RLS, people with secondary forms and those with a lower body mass index appear to find physical exercise more effective for relief [2].

Over the years, several non-invasive devices have been developed, including those that provide vibrational or compressive stimulation to the legs. However, none of these devices have yet been proven effective in a randomized, placebo-controlled trial [31].

Peripheral peroneal nerve stimulation is a non-pharmacological treatment for moderate-to-severe RLS that does not respond to drug therapy. It involves the use of a bilateral wearable device that applies high-frequency electrical stimulation to the peroneal nerve.

This stimulation activates a therapeutic mechanism, reducing discomfort during sleep and improving sleep quality. This treatment method has been shown to reduce RLS symptoms as an adjunct to other treatments or as a monotherapy [28].

Transcranial magnetic stimulation is a technique that alters cortical excitability and has been suggested as an effective treatment for conditions related to hyper- or hypo-excitability in the brain. This technique can significantly alleviate motor symptoms, sleep disturbances, and anxiety in patients with RLS [29].

Superficial application of heat and cold involves exposing the skin and underlying tissues to different temperatures in order to alter their temperature. This therapeutic method is based on superficial techniques that use conduction or convection mechanisms. The application of heat improves blood circulation by promoting the delivery of nutrients and oxygen to cells. Conversely, cold application enhances the production of white blood cells, reduces the levels of histamine, and strengthens the body's immune response. There is some evidence to suggest that this technique also has antioxidant effects and influences plasma norepinephrine levels, which may lead to increased neurotransmitter activity [32].

Massage has been shown to be beneficial for patients' well-being, both physically and psychologically. On the one hand, it can relieve tension in the skin and muscles, improving blood circulation and boosting the immune system. This, in turn, can lead to a sense of relaxation and contentment. However, despite the common use of massage therapy in patients with RLS, its effectiveness in treating this condition remains a topic of debate [33].

Patients with RLS may require iron replacement therapy, either through increased intake of iron-rich foods or initiation of oral or intravenous iron therapy, if blood ferritin levels are below 75 ng/mL or transferrin saturation is < 20 %. Moreover, studies conducted on RLS patients have shown that intravenous iron therapy is effective for RLS patients who do not have iron overload, with serum ferritin levels ranging from 100–300 µg/L. These data challenge current recommendations and suggest that intravenous iron administration could be considered for a wider range of RLS patients [15].

Several treatment strategies may need to be implemented simultaneously, including pharmacogenomic testing, strategies to improve treatment adherence, optimization of drug therapy, cognitive-behavioral therapy for insomnia, and management of concurrent chronic conditions [12].

## CONCLUSION

Restless legs syndrome is a common neurological disorder that significantly affects quality of life and is a complex condition that remains inadequately understood from a therapeutic standpoint.

The significance of timely diagnosis and effective management is emphasized by studies examining the associations between RLS and somatic and psychological disorders.

At present, there are no established laboratory or instrumental diagnostic methods that can confirm a diagnosis of RLS. Diagnosis is currently based solely on clinical criteria, such as patient complaints and medical history, which may be insufficient, especially when dealing with pediatric and geriatric patients.

Due to the contraindications of most RLS medications during pregnancy and breastfeeding, further research into non-pharmacological treatments is needed.

Available pharmacological treatment options for RLS appear to be insufficient, and there is a need for further research into the pathophysiology of RLS in order to develop more effective therapies. Additionally, increased public awareness about RLS is also necessary.

Reducing RLS symptoms through medication and creating a calm sleeping environment meets psychological needs. Controlling RLS symptoms, engaging in activities, having trust in treatment, and receiving social support contribute to a high quality of life. A holistic and patient-centered approach that addresses physiological, psychological, and social aspects can help healthcare professionals provide comprehensive care for patients with RLS.

Analysis of the literature over recent years suggests that RLS is a manageable condition, but the risk of augmentation during drug therapy necessitates the development of effective non-pharmacological treatment methods.

## Conflicts of interest

The authors declare no conflicts of interest.

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## ONCOLOGY

### RATIONALE FOR THE USE OF LOW-INTENSITY PHOTODYNAMIC THERAPY IN STAGE I BASAL CELL SKIN CARCINOMA

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#### ABSTRACT

**Background.** The paper describes a method of low intensity photodynamic therapy (LIPDT) for the treatment of superficial basal cell skin cancer (SBCSC) stage I using topical application of the photosensitizer Photoditazine gel<sup>®</sup> or intravenous Photoditazine<sup>®</sup> solution. LIPDT is the treatment of choice in medical organizations lacking laser equipment, specialized facilities, or for remote use (in distant areas). It can also be used as an adjunct to other treatment methods.

**Aim.** To provide a theoretical rationale for the use of a LIPDT device with Photoditazine<sup>®</sup> and Photoditazine gel<sup>®</sup> for the treatment of SBCSC.

**Materials and methods.** The developed device consists of the following elements: a battery, a control board, a DC-DC voltage converter, a microcontroller, a driver, and an LED matrix. The mechanism of the device is based on the classical PDT method but has several key differences: automatic mode (eliminating the need for manual adjustment), low output radiation power (which prevents oxygen depletion in tumor tissue), and the ability to function under conditions unsuitable for standard PDT.

**Conclusion.** The proposed LIPDT method for treating stage I SBCSC will reduce treatment costs while maintaining the effectiveness of the existing standard PDT method.

**Key words:** basal cell skin cancer (BCSC), low-intensity phototherapy therapy (LIPDT), photosensitizer (PS), Photoditazine<sup>®</sup>, Photoditazine-gel<sup>®</sup>

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## ОБОСНОВАНИЕ ПРИМЕНЕНИЯ СПОСОБА НИЗКОИНТЕНСИВНОЙ ФОТОДИНАМИЧЕСКОЙ ТЕРАПИИ ПРИ БАЗАЛЬНОКЛЕТОЧНОМ РАКЕ КОЖИ I СТАДИИ

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### РЕЗЮМЕ

**Обоснование.** В работе представлено описание методики проведения низкоинтенсивной фотодинамической терапии (НФДТ). При лечении поверхностного базальноклеточного рака кожи (БКРК) I стадии по системе AJCC 8-го пересмотра (2017 г.) оценен результат наружного применения препарата «Фотодитазин-гель»<sup>®</sup> или раствора «Фотодитазин»<sup>®</sup> для внутривенного введения. НФДТ является выбором метода лечения в условиях медицинских организаций, не имеющих лазерного оснащения, специализированного помещения или возможности дистанционного контроля лечения (на отдаленных территориях). Может быть применен в дополнение к другим способам лечения.

**Цель.** Теоретически обосновать применение аппарата НФДТ с препаратами «Фотодитазин»<sup>®</sup> и «Фотодитазин-гель»<sup>®</sup> для лечения поверхностного БКРК.

**Материалы и методы.** Разработанный аппарат состоит из следующих элементов: аккумулятор, плата контроля, DC-DC преобразователь напряжения, микроконтроллер, драйвер и светодиодная матрица. Механизм работы аппарата базируется на классическом методе фотодинамической терапии (ФДТ), имея при этом ключевые отличия: автоматический режим (без необходимости ручной настройки), низкую выходную мощность излучения (благодаря чему не происходит истощение кислорода в опухолевой ткани), возможность работы в условиях, не приспособленных для стандартной ФДТ.

**Заключение.** Способ лечения НФДТ позволит снизить стоимость лечения поверхностного БКРК I стадии, сохранив при этом эффективность уже существующего стандартного метода ФДТ.

**Ключевые слова:** базальноклеточный рак кожи (БКРК), низкоинтенсивная фотодинамическая терапия (НФДТ), фотосенсибилизатор (ФС), препараты «Фотодитазин»<sup>®</sup>, «Фотодитазин-гель»<sup>®</sup>

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## INTRODUCTION

In 2023, a total of 76,081 patients with skin malignancies (excluding melanoma) were registered in the Russian Federation with a first-ever diagnosis of a malignant neoplasm, thereby ranking first in cancer detectability. Malignant neoplasms of the breast ranked second (74,008 patients). Between 2013 and 2023, the prevalence of skin malignancies increased by 18.3 % [1]. Photodynamic therapy (PDT) is successfully used to treat various precancerous conditions and malignant neoplasms [2–4].

However, research continues to identify the optimal treatment modality to achieve complete tumor eradication. Reducing treatment intensity is also of great importance, given that pain remains the most common adverse effect [5].

## AIM

To provide a theoretical rationale for the use of a LIPDT device with the photosensitizer “Photoditazine”® and “Photoditazine-gel”® for the treatment of superficial basal cell skin carcinoma (SBCSC), which will reduce treatment costs, simplify the procedure, while maintaining the effectiveness of photodynamic therapy.

## MATERIALS AND METHODS

The developed device consists of the following components: a battery, a control board, a DC-DC voltage converter, a microcontroller, a driver, and an LED matrix. The operating principle of the device is based on the classical PDT method but has several key differences: an automatic mode (eliminating the need for manual adjustment), low output radiation power (which prevents oxygen depletion in tumor tissue), and the ability to function under conditions unsuitable for standard PDT.

The difference between LIPDT and conventional laser PDT lies in the fact that LIPDT uses an LED emitter based on a single LED or an LED matrix consisting of several LEDs, powered autonomously by a lithium-ion battery (power bank).

The LIPDT method for patients with malignant neoplasms is performed by applying “Photoditazine-gel”® or by intravenous administration of “Photoditazine”®, followed by exposure of the tumor growth zone to light radiation at a wavelength within the photosensitizer absorption range, with an energy fluence of 300 to 600 J/cm<sup>2</sup>. The irradiated field area is selected to match the tumor area, extending 0.5 mm beyond the clinically evident tumor margin.

In practice, “Photoditazine-gel”®, a light penetrator, was used at a concentration of 0.5 % and a volume of 1 mL. This preparation is intended to enhance the penetrating effect of the light energy source used in phototherapy, including photodynamic therapy, under various light exposure conditions. The gel was applied topically to the skin

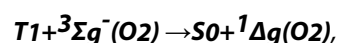
topically 30 minutes prior to light exposure, using 1 mL of gel for every 3–5 cm<sup>2</sup> of the treated surface. After 30 minutes, the applied gel was removed with a cotton swab moistened with distilled water, followed by light exposure.

The irradiation session is performed using LED emitters that provide a light flux in the 650 nm wavelength range.

LIPDT is performed using an LED emitter with a radiation wavelength of 650 nm, powered autonomously by a portable power bank. The size of the LED emitter is selected according to the irradiated field area, matching the tumor area circumferentially, extending 5 mm beyond the clinically evident tumor margin. The LED emitter (matrix) is fixed to the tumor surface using standard fixation methods (adhesive tape, securing bandage), allowing the patient to move freely and perform self-care during the session.

## RESULTS AND DISCUSSION

The developed LIPDT method is based on the photodynamic effect on malignant cells following the topical application or systemic administration of photosensitizers (PS), which are substances that increase the sensitivity of tumor tissues to light. The LIPDT procedure can be divided into the following stages: administration of the PS into the body; preferential accumulation of the PS in the tumor area; excitation of the PS at the target area by LIPDT, resulting in the formation of reactive compounds; cell death; tissue destruction; elimination of the PS; and wound healing. The preferential accumulation of the PS in the tumor is a specific property of the photosensitizers used and is based on differences in oxidative processes between cancer cells and healthy tissues. Excitation of the PS occurs when the tumor area containing the accumulated PS is irradiated with light at a wavelength corresponding to the optical absorption band of the PS. The excitation energy of the PS is transferred to oxygen molecules present in the tumor cells, converting them from the ground triplet quantum state to the singlet state, resulting in the formation of reactive oxygen species (ROS). Singlet oxygen (<sup>1</sup>O<sub>2</sub>) is highly reactive and oxidizes cell molecules, leading to cell death and necrosis of tumor tissue. This constitutes the essence of both PDT and LIPDT. The photodynamic therapy process can be described by the following reaction:



where *T1* – PS in the excited triplet state, into which it transitions as a result of light exposure within the absorption band; <sup>3</sup>Σ<sub>g</sub><sup>−</sup>(O<sub>2</sub>) – molecular oxygen in the ground triplet state in the cells of the tumor tissue; *S0* – PS in the ground singlet state, into which it transitions after interacting with oxygen; <sup>1</sup>Δ<sub>g</sub>(O<sub>2</sub>) – molecular oxygen in the excited active singlet state after interacting with the PS.

Singlet oxygen <sup>1</sup>Δ<sub>g</sub>(O<sub>2</sub>), having paired electrons on one orbital and a vacant second orbital, readily interacts

with electron-rich molecules [6]. The photosensitizer itself causes damage to specific parts of cells, inducing apoptosis, necroptosis, autophagy, and paraptosis, which can occur as a result of damage to mitochondria, lysosomes, and the endoplasmic reticulum [7]. An important factor for overall treatment effectiveness is the balance between these processes [8].

Under intense light irradiation, the primary PDT effect occurs at the onset of laser exposure, when the maximum number of oxygen molecules participates in the generation of singlet states. Subsequently, both the intensive consumption of oxygen in its active forms and the slow diffusion of free oxygen into the photoactive zone inevitably lead to a decrease in oxygen concentration around the photosensitizing molecules. This diminishes the photodynamic effect and, consequently, reduces treatment effectiveness. Studies conducted in several works, such as [4], have confirmed that singlet oxygen generation during PDT can deplete photochemical oxygen within the tumor, resulting in local hypoxia and reduced treatment effectiveness.

The rate of oxygen supply to the tissue after its consumption during the photodynamic reaction is relatively low. In practice, the time constant for tissue reoxygenation varies across different tissues, ranging approximately from two to eight seconds [9].

In this regard, solving the problem of tissue deoxygenation within the irradiated area is possible through reducing the power density (irradiance) of the light exposure, while simultaneously extending the exposure time to achieve the required total energy fluence. Addressing the issue of tissue deoxygenation during PDT in the irradiated area may be achieved at deliberately low light intensity levels, at which the replenishment of the tissue oxygen content is maintained at an adequate level due to the low rate of its conversion and the extended time required for its transition to the active singlet state.

The LIPDT method simplifies the PDT procedure, reduces the risk of thermal injury to the treated area, and eliminates the risk of damage to the optical fiber cable used for laser PDT. Ongoing physician monitoring of the current state of the treated area allows the therapy to be administered on an outpatient basis using a portable power supply, as well as remote monitoring. Unlike PDT using laser radiation sources, this method does not require specialized rooms (with special wall coverings to prevent light reflection) or eyewear with optical filters for protection.

Due to the low radiation intensity of LEDs compared to lasers, LIPDT requires a longer treatment interval following photosensitizer application. However, the use of LIPDT reduces the risk of thermal injury to surrounding tissues.

The rate and extent of preferential PS accumulation in tumors compared to surrounding tissues can be 3–10 times higher, depending on the tumor type and the PS employed.

Second-generation photosensitizers, commonly used in medicine, such as "Photoditazine"<sup>®</sup> for intravenous

administration and "Photoditazine-gel"<sup>®</sup> for topical application, developed by Russian companies, belong to the chlorin family. "Photoditazine"<sup>®</sup> is the di-N-methylglucamine salt of chlorin E6 and a derivative of chlorophyll *a* [10]. "Photoditazine"<sup>®</sup> is produced from the biomass of the microalgae *Spirulina platensis*, which is used to extract chlorin E6.

"Photoditazine"<sup>®</sup> exhibits high photodynamic activity for singlet oxygen generation and has several advantages compared to other second-generation photosensitizers. These advantages make it a preferred choice for PDT, as they help to minimize damaging effects on healthy tissues and organs, as well as skin damage caused by light exposure [11].

The LED emitter power and irradiation time are set to provide a total energy fluence of 300–600 J/cm<sup>2</sup>, depending on the specific requirements. Figure 1 shows the spectral correlation between the LED emission and "Photoditazine"<sup>®</sup>.

A key parameter for calculating the light dose is the energy fluence, which is the total light energy delivered to a unit area of irradiated pathological tissue in the tumor area.

The irradiation dose is calculated using the following formula:

$$E = P \cdot t, \quad (1)$$

where **E** – total irradiation energy (J), **P** – output power of the LED emitter (W), **t** – irradiation time (exposure), seconds.

To calculate the energy fluence (**Q**), the total irradiation energy must be divided by the area (**S**) of the light spot on the irradiated surface

$$Q = E / S = P \cdot t / S. \quad (2)$$

Accordingly, the following formula can be used to calculate the irradiation time (**t** in seconds):

$$t = Q \cdot S / P, \quad (3)$$

where **Q** – energy fluence (dose), J/cm<sup>2</sup>, **S** – area of the light spot on the irradiated surface, cm<sup>2</sup>, **P** – output power of the LED emitter, W.

Table 1 shows the correlation between the irradiation dose of light, the irradiation time, and the emitter power, assuming that the light spot area matches the tumor area. To determine the key parameter, the energy fluence, the total irradiation energy (**E** in J) from the table must be divided by the surface area of the irradiated tissue in the tumor area.

If the size of the light spot does not match the tumor size, two scenarios are possible: the tumor is smaller than the light spot size, or the tumor is larger than the light spot size.

When the tumor size is smaller than the light spot size, the irradiation dose delivered to the tumor must be calculated using the following formula:

$$E_{\text{tumor}} = E_{\text{total}} * S_{\text{light}} / S_{\text{tumor}} \quad (4)$$

where  $E_{\text{tumor}}$  – irradiation dose received by a particular area of the tumor,  $E_{\text{total}}$  – total irradiation dose from the table,  $S_{\text{light}}$  – light spot area,  $S_{\text{tumor}}$  – tumor area.

In this case, the energy fluence (dose density)  $Q$  can be calculated using the following formula:

$$Q = E_{\text{tumor}} / S_{\text{tumor}} \quad (5)$$

or:

$$Q = E_{\text{total}} / S_{\text{light}} \quad (6)$$

If the light spot size is smaller than the tumor size, the dose can only be calculated for the part of the tumor that has been irradiated, using Formula 6. The light spot can be moved across the tumor for subsequent irradiation

sessions, resulting in the summation of irradiation doses:  $E_{\text{total}} = E_{\text{total1}} + E_{\text{total2}}$ . In this case, the average dose per unit area can be calculated as  $E_{\text{total}} / S_{\text{light}} = (E_{\text{total1}} + E_{\text{total2}}) / (S_{\text{light1}} + S_{\text{light2}})$ .

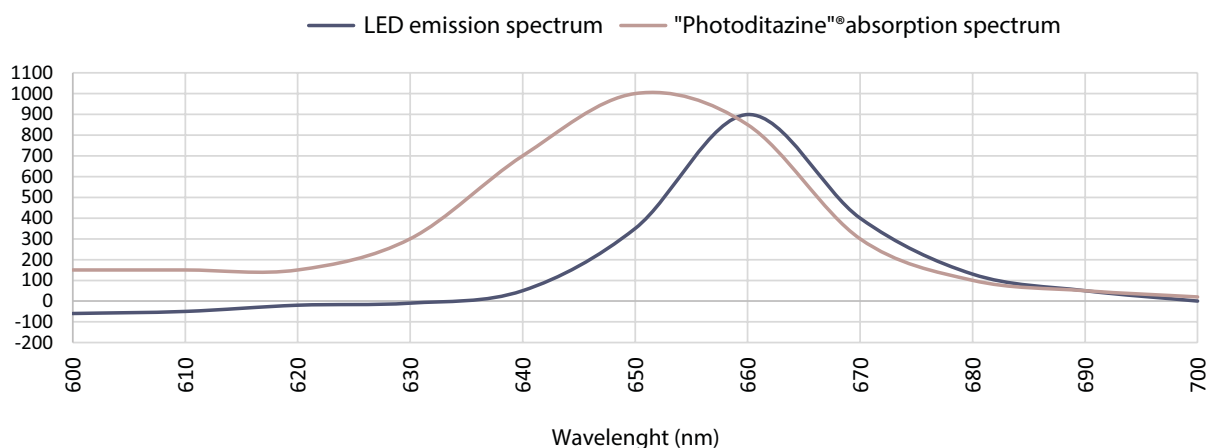
**Example 1.** For a tumor size of up to 1 cm ( $E = 200$  J/cm<sup>2</sup>) and an LED power of 0.05 W, the power density ( $P$ ) is calculated as follows:

$$0.05 \text{ W} / 0.78 \text{ cm}^2 = 0.064 \text{ W/cm}^2$$

$$\text{W/cm}^2 = 3125 \text{ sec. or } 52 \text{ min.}$$

Calculation of the LIPDT procedure time ( $t$ ) as a function of the diameter ( $D$ ) and area ( $S$ ) of the light spot at a given energy fluence ( $Q$ ) and an LED power of 0.07 W (Table 2).

The active ingredient in "Photoditazine"<sup>®</sup> (chlorin E6 dimeglumine) has a selective accumulation in tumor cells. When the tumor area is subsequently irradiated with monochromatic light at a wavelength of 650–670 nm,



**FIG. 1.**  
Spectral correlation between the LED emission spectrum and the "Photoditazine"<sup>®</sup> absorption spectrum

**TABLE 1**

**DEPENDENCE OF THE IRRADIATION DOSE ON EXPOSURE TIME AND EMITTER POWER WHEN THE LIGHT SPOT AREA MATCHES THE TUMOR AREA**

| Power, mW → |         | 5                   | 10  | 20  | 30  | 40  | 50  | 60  | 70  | 80  | 90  | 100 |
|-------------|---------|---------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| t, sec. ↓   | t, min. | Irradiation dose, J |     |     |     |     |     |     |     |     |     |     |
| 60          | 1       | 0.3                 | 0.6 | 1.2 | 1.8 | 2.4 | 3   | 3.6 | 4.2 | 4.8 | 5.4 | 6   |
| 300         | 5       | 1.5                 | 3   | 6   | 9   | 12  | 15  | 18  | 21  | 24  | 27  | 30  |
| 600         | 10      | 3                   | 6   | 12  | 18  | 24  | 30  | 36  | 42  | 48  | 54  | 60  |
| 900         | 15      | 4.5                 | 9   | 18  | 27  | 36  | 45  | 54  | 63  | 72  | 81  | 90  |
| 1200        | 20      | 6                   | 12  | 24  | 36  | 48  | 60  | 72  | 84  | 96  | 108 | 120 |
| 1500        | 25      | 7.5                 | 15  | 30  | 45  | 60  | 75  | 90  | 105 | 120 | 135 | 150 |
| 1800        | 30      | 9                   | 18  | 36  | 54  | 72  | 90  | 108 | 126 | 144 | 162 | 180 |
| 2100        | 35      | 10.5                | 21  | 42  | 63  | 84  | 105 | 126 | 147 | 168 | 189 | 210 |
| 2400        | 40      | 12                  | 24  | 48  | 72  | 96  | 120 | 144 | 168 | 192 | 216 | 240 |

a photosensitization effect occurs. This effect involves the generation of reactive oxygen species in the tumor tissue, including singlet oxygen and other free radicals. These species cause structural and functional changes to tumor cells, ultimately leading to their death.

The duration of the treatment after the application of the photosensitizer is determined by the calculated dose (Table 2).

The light-emitting device based on an LED matrix and power supply is schematically illustrated in Figure 2. A lithium-ion battery (1) is used to power the device. A battery charge/discharge controller (2) is used for charging and monitoring the battery. A DC-DC voltage converter (3) increases and maintains the voltage from the battery at a constant level. A microcontroller (4) generates a PWM signal, which is sent to a driver (5) to regulate the operation of the emitter (LED matrix) (8). The emitter operating mode is set using control buttons (7) and the status is displayed on the display (6).

The light-emitting matrix, comprising at least four LEDs, is mounted on a heat sink and enclosed within a plastic casing (Fig. 3). The casing has lugs for securing the emitter to the patient's body via elastic straps

or adhesive tape. On the external side of the casing, there are openings for heat dissipation from the heat sink and an opening for the LED power cable.

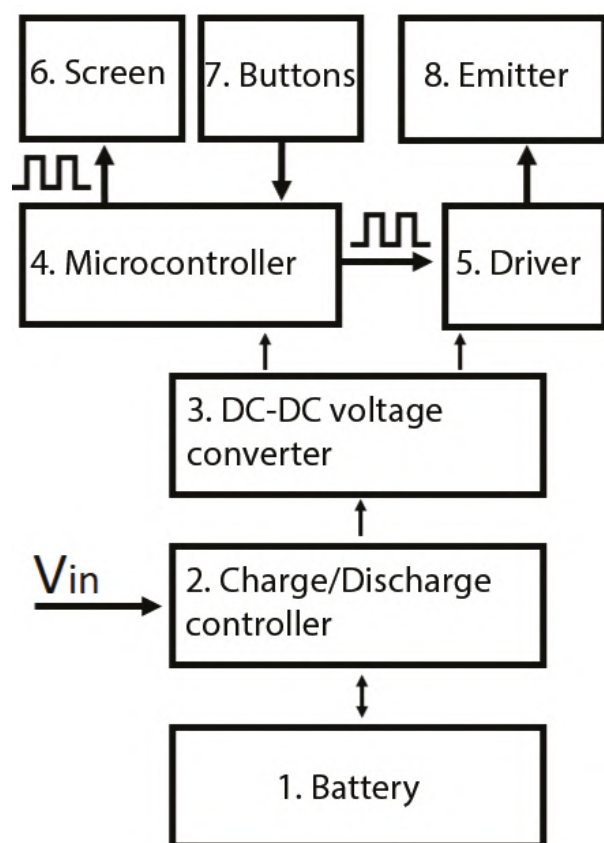
The light-emitting matrix is connected to a power supply and control unit through a flexible cable. The external view of the unit is shown in Figure 4. The driver stabilizes the current through the LEDs and, under control of the microcontroller, delivers current pulses to the LEDs at a specified frequency. The microcontroller controls the LED emission modes by sending control signals to the driver using pulse-width modulation (PWM), enabling the LEDs to emit in a pulsed mode with adjustable frequency. This allows treatment procedures to be performed in an optimal mode, reducing the thermal load on the irradiated area and allowing the selection of irradiation frequency and power for affected areas according to the most effective therapeutic protocol.

The power supply unit has control buttons for setting the emission mode, and parameters displayed on an integrated screen (Fig. 4).

Therefore, the proposed device allows for the treatment of skin neoplasms using the LIPDT method, resulting in good cosmetic outcomes.

**TABLE 2**  
**CALCULATION OF THE LIPDT PROCEDURE TIME (t)**

| Energy fluence, J/cm <sup>2</sup> | 100                    | 200 | 300 | 400 | 500 | 600 |
|-----------------------------------|------------------------|-----|-----|-----|-----|-----|
| Light spot diameter, cm           | Irradiation time, min. |     |     |     |     |     |
| 0.5                               | 7                      | 13  | 20  | 26  | 33  | 39  |
| 0.6                               | 9                      | 19  | 28  | 38  | 47  | 57  |
| 0.7                               | 13                     | 26  | 38  | 51  | 64  | 77  |
| 0.8                               | 17                     | 33  | 50  | 67  | 84  | 100 |
| 0.9                               | 21                     | 42  | 64  | 85  | 106 | 127 |
| 1.0                               | 26                     | 52  | 79  | 105 | 131 | 157 |
| 1.1                               | 32                     | 63  | 95  | 127 | 158 | 190 |
| 1.2                               | 38                     | 75  | 113 | 151 | 188 | 226 |
| 1.3                               | 44                     | 88  | 133 | 177 | 221 | 265 |
| 1.4                               | 51                     | 103 | 154 | 205 | 256 | 308 |
| 1.5                               | 59                     | 118 | 177 | 236 | 294 | 353 |
| 1.6                               | 67                     | 134 | 201 | 268 | 335 | 402 |
| 1.7                               | 76                     | 151 | 227 | 302 | 378 | 454 |
| 1.8                               | 85                     | 170 | 254 | 339 | 424 | 509 |
| 1.9                               | 94                     | 189 | 283 | 378 | 472 | 567 |
| 2.0                               | 105                    | 209 | 314 | 419 | 523 | 628 |



**FIG. 2.**  
Block diagram of a light-emitting device based on an LED matrix and a power supply

## CONCLUSION

The developed new treatment method (LIPDT) will reduce the cost of treatment for patients with stage I BCSC by simplifying the procedure while maintaining comparable effectiveness to conventional methods. This method does not require the use of specialized rooms or reflective-coated walls.

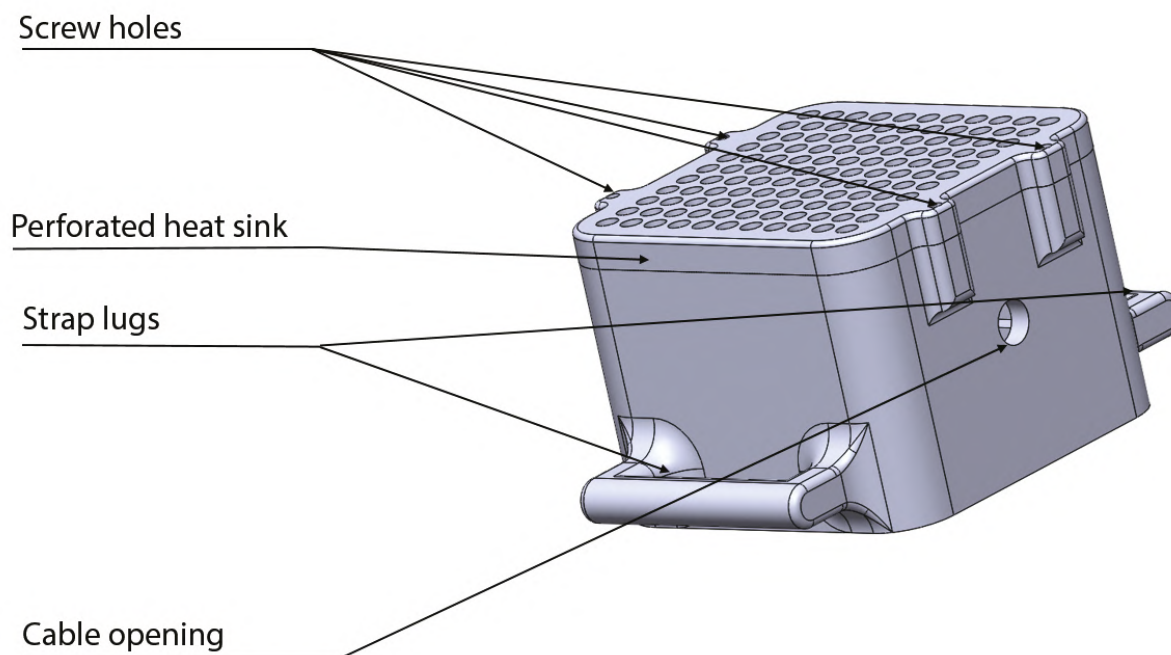
In medical organizations that lack laser equipment, LIPDT can serve as the treatment of choice. It can also be used in conjunction with PDT for cases of multiple primary tumors, as well as for remote monitoring in hospice settings. Simultaneous application of multiple LEDs can be utilized with this method.

## Conflicts of interest

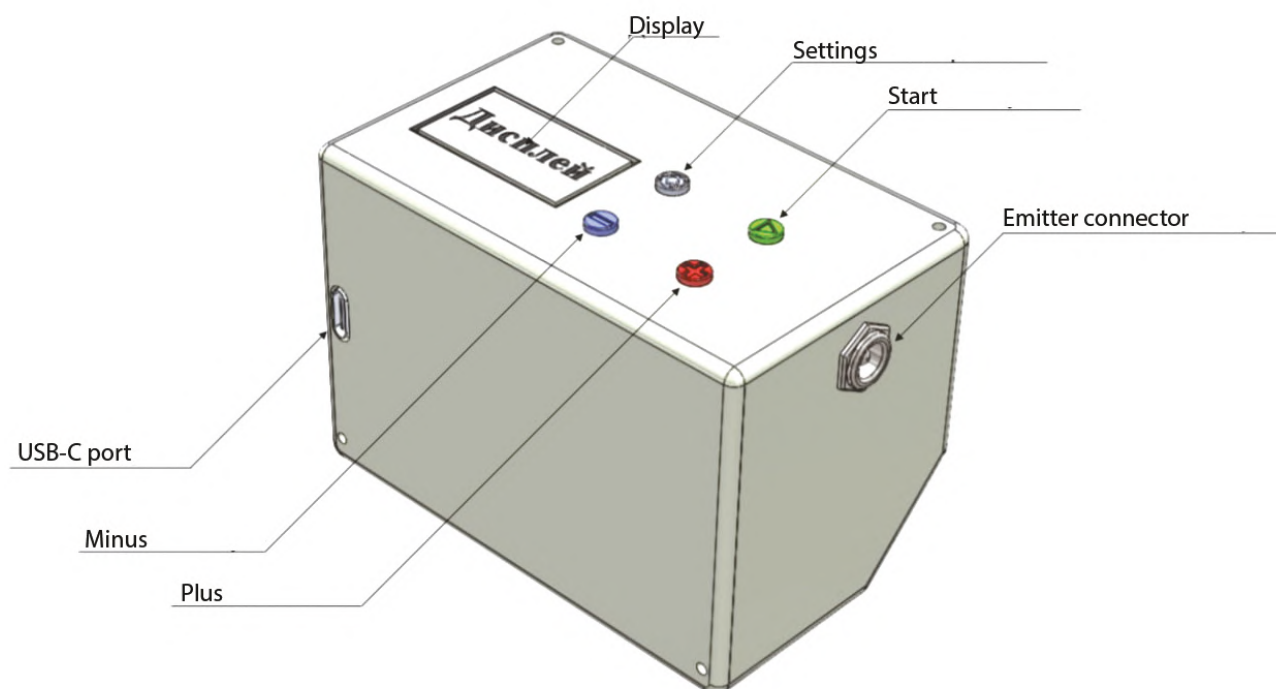
The authors declare no conflicts of interest.

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**FIG. 3.**  
Light-emitting matrix based on LEDs for LIPDT



**FIG. 4.**  
Power supply and control unit for the LED-based emitter for LIPDT

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## PEDIATRICS

### LONGITUDINAL CHANGES IN SERUM INTESTINAL FATTY ACID-BINDING PROTEIN AND CLAUDIN-2 LEVELS IN CHILDREN DURING THE FIRST 24 MONTHS OF LIFE: A PROSPECTIVE OBSERVATIONAL COHORT STUDY

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#### ABSTRACT

**Background.** Maturation of the intestinal epithelial barrier in early life is accompanied by variability in biomarkers of enterocyte injury and permeability. However, longitudinal serum profiles of I-FABP and claudin-2 in seemingly healthy children, including those born preterm, remain insufficiently studied.

**Aim.** To assess the dynamics of serum I-FABP and claudin-2 concentrations in children born at 34–41 weeks' gestation during the first 24 months of life, with consideration of gestational age.

**Methods.** This prospective observational cohort study included three time points: up to 7 days; 1–1.5 months/postmenstrual age (PMA) 40–41 weeks; and 18–24 months. At time points 1–2, 98 newborns were enrolled; by time point 3, follow-up was completed in 88 children: preterm (34–36 weeks,  $n = 30$ ) and term (37–41 weeks,  $n = 58$ ). Loss to follow-up was 10.2 % ( $n = 10$ ). I-FABP and claudin-2 were measured by ELISA.

**Results.** I-FABP remained stable during the first 1–1.5 months and increased by the second year of life: in preterm children 1.12 [1.07; 1.30] – 1.30 [0.97; 1.87] – 6.09 [3.18; 6.65] ng/mL ( $p < 0.001$ ), and in term children 1.10 [0.80; 1.95] – 1.21 [0.92; 1.73] – 3.89 [2.33; 6.10] ng/mL ( $p < 0.001$ ); no between-group differences were observed at the respective time points ( $p = 0.738$ ; 0.610; 0.131). In preterm children, claudin-2 did not change significantly: 6.76 [3.00; 9.14] – 7.59 [5.20; 10.96] – 7.77 [6.33; 9.20] ng/mL ( $p = 0.497$ ). In term children, claudin-2 increased: 1.85 [1.02; 5.22] – 5.10 [3.47; 8.31] – 7.95 [6.32; 11.28] ng/mL ( $p < 0.001$ ). At time points 1–2, claudin-2 values were higher in preterm than in term children ( $p = 0.006$ ;  $p = 0.009$ ), whereas at time point 3 they were comparable ( $p = 0.326$ ).

**Conclusions.** Within the 34–41-week gestational range, gestational age does not determine I-FABP levels, whereas claudin-2 is gestational age-dependent. Interpretation of these biomarkers requires consideration of both chronological age and gestational age.

**Keywords:** intestinal permeability, preterm infants, I-FABP, claudin-2, intestinal epithelial barrier, early childhood

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# ДИНАМИКА ИНТЕСТИНАЛЬНОГО БЕЛКА, СВЯЗЫВАЮЩЕГО ЖИРНЫЕ КИСЛОТЫ И КЛАУДИН-2 В СЫВОРОТКЕ КРОВИ У ДЕТЕЙ В ПЕРВЫЕ 24 МЕСЯЦА ЖИЗНИ: ПРОСПЕКТИВНОЕ ОБСЕРВАЦИОННОЕ КОГОРТНОЕ ИССЛЕДОВАНИЕ

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## РЕЗЮМЕ

**Обоснование.** Созревание кишечного эпителиального барьера в раннем возрасте сопровождается вариабельностью маркеров повреждения и проницаемости энтероцитов; продольные профили сывороточных I-FABP (Intestinal fatty acid-binding protein) и клаудин-2 у условно здоровых детей, включая поздних недоношенных, изучены недостаточно.

**Цель.** Оценить динамику концентраций I-FABP и клаудин-2 в сыворотке крови у детей, рождённых на сроке 34–41 недели гестации, в первые 24 месяца жизни с учётом гестационного возраста.

**Методы.** Проспективное обсервационное когортное исследование (3 этапа: до 7 суток; 1–1,5 месяца/ПМВ 40–41 нед.; 18–24 месяца). На 1–2 этапах включено 98 новорождённых; к 3 этапу завершили наблюдение 88 детей: недоношенные 34–36 нед. ( $n = 30$ ) и доношенные 37–41 нед. ( $n = 58$ ); потери 10,2 % ( $n = 10$ ). I-FABP и клаудин-2 определяли методом ИФА.

**Результаты.** I-FABP был стабильным в первые 1–1,5 месяца и увеличивался ко 2-му году жизни: у недоношенных 1,12 [1,07;1,30] – 1,30 [0,97;1,87] – 6,09 [3,18;6,65] нг/мл ( $p < 0,001$ ), у доношенных 1,10 [0,80;1,95] – 1,21 [0,92;1,73] – 3,89 [2,33;6,10] нг/мл ( $p < 0,001$ ); межгрупповых различий не выявлено ( $p = 0,738$ ; 0,610; 0,131). Для клаудин-2 у недоношенных изменений не отмечено: 6,76 [3,00;9,14] – 7,59 [5,20;10,96] – 7,77 [6,33;9,20] нг/мл ( $p = 0,497$ ). У доношенных клаудин-2 возрастал: 1,85 [1,02;5,22] – 5,10 [3,47;8,31] – 7,95 [6,32;11,28] нг/мл ( $p < 0,001$ ); на 1–2 этапах значения были выше у недоношенных ( $p = 0,006$ ;  $p = 0,0009$ ), на 3-й — сопоставимы ( $p = 0,326$ ).

**Заключение.** В диапазоне 34–41 недели гестации гестационный возраст не определяет уровень I-FABP, тогда как клаудин-2 зависит от гестационного возраста; интерпретация маркеров требует учёта возраста и срока гестации.

**Ключевые слова:** кишечная проницаемость, недоношенные, I-FABP, клаудин-2, кишечный эпителиальный барьер, ранний возраст

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## BACKGROUND

The maturation of the intestinal wall determines the formation of a barrier function and intestinal permeability, which are key factors for efficient nutrient absorption, immune defense, and, consequently, resistance to infections in children during the first years of life. Results from modern cohort studies provide compelling evidence for an association between impaired intestinal barrier function in infancy and reduced growth rates [1], systemic inflammatory response [2, 3], and subsequent health outcomes in later childhood, with the severity of pathological processes depending on age, feeding type, antigenic load, and disease frequency [2–4].

Intestinal fatty acid-binding protein (I-FABP) is a cytosolic protein of small intestinal epithelial cells that is detected in biological fluids (blood and urine) only upon enterocyte damage [5, 6]. Consequently, in the current literature, I-FABP is considered primarily as a marker of intestinal epithelial cell injury rather than a direct indicator of intestinal permeability [5, 7]. In contrast, Claudin-2, a tight junction protein responsible for paracellular transport of water and cations, is regarded as a non-invasive marker of intestinal permeability [8, 9].

The high diagnostic and prognostic value of I-FABP for early detection and prediction of conditions associated with enterocyte death in neonatal and pediatric practice has been convincingly demonstrated [5, 8, 10]. For example, Prikhodchenko N.G. et al. demonstrated the diagnostic significance of I-FABP in children with protein-induced enteropathy [11] and gastrointestinal forms of food allergy [12]. The prognostic and diagnostic potential of I-FABP in necrotizing enterocolitis has been confirmed not only in original studies [7], but also in a meta-analysis [13]. The specificity of I-FABP as a marker of intestinal injury is clearly illustrated by the results of a study assessing intestinal permeability via serum biomarkers upon early introduction of allergens, particularly peanuts, into the diet: no changes in I-FABP concentration were detected in cases of predominant skin involvement without clinically significant gastrointestinal symptoms [14].

The diagnostic potential of Claudin-2 is currently less well studied. However, publications are emerging that demonstrate alterations in tight junction protein expression, particularly Claudin-2, in children with conditions associated with increased intestinal permeability [15, 16].

Although the first studies on intestinal permeability in children were published more than 30 years ago [17], a significant knowledge gap remains regarding age-related variability of biomarkers of intestinal injury and permeability in apparently healthy infants. Most studies on I-FABP and Claudin-2 concentrations in biological fluids have been conducted in high-risk groups, such as NEC [7, 13], are experimental in nature [16], or are limited to specific disease entities [11, 12, 14].

At the same time, it has been established that intestinal barrier function indices in early life undergo dynamic changes depending on nutrition, infectious diseases, and social factors [2–4, 18, 19]. However, longitudinal profiles of I-FABP and Claudin-2 concentrations during the first 24 months of life in apparently healthy children, including preterm infants, remain insufficiently characterized.

The gestational age range of 34–41 weeks includes late preterm, early term, and full-term infants. These groups exhibit significant differences in morphofunctional maturity of the intestinal barrier, postnatal adaptation characteristics, and nutritional requirements. The absence of age-specific reference values and longitudinal profiles of I-FABP and Claudin-2 concentrations complicates the differentiation of physiological variability from changes in permeability and enterocyte death in pathological conditions accompanied by biomarker elevation, thereby limiting the clinical interpretation of results.

## AIM

To determine the levels of I-FABP and Claudin-2 in apparently healthy children during the first 2 years of life in a longitudinal manner.

## METHODS

### Study Design

To achieve the aim, a prospective observational cohort study with longitudinal follow-up of patients was conducted. A flowchart of the study design is presented in Figure 1.

**Eligibility Criteria:** gestational age 34–41 weeks; postnatal age less than 7 days at the first assessment; at the second assessment: for Group 1 patients – postmenstrual age (PMA) 40–41 weeks; for Group 2 – postnatal age 1–1.5 months; at the third assessment – less than 24 months; written informed consent for participation in the study provided by the child's legal representative.

**Exclusion Criteria:** surgical pathology and/or congenital gastrointestinal malformations; clinical manifestations of perinatal infection; concomitant genetic syndromes, chromosomal abnormalities, metabolic disorders; severe congenital malformations of other organs and systems requiring surgical intervention in the neonatal period; established diagnosis of disaccharidase deficiency or other enteropathies; need for total parenteral nutrition; loss of contact with the family or refusal of the legal representative to continue participation; development during follow-up of a severe disease or condition potentially affecting gastrointestinal function and/or prescription of long-term specific therapy potentially affecting intestinal permeability markers.

### Study Setting

Patient enrollment and subsequent follow-up were conducted at the Voronezh Regional Children's Clinical Hospital No. 1. Laboratory analyses were performed at the Research Institute of Experimental Biology and Medicine of the Burdenko Voronezh State Medical University, Voronezh, Russia.

### Study Duration

The initial study cohort was formed from March 2023 to May 2024. Children were examined at three time points: within the first 7 days of life; upon reaching postmenstrual age 40–41 weeks in Group 1, and at age

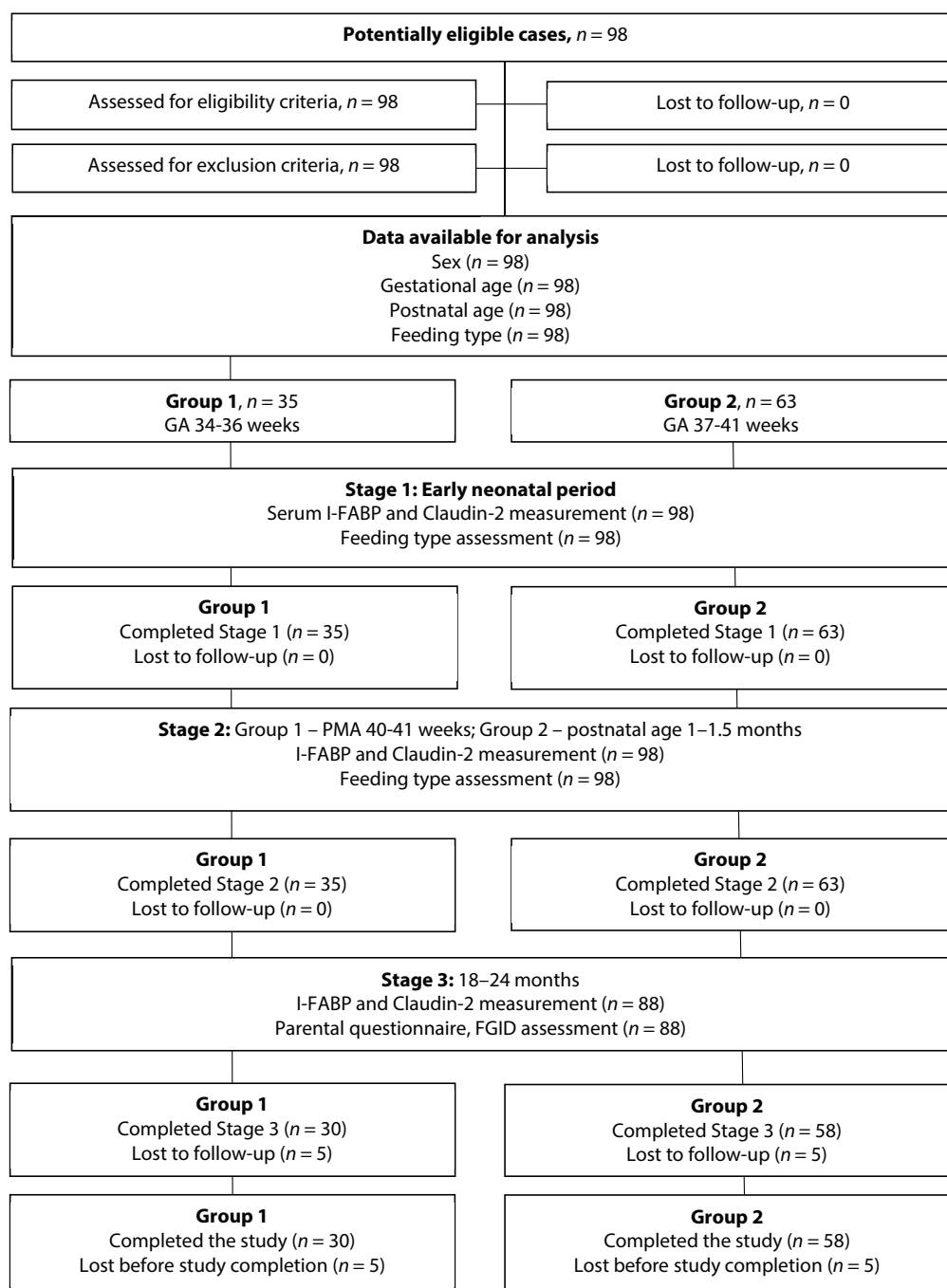
1–1.5 months in Group 2; and at age 18–24 months. The third follow-up was conducted from March 2025 to January 2026.

### Description of Medical Intervention

Blood sampling was performed by peripheral venipuncture by a trained nurse using standard technique. Blood was collected in the morning, on an empty stomach.

### Study Outcomes

**Primary Outcome:** dynamics of serum I-FABP and Claudin-2 levels during the first 24 months of life in children depending on gestational age.



**FIG. 1.**  
Study design

**Secondary Outcomes:** type of feeding, frequency of functional gastrointestinal disorders in infants.

### Subgroup Analysis

Subgroups were formed based on gestational age and feeding type.

### Outcome Registration Methods

I-FABP levels were measured using the Human IFABP/FABP2 (Intestinal Fatty Acid Binding Protein) ELISA Kit, and Claudin-2 levels using the Human CLDN2 (Claudin-2) ELISA Kit on a Multiskan Go microplate reader (Thermo Fisher Scientific, Finland), with a Wellwash microplate washer (Thermo Fisher Scientific, Finland) and a PST-60HL-4 thermoshaker (Biosan, Latvia). Serum was obtained by standard centrifugation (3000 rpm, 10 minutes).

To identify children who developed functional gastrointestinal disorders during the first 24 months of life, parents completed a questionnaire for detecting functional gastrointestinal disorders in children aged 0 months to 3 years 11 months prior to the third assessment [20, 21]. The questionnaire consists of two sections: the first for parents of children under one year, and the second for parents of children over one year, and enables diagnosis of the following functional gastrointestinal disorders: regurgitation, dyschezia, infantile colic, constipation and diarrhea, cyclic vomiting syndrome, and rumination. The questionnaire items are presented as questions with multiple-choice answers. Interpretation is performed using a pre-established key. For each functional gastrointestinal disorder, diagnostic criteria are considered met if all key-specified answers are obtained, except for functional constipation, which requires the presence of 2 or more criteria. "Red flags" requiring additional examination are separately indicated.

Stool consistency was assessed using the Amsterdam Stool Scale (Bekkali scale) for infants. The scale evaluates stool frequency, consistency, and color.

Feeding type and dietary characteristics at each follow-up stage were ascertained from parents during history taking.

### Ethical Approval

The study was approved by the local ethics committee of the Burdenko Voronezh State Medical University (Protocol No. 4, dated September 21, 2020).

### Statistical Analysis

**Sample Size Calculation Principles.** Sample size was not calculated a priori.

Statistical analysis was performed using Stat-Tech v. 4.11.1 (Stattech LLC, Russia). Effect size calculations were performed using the Python programming language in the Jupyter Notebooks environment with pandas and numpy libraries. Normality of quantitative variable distributions was assessed using appropriate tests (Kolmogorov – Smirnov for  $n > 50$ , Shapiro – Wilk for  $n < 50$ ). For normally distributed data, quantitative variables are presented as arithmetic means (M) and standard deviations (SD), with 95 % confidence intervals (95% CI). For non-normally distributed data,

medians (Me) with lower and upper quartiles (Q1–Q3) were used. Categorical data are presented as absolute numbers and percentages. Comparison of two independent groups for quantitative variables with normal distribution and equal variances was performed using Student's *t*-test; with non-normal distribution and equal variances – the Mann – Whitney U test; with unequal variances – the Brunner – Munzel test. Analysis of 2×2 contingency tables was performed using Pearson's chi-square test (when expected frequency  $> 10$ ) or Fisher's exact test (when expected frequency  $< 10$ ); for larger tables – Pearson's chi-square test.

Comparison of three or more dependent groups with non-normal distribution was performed using the Friedman test, with post-hoc comparisons using the Conover – Iman test with Holm correction. Differences were considered statistically significant at  $p < 0.05$ .

To assess the practical and clinical significance of identified differences, effect sizes were additionally calculated. For non-parametric tests of independent groups, the effect size was used. For the Friedman test (multiple related comparisons), Kendall's coefficient of concordance *W* was calculated. Interpretation of *r* and *W* values followed Cohen's criteria: 0.1 – small, 0.3 – medium,  $\geq 0.5$  – large effect.

## RESULTS

### Study Participants

The initial cohort included 98 newborns with gestational age (GA) 34–41 weeks, median GA 38.0 [36.0–39.0] weeks, of whom 62 (63.3 %) were male and 36 (36.7 %) female. All children completed the second follow-up. The third study stage represented continued follow-up of the formed cohort up to 24 months of age (median 21.0 [20.0–23.0]). The final stage was completed by 88 children, including 30 preterm and 58 full-term infants. Loss to follow-up was 10.2 % ( $n = 10$ ), due to withdrawal of consent by legal representatives or loss of contact with the family. Thus, the final study sample comprised 88 children, divided into two clinical groups: Group 1 – newborns with gestational age 34–36 weeks ( $n = 30$ ), Group 2 – newborns with gestational age 37–41 weeks ( $n = 58$ ). Age at enrollment was 3 [3; 6] days in Group 1 and 4 [2; 6] days in Group 2 ( $p = 0.157$ ), and at the end of follow-up – 20 [19; 22] months and 21 [20; 23] months, respectively ( $p = 0.268$ ).

To assess the risk of selection bias due to loss to long-term follow-up, a comparative analysis of patients who completed the study and those lost before the third follow-up was performed. Due to significant heterogeneity of the overall cohort, the analysis was stratified by preterm and full-term groups. Results are presented in Tables 1 and 2.

In Group 1, the proportion of girls among those lost before the third stage was higher compared to those who completed the study. Notably, sex is not

a primary exposure in this study and has a limited influence on the main outcome – serum I-FABP and Claudin-2 concentrations.

In the full-term group, no statistically significant differences in baseline characteristics were found between children who completed follow-up and those lost to follow-up. Thus, differences between children who completed the study and those lost before the end of follow-up have limited impact on the results and are unlikely to introduce selection bias.

### Primary Outcomes

Dynamics of I-FABP in the study groups are presented in Table 3. Notably, serum I-FABP concentration increased significantly at the third time point, while remaining relatively stable during the first 1–1.5 months of life (first and second time points) (Table 3). At the third follow-up, median serum I-FABP levels in preterm infants increased 4.7-fold compared to levels at PMA 40–41 weeks, and in full-term infants – 3.2-fold compared to the median concentration at 1–1.5 months of age. Notably, the longitudinal profile of I-FABP in preterm infants during the first 24 months corresponds to that of full-term infants: no statistically significant differences in biomarker concentration between groups were observed at any follow-up stage.

Dynamics of Claudin-2 in the study groups are presented in Table 4. Notable differences in longitudinal

profiles of Claudin-2 between groups were observed: for preterm infants, no significant fluctuations in biomarker concentration were noted during the first 24 months. In contrast, for full-term infants, Claudin-2 increased with growth – median levels at the second time point increased 2.7-fold and at the third time point 4.3-fold compared to the early neonatal period.

Comparative analysis of biomarker concentrations between groups showed that at the first two time points, serum Claudin-2 concentration was significantly higher in preterm infants compared to full-term infants; at the third time point, biomarker levels were comparable between groups.

### Secondary Outcomes

All children included in the study tolerated enteral feeding. None required total parenteral nutrition. During the follow-up period, no child developed a severe disease or condition potentially affecting gastrointestinal function and/or requiring long-term specific therapy potentially affecting intestinal permeability markers. Stool consistency, frequency, and color assessed by the Bekkali scale were within normative values for each age group.

At the first I-FABP and Claudin-2 assessment, the proportion of exclusively breastfed children in Group 1 was 16.7 % ( $n = 5$ ) vs. 20.7 % ( $n = 12$ ) in Group 2 ( $p = 0.881$ ); at the second assessment – 26.7 % ( $n = 8$ )

**TABLE 1**

**COMPARISON OF BASELINE CHARACTERISTICS BETWEEN PRETERM CHILDREN WHO COMPLETED THE THIRD FOLLOW-UP VISIT AND THOSE LOST TO FOLLOW-UP**

| Parameter                                 | Lost to follow-up, $n=5$            | Completed the study, $n=30$         | $p$    |
|-------------------------------------------|-------------------------------------|-------------------------------------|--------|
| Female, abs. (%)                          | 4 (80.0)                            | 6 (20.0)                            | 0.017* |
| Male, abs. (%)                            | 1 (20.0)                            | 24 (80.0)                           |        |
| Neonatal antibiotic exposure, abs. (%)    | 1 (20.0)                            | 19 (63.3)                           | 0.141  |
| No neonatal antibiotics, abs. (%)         | 4 (80.0)                            | 11 (36.7)                           |        |
| Vaginal delivery, abs. (%)                | 4 (80.0)                            | 17 (56.7)                           | 0.627  |
| Cesarean delivery, abs. (%)               | 1 (20.0)                            | 13 (43.3)                           |        |
| Gestational age, weeks, Me [IQR]          | 35.0 [35; 35]                       | 35.0 [34; 36]                       | 0.689  |
| Apgar score at 1 min, points, Me [IQR]    | 7.0 [7.0; 7.0]                      | 7.0 [6.0; 7.0]                      | 0.575  |
| Apgar score at 5 min, points, Me [IQR]    | 8.0 [8.0; 8.0]                      | 8.0 [8.0; 8.0]                      | 0.533  |
| Birth weight, g, $M \pm SD$ 95% CI        | 2528.0 $\pm$ 343.3<br>2102.1–2953.9 | 2595.8 $\pm$ 584.2<br>2377.7–2813.9 | 0.804  |
| Birth length, cm, $M \pm SD$ 95% CI       | 48.8 $\pm$ 1.6<br>46.8–50.8         | 49.1 $\pm$ 3.5<br>47.7–50.4         | 0.869  |
| Head circumference at birth, cm, Me [IQR] | 33.0 [33.0; 34.0]                   | 32.0 [30.25; 33.0]                  | 0.050  |
| Maternal age, years, $M \pm SD$ 95% CI    | 25.8 $\pm$ 5.6<br>18.9–32.7         | 30.4 $\pm$ 5.7<br>28.3–32.5         | 0.103  |

**Note.** \* – differences between groups are statistically significant ( $p < 0.05$ ).

and 39.7 % ( $n = 23$ ), respectively ( $p = 0.289$ ). Median duration of breastfeeding in Group 1 was 8 [1; 18] months, in Group 2 – 4 [1; 15] months ( $p = 0.465$ ). At the third biomarker assessment, all children were receiving solid foods. No statistically significant differences in serum I-FABP and Claudin-2 concentrations were found in groups stratified by feeding type during the first months of life.

According to parental questionnaire results, no child was diagnosed with functional gastrointestinal disorders. No respondent reported complaints of recurrent

vomiting, regurgitation, straining during defecation, hard stools, or pathological impurities in stool. Most respondents reported regular bowel movements: 1–3 times/day in 63.6 % ( $n = 56$ ), 3–6 times/week in 36.4 % ( $n = 32$ ). Regarding stool consistency, most parents chose “not very hard, but not soft” – 96.6 % ( $n = 85$ ); 3.4 % ( $n = 3$ ) reported inconsistent stool consistency (“different each time”). Diagnostic criteria for functional gastrointestinal disorders were met in none of the questionnaires.

#### Adverse Events

No adverse events were recorded during follow-up.

**TABLE 2**

**COMPARISON OF BASELINE CHARACTERISTICS BETWEEN TERM CHILDREN WHO COMPLETED THE THIRD FOLLOW-UP VISIT AND THOSE LOST TO FOLLOW-UP**

| Parameter                                 | Lost to follow-up, $n=5$            | Completed the study, $n=58$           | $p$   |
|-------------------------------------------|-------------------------------------|---------------------------------------|-------|
| Female, abs. (%)                          | 2 (40.0)                            | 24 (41.4)                             | 1.00  |
| Male, abs. (%)                            | 3 (60.0)                            | 34 (58.6)                             |       |
| Neonatal antibiotic exposure, abs. (%)    | 1 (20.0)                            | 17 (29.3)                             | 1.00  |
| No neonatal antibiotics, abs. (%)         | 4 (80.0)                            | 41 (70.7)                             |       |
| Vaginal delivery, abs. (%)                | 2 (40.0)                            | 41 (70.7)                             | 0.315 |
| Cesarean delivery, abs. (%)               | 3 (60.0)                            | 17 (29.3)                             |       |
| Gestational age, weeks, Me [IQR]          | 39.0 [38; 40]                       | 39.0 [38; 40]                         | 0.831 |
| Apgar score at 1 min, points, Me [IQR]    | 7.0 [7.0; 7.0]                      | 7.0 [7.0; 8.0]                        | 0.308 |
| Apgar score at 5 min, points, Me [IQR]    | 8.0 [8.0; 9.0]                      | 9.0 [8.0; 9.0]                        | 0.084 |
| Birth weight, g, $M \pm SD$ 95% CI        | 3150.0 $\pm$ 540.1<br>2479.4–3820.6 | 3374.7 $\pm$ 484.0<br>3247.4 – 3501.9 | 0.327 |
| Birth length, cm, $M \pm SD$ 95% CI       | 53.0 [53.0; 54.0]                   | 53.0 [52.0; 55.0]                     | 0.969 |
| Head circumference at birth, cm, Me [IQR] | 34.0 [33.5; 36.0]                   | 35.0 [34.0; 36.0]                     | 0.633 |
| Maternal age, years, $M \pm SD$ 95% CI    | 33.6 $\pm$ 2.8<br>30.1–37.1         | 28.56 $\pm$ 5.92<br>26.99–30.13       | 0.066 |

**TABLE 3**

**I-FABP DYNAMICS IN CHILDREN BORN AT 34–41 WEEKS OF GESTATION DURING THE FIRST 24 MONTHS OF LIFE**

| Group /<br>Gestational age | I-FABP, 1 <sup>st</sup><br>measurement, ng/mL | I-FABP, 2 <sup>nd</sup><br>measurement, ng/mL | I-FABP, 3 <sup>rd</sup><br>measurement, ng/mL | $p$                                                   | $W$   |
|----------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-------------------------------------------------------|-------|
|                            | Me [Q <sub>1</sub> ; Q <sub>3</sub> ]         | Me [Q <sub>1</sub> ; Q <sub>3</sub> ]         | Me [Q <sub>1</sub> ; Q <sub>3</sub> ]         |                                                       |       |
| Group 1<br>(34–36 weeks)   | 1.12<br>[1.07; 1.30]<br>( $n=30$ )            | 1.30<br>[0.97; 1.87]<br>( $n=30$ )            | 6.09<br>[3.18; 6.65]<br>( $n=30$ )            | <0.001*<br>$p_{(1-3)} < 0.001$<br>$p_{(2-3)} < 0.001$ | 0.573 |
| Group 2<br>(37–41 weeks)   | 1.10<br>[0.80; 1.95]<br>( $n=58$ )            | 1.21<br>[0.92; 1.73]<br>( $n=58$ )            | 3.89<br>[2.33; 6.10]<br>( $n=58$ )            | <0.001*<br>$p_{(1-3)} < 0.001$<br>$p_{(2-3)} < 0.001$ |       |
| $p$                        | 0.738                                         | 0.610                                         | 0.131                                         | –                                                     |       |
| $r$                        | 0.036                                         | 0.055                                         | 0.161                                         | –                                                     |       |

**Note.** \* – differences between groups are statistically significant ( $p < 0.05$ ).

TABLE 4

CLAUDIN-2 DYNAMICS IN CHILDREN BORN AT 34–41 WEEKS OF GESTATION DURING THE FIRST 24 MONTHS OF LIFE

| Group /<br>Gestational age | Claudin-2 1 <sup>st</sup><br>measurement, ng/mL | Claudin-2 2 <sup>nd</sup><br>measurement, ng/mL | Claudin-2 3 <sup>rd</sup><br>measurement, ng/mL | <i>p</i>                                                                                                               | <i>W</i> |
|----------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|------------------------------------------------------------------------------------------------------------------------|----------|
|                            | Me [Q <sub>1</sub> ; Q <sub>3</sub> ]           | Me [Q <sub>1</sub> ; Q <sub>3</sub> ]           | Me [Q <sub>1</sub> ; Q <sub>3</sub> ]           |                                                                                                                        |          |
| Group 1<br>(34–36 weeks)   | 6.76<br>[3.00–9.14]<br>( <i>n</i> =30)          | 7.59<br>[5.20–10.96]<br>( <i>n</i> =30)         | 7.77<br>[6.33–9.20]<br>( <i>n</i> =30)          | 0.497                                                                                                                  | 0.023    |
| Group 2<br>(37–41 weeks)   | 1.85<br>[1.02–5.22]<br>( <i>n</i> =58)          | 5.10<br>[3.47–8.31]<br>( <i>n</i> =58)          | 7.95<br>[6.32–11.28]<br>( <i>n</i> =58)         | <0.001*<br><i>p</i> <sub>(1-2)</sub> = 0.007<br><i>p</i> <sub>(1-3)</sub> < 0.001<br><i>p</i> <sub>(2-3)</sub> = 0.017 | 0.251    |
| <i>p</i>                   | 0.006*                                          | 0.009*                                          | 0.326                                           | –                                                                                                                      |          |
| <i>r</i>                   | 0.296                                           | 0.277                                           | 0.105                                           | –                                                                                                                      | –        |

Note. \* – differences between groups are statistically significant (*p* < 0.05).

## DISCUSSION

### Summary of Primary Outcomes

This study examined the dynamics of serum I-FABP and Claudin-2 levels in children with gestational age 34–41 weeks during the first 24 months of life. The results demonstrate that I-FABP concentration remains stable during the first 1–1.5 months of life, is independent of gestational age, and increases by 24 months of age in both full-term and preterm infants.

Dynamics of serum Claudin-2 levels differ between children with varying gestational age within the 34–41 week range: late preterm infants exhibit stable biomarker concentrations during the first 24 months, while full-term infants show an increase in serum Claudin-2 with postnatal growth. Thus, gestational age within the 34–41 week range is not a determinant of I-FABP levels during the first 24 months of life. For Claudin-2, variability depending on the degree of gestational maturity is characteristic.

### Discussion of Primary Outcomes

Our data complement the findings of international studies emphasizing the need for age-specific interpretation of I-FABP and longitudinal monitoring to enhance diagnostic value [10]. Shores D.R. et al. proposed normative I-FABP values considering gestational age, noting significant variability even in clinically stable children and an increase in I-FABP with postnatal growth, which the authors attribute to changes in intestinal epithelial load and enterocyte vulnerability to external agents [10].

An age-dependent trend in intestinal barrier and permeability markers was also demonstrated in another cohort study [4] and is generally consistent with our findings: from 12 to 24 months of age, a trend toward increased markers of intestinal epithelial injury

and permeability was observed, which may be related to active dietary changes, microbiome shifts, and increased antigenic load in children of this age [4]. Thus, the increase in I-FABP concentration we observed may reflect these processes. Hasan M.M. et al. showed that I-FABP levels in young children are associated with markers of environmental enteropathy and stunting. The authors emphasize that I-FABP elevation is not always accompanied by clinically significant gastrointestinal symptoms and may reflect chronic and/or recurrent subclinical enterocyte injury, suggesting that this marker may indicate increased intestinal epithelial load rather than overt injury [1].

Therefore, elevation of intestinal wall markers, including I-FABP, may be interpreted as reflecting the load on the intestinal epithelium during periods of active dietary and microbiota changes occurring during the first 24 months of life.

It is important to emphasize that in the present study, I-FABP elevation at 24 months of age was not accompanied by clinical signs of gastrointestinal pathology. Thus, it is reasonable to assume that the observed dynamics reflect a physiological response of the intestinal epithelium to changes in diet, feeding patterns, and increased antigenic load occurring during the second year of life.

Several studies have demonstrated I-FABP elevation in enteropathies and gastrointestinal forms of food allergy [11, 12], whereas in allergy cohorts without clinically significant gastrointestinal involvement, no differences in I-FABP levels were detected [14]. The results of the present study complement the knowledge on I-FABP variability across different clinical groups with data on age-related variability of the marker. Thus, I-FABP levels should be interpreted considering the patient's clinical status and age.

Claudin-2 dynamics differ significantly between groups and likely reflect morphofunctional differences in the intestinal wall of preterm versus full-term infants. Claudin-2, a tight junction protein capable of increasing intestinal permeability to water and cations, may be considered an adaptive mechanism in the context of increased nutritional demands in preterm infants. On the other hand, this characteristic, in the presence of risk factors such as intestinal hypoxia/ischemia, ICU hospitalization, hemodynamic instability, and colonization with hospital flora, may serve as a substrate for progression of necrotizing enterocolitis or reduced enteral feeding tolerance. Based on the obtained results, it can be concluded that interpretation of Claudin-2 concentrations during the first 24 months should consider the child's gestational and postnatal age.

The changes in biomarkers observed during the first 24 months of life should not be interpreted as evidence of pathological intestinal permeability or impaired intestinal barrier integrity due to enterocyte damage. In our view, this represents age-related variability of these markers.

#### Study Limitations

The limitations of this study are, on the one hand, related to the absence of direct intestinal permeability assessment using gold-standard methods (e.g., lactulose/mannitol test), which precludes comparison of I-FABP and Claudin-2 levels with direct permeability measurements. Additionally, data on the intestinal microbiome of the children, which may have influenced I-FABP and Claudin-2 levels, were not available. Investigation of these parameters was not within the scope of the study, but is noted as a limitation and potential direction for future research.

On the other hand, the study was subject to loss to follow-up and involved a relatively small number of patients, which may have affected the statistical power of the analysis.

## CONCLUSION

In children born at 34–41 weeks of gestation, the age-related dynamics of I-FABP during the first 24 months of life are characterized by an increase by the second year of life, with comparable longitudinal I-FABP concentration profiles in preterm and full-term children. Gestational age within the 34–41 week range does not independently influence I-FABP levels.

In preterm infants born at late gestational ages, longitudinal Claudin-2 profiles are characterized by the absence of significant fluctuations in concentration during the first 24 months. In full-term infants, conversely, Claudin-2 increases with postnatal growth.

Accumulation of data on I-FABP and Claudin-2 dynamics allows for more accurate interpretation of biomarker values and facilitates differentiation between physiological age-related changes and pathological intestinal epithelial injury in early childhood.

## Conflict of interest

The authors declare no conflict of interest.

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## CLINICAL CHARACTERISTICS OF CHRONIC ADENOIDITIS IN CHILDREN AGED 3–6 YEARS WITH PROBIOTIC SUPPORT

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### ABSTRACT

**Background.** The prevalence of chronic tonsil diseases is constantly increasing among children of different ages. To date, the treatment of pediatric patients with chronic diseases of the upper respiratory tract remains controversial and requires new therapeutic approaches.

**Aim.** To study the clinical and anamnestic characteristics of chronic adenoiditis in children aged 3–6 years and to evaluate the effectiveness of including a probiotic drug in postoperative rehabilitation.

**Methods.** A total of 100 children aged 3–6 years with chronic inflammatory processes of the nasopharynx were examined. The patients were divided into 2 comparable groups, depending on the treatment and rehabilitation measures received. A parent survey was conducted to assess the severity of clinical symptoms before treatment and 30 days after adenotomy.

**Results.** The frequency and duration of viral infections in children with chronic adenoiditis were assessed. Before surgery, 62 % of children had acute respiratory infections (ARIs) once every 2 months or more, and 62 % had ARI episodes lasting more than 7 days. According to the survey results, after treatment there was a statistically significant decrease in the frequency of the following symptoms in children in both study groups: difficulty in nasal breathing, snoring, cough, and bad breath. In the main group, there was a marked decrease in the intensity of nasal breathing difficulty (by 84 %) and in the frequency of sleep disorders (by 58 %) after probiotic treatment.

**Conclusion.** The post-treatment survey data demonstrate regression of clinical symptoms in both groups. However, with respect to symptoms such as decreased intensity of nasal breathing and reduced frequency of sleep disorders, higher therapeutic efficacy was observed when a probiotic was used as an adjunct to adenotomy.

**Keywords:** chronic adenoiditis, adenotomy, probiotics, *Bifidobacterium longum* MC-42, clinical manifestations

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## КЛИНИЧЕСКАЯ ХАРАКТЕРИСТИКА ТЕЧЕНИЯ ХРОНИЧЕСКОГО АДЕНОИДИТА У ДЕТЕЙ 3–6 ЛЕТ НА ФОНЕ ПРОБИОТИЧЕСКОЙ ПОДДЕРЖКИ

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### РЕЗЮМЕ

**Обоснование.** Распространенность хронических заболеваний миндалин постоянно увеличивается среди детей различных возрастов. На сегодняшний день вопросы лечения пациентов детского возраста с хроническими заболеваниями верхних дыхательных путей остаются дискуссионными и требуют новых подходов к терапии.

**Цель исследования.** Изучить клинико-анамнестические характеристики течения хронических воспалительных заболеваний аденоидов у детей 3–6 лет с оценкой эффективности включения в программу послеоперационной реабилитации пробиотического средства.

**Методы.** Обследовано 100 детей в возрасте 3–6 лет с хроническими воспалительными процессами носоглотки. Пациентов разделили на 2 сопоставимые группы в зависимости от проводимых лечебных и реабилитационных мероприятий. Проведено анкетирование родителей для определения степени выраженности клинических симптомов до лечения и через 30 дней после проведенной аденотомии.

**Результаты.** Оценивалась частота возникновения и длительность течения вирусных инфекций у детей с хроническим аденоидитом, которые до операции: 80 % переносили острые респираторные инфекции (ОРИ) 1 раз в 2 месяца и чаще, 62 % выздоравливали более чем за 7 дней. По результатам анкетирования после лечения наблюдали статистически значимое снижение частоты встречаемости следующих симптомов у детей в исследуемых группах: затрудненное носовое дыхание, храп, кашель, неприятный запах изо рта. В основной группе наблюдается выраженное снижение интенсивности затруднения дыхания через нос (на 84 %) и частоты встречаемости нарушений сна (на 58 %) после лечения пробиотиком.

**Заключение.** Данные анкетирования после лечения демонстрируют эффективность в аспекте регресса клинической симптоматики в обеих группах. Однако по таким симптомам, как снижение интенсивности затруднения дыхания через нос и частота встречаемости нарушений сна, определена более высокая лечебная эффективность применения пробиотического препарата на фоне аденотомии.

**Ключевые слова:** хронический аденоидит, аденотомия, пробиотические препараты, *Bifidobacterium longum* MC-42, клинические проявления

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## BACKGROUND

Adenoids (pharyngeal tonsil) and tonsils (palatine, tubal, and lingual) are accumulations of lymphoid tissue that form the Pirogov – Waldeyer ring. These structures play a crucial role in screening microorganisms that enter the human body through the upper respiratory tract, as well as in eliminating local pathogens by activating immune system effector cells and memory cells [1]. However, these anatomical structures can also act as reservoirs for pathogenic and opportunistic bacteria. This can lead to the development of recurrent and chronic respiratory tract infections, through the formation of persistent bacterial biofilms. In this process, microorganisms attach to the tonsillar surfaces and create a matrix that protects them from the host immune response and makes them resistant to antibiotics [2].

The prevalence of both allergic and chronic inflammatory tonsil diseases is increasing in most countries, affecting adult patients and children from all age groups [3]. Chronic diseases of the nasal and oropharyngeal regions are a result of persistent infections in the tonsils. This may be due to either high virulence of the respiratory tract microbiome, or to a combination of sensitization (or sensitization alone), primary ciliary dyskinesia, or congenital immune system defects. To date, there is a lack of reliable statistical data on the actual prevalence of chronic inflammatory conditions involving the palatine tonsils and adenoids in the Russian Federation.

Particular attention is given to the issue of children with frequent and prolonged recurring infections, which is particularly relevant for preschool and early school-age children (up to the age of 10) [4]. The high incidence of acute respiratory infections (ARIs) at this age is due to the anatomical features of the upper respiratory tract (in particular, the narrowness of the lumen) and the excessive hypertrophy of lymphoid tissue associated with the development of the immune system [5]. It has been shown that the presence of chronic inflammatory processes in the nasal cavity of children is also linked to changes in the composition of the local microbiota. Local inflammation limits the range of microbial species capable of colonizing the nasopharynx, and a dysbiotic environment may promote the adhesion and proliferation of new specific bacterial groups [6]. It is known that opportunistic bacteria may be transiently or persistently present on the mucosal surfaces of various parts of the pharyngeal region even in healthy children. These microorganisms include representatives of the following genera and species: *Neisseria*, *Streptococcus pyogenes*, *Haemophilus influenzae*, *Staphylococcus aureus*, *Actinomyces*, *Bacteroides*, *Prevotella*, and others [2].

Chronic inflammatory diseases in children often manifest as recurrent sore throat, difficulty swallowing, snoring, mouth breathing, and sleep disturbances. The most severe manifestation of these conditions is obstructive sleep apnea. As children grow older, these symptoms are accompanied by persistent bad breath, changes in voice, and difficulty swallowing [2]. These clinical manifestations

significantly reduce the quality of life for children, negatively affecting their social development and ability to participate in school activities. This justifies the need for more effective treatment methods.

To date, the diagnosis and management of pediatric patients with chronic tonsil diseases remains a controversial area and requires innovative therapeutic approaches. Traditional treatment of children with persistent infections associated with tonsil hypertrophy often involves surgical intervention, although this is not always successful [5]. Accumulated knowledge of the composition and physiological characteristics of the indigenous microbiota of the oropharyngeal region supports the use of drugs that maintain the balance of the upper airway microbiocenosis. These drugs can not only alleviate or eliminate symptoms of the disease, but also stimulate and maintain local reparative processes and prevent recurrence of the underlying condition [7]. Previous studies have demonstrated the positive impact of probiotics, in particular *S. salivarius*, on the clinical symptoms of children with tonsillitis [8]. This led to the search for other probiotic agents that could influence the dynamics of the clinical course of inflammatory diseases of the upper respiratory tract. The effectiveness of the *Bifidobacterium longum* MC-42 strain has been shown in the practice of otolaryngologists and is included in the methodological recommendations for physicians and otolaryngologists "Chronic Pharyngeal Infection Foci and Associated Somatic Pathology" [9]. However, the authors have not found any studies in the Russian or international literature on the use of this *Bifidobacterium* strain in the postoperative period as part of complex therapy for children with chronic upper respiratory tract inflammation, which confirms the novelty of this study.

## AIM

To study the clinical and anamnestic characteristics of chronic adenoiditis in children aged 3–6 years and to evaluate the effectiveness of including a probiotic drug in the postoperative rehabilitation program.

## METHODS

### Study Conditions

The study involved 100 children aged 3 to 6 years (57 % male, 43 % female) with chronic inflammatory processes of the nasopharynx (ICD-10 code: J35.9 Chronic disease of tonsils and adenoids). The diagnosis was confirmed by otorhinolaryngology specialists. All 100 participants received surgical treatment at the City Children's Emergency Clinical Hospital in the Novosibirsk region.

### Group Characteristics

Prior to treatment, patients were randomly assigned into two groups using a computer-generated randomization process. The two groups were equal in size (1:1 ratio).

Group 1 (the control group) consisted of patients who underwent adenoidectomy surgery under general anesthesia.

Group 2 (the main group) included patients who underwent adenoidectomy surgery under general anesthesia and received postoperative nasopharyngeal irrigation with a probiotic solution for a period of 30 days after surgery. This irrigation was administered for 14 days transnasally and orally and for 16 days only orally.

#### Description of the Medical Intervention

To provide probiotic support, patients were administered a liquid dietary supplement (food additive) containing *Bifidobacterium longum* MC-42, which is recommended for use in infants and children (SGR RU.77.99.88.003. E00157304.16 dated April 12, 2016, produced in accordance with TU 9224-010-70517093-2015). The number of active bifidobacteria *B. longum* MC-42 in 1 cm<sup>3</sup> of the product at the end of its shelf life is  $5 \times 10^8$  CFU/g in the natural environment of their metabolites (volatile short-chain fatty acids, etc.). The product was stored at  $4 \pm 2^\circ\text{C}$ . The probiotic was administered to patients *per os* and intranasally at a dose appropriate for pediatric use. The dose corresponded to the recommended daily intake of the probiotic supplement for children aged 3 to 6 years, ranging from 4 to 6 mL per day. The use of the dietary supplement was conducted in accordance with Methodological Recommendations approved by the Coordination and Methodology Council for Postgraduate Education of the Novosibirsk State Medical University on April 6, 2018 (Protocol No. 98) [9]. According to the results of laboratory tests, the dietary supplement meets the standards of the Voluntary Certification System for Food Products and Dietary Supplements established by the GOST R Certification System under the Federal Agency for Technical Regulation and Metrology. The product is intended to normalize the microbiota and improve the barrier function of the human mucous membrane.

#### Survey Administration

Parental interviews were conducted, complaints and medical histories were collected, and the children were clinically examined by an otorhinolaryngologist. A parental survey was conducted to determine the severity of clinical symptoms prior to treatment and 30 days after rehabilitation measures to clarify the medical history. The survey included the following parameters: difficulty in nasal breathing, coughing, hearing loss, runny nose, nasal voice, sleep disturbances, snoring, and bad breath. Each proposed criterion was rated by the parents before and after treatment on a scale of 0 to 10, with 0 indicating absence and 10 indicating a pronounced symptom. In addition, the frequency and average duration of acute respiratory infections among children of all study groups was analyzed.

#### Eligibility Criteria

##### Inclusion Criteria:

- Age 3 to 6 years;
- Previously diagnosed with chronic inflammatory disease of the tonsils and adenoids, with grades 2 and 3 hypertrophy (ICD 10 code: J35.9).

##### Non-inclusion Criteria:

- Presence of allergic rhinitis, bronchial asthma, acute viral infections, primary ciliary dyskinesia, congenital immune response defects, systemic and oncological diseases, or severe somatic conditions in the acute phase.

#### Study Outcome Measures

##### Primary Outcome Measure

Anamnestic and clinical-functional features of the course of chronic inflammatory diseases of the palatine tonsils and adenoids in children.

##### Statistical Procedures

##### Sample Size Calculation

A preliminary calculation of the required sample size was not performed.

##### Statistical Methods

Statistical analysis was conducted using the STATISTICA v.10 software (StatSoft Inc, USA). Descriptive statistics included the number of individuals and the percentage of the sample (%). Differences between groups were analyzed using the two-tailed Fisher's exact test, with multiple comparisons adjusted for the Bonferroni correction. Repeated observations before and after treatment were analyzed using the two-tailed z-test for related samples. The significance level was set at 5 % ( $p \leq 0.05$ ), with the minimum probability of the null hypothesis being true.

##### Ethical Review

All patient information was anonymized and access to the original data was limited. The study was conducted as part of a thesis and was approved by the Local Ethics Committee of the Novosibirsk State Medical University (Protocol No. 111, dated November 29, 2018).

## RESULTS

#### Study Sample Formation

The study involved 100 children aged 3 to 6 years with chronic tonsil and adenoid diseases, verified by otolaryngologists. The groups were comparable in terms of sex and age. In Group 1 (the main group), 58 % of the participants were male and 42 % were female, with a mean age of 5.42 years. In Group 2 (the control group), 56 % of the participants were male and 44 % female, with a mean age of 5.2 years.

The indications for surgical treatment were determined by attending physicians based on the following criteria: persistent difficulty in nasal breathing for more than 3 months, despite conservative treatment; snoring with apnea (respiratory arrest against the background of snoring with continued attempts at chest excursion followed by a noisy inhalation) as reported by legal representatives; and prolonged exudative otitis media lasting more than 3 months, also despite conservative treatment.

#### Main Results of the Study

*The frequency and duration of acute respiratory infections (ARIs) in children with chronic inflammatory conditions of the tonsils and adenoids*

All legal representatives reported difficulty attending organized children's groups due to recurrent diseases.

Prior to treatment and rehabilitation measures, there was no statistically significant difference in the frequency of viral infections of the upper respiratory tract among patients in all groups ( $p > 0.05$ ): 80 % of patients experienced ARIs once every 2 months or more frequently (Table 1).

The duration of acute respiratory infections (ARIs) in the children of all examined groups varied from 3–4 days to 14 days or more: in 62 % of cases, the recovery from respiratory tract infections required more than 7 days (Table 2).

#### *Clinical Symptoms and Their Severity*

Before treatment, 98 % of the individuals included in the study reported nasal breathing difficulty, with no significant difference in the severity of this symptom between the compared groups ( $p > 0.05$ ). Of these, 18 % rated the severity at 7–8 points and 7 % rated it at 9–10 points. After treatment, there was a significant increase in the number of participants reporting complete absence of nasal breathing difficulty (0 points): 52 % in Group 1 ( $p < 0.0001$ ) and 84 % in Group 2 ( $p < 0.0001$ ), respectively (Fig. 1). Ratings of 7–10 for this symptom were not observed after treatment. The most significant reduction in the severity of nasal breathing difficulty after treatment was seen in Group 2 ( $p_{1-2} = 0.0027$ ).

Before treatment, snoring was observed in 81 % of all patients. A high severity of snoring (9–10 on the scale) was reported by 7 % of study participants, and 17 % reported a moderate severity (7–8). After surgical intervention, there was a significant reduction in snoring severity in both groups. After treatment, 78 % of patients in Group 1 reported the absence of snoring, compared to 4 % prior to treatment ( $p < 0.0001$ ), and 66 % of patients in Group 2 reported no snoring after treatment, compared to 8 % before treatment ( $p < 0.0001$ ). None of the patients reported a severity of 7–10 for snoring after the treatment.

Before any treatment, 80 % of the examined children exhibited nasal voice. After therapy, there was a significant increase in the number of respondents who reported the absence of this symptom in each group. In Group 1, this number increased from 14 % prior to treatment to 76 % 30 days following the intervention ( $p < 0.0001$ ). In Group 2, it increased from 18 % to 86 % ( $p < 0.0001$ ). Before treatment, 48 % of respondents reported nasal voice severity of 4 or higher. After treatment, only 3 % of participants provided such a rating.

Before treatment, sleep disturbances were present in 75 % of all patients. This number decreased significantly after therapy, with a distribution as follows. Before interventions, this symptom was observed in 14 % of patients in Group 1 and in 20 % of patients in Group 2. After treatment, these figures were 78 % ( $p < 0.0001$ ) and 98 % ( $p < 0.0001$ ), respectively (Fig. 2). The severity of sleep disturbances was low: before treatment, 45 %

**TABLE 1**

#### **FREQUENCY OF ACUTE RESPIRATORY VIRAL INFECTIONS IN PRESCHOOL CHILDREN WITH CHRONIC INFLAMMATORY DISEASES OF THE TONSILS AND ADENOIDS**

| Group               | Group 1<br>(n=50) [1] | Group 2<br>(n=50) [2] |
|---------------------|-----------------------|-----------------------|
| Frequency of ARIs   | n (%)                 | n (%)                 |
| once every 2 weeks  | 6 (12%)               | 10 (20%)              |
| once a month        | 14 (28%)              | 19 (38%)              |
| once every 2 months | 20 (40%)              | 11 (22%)              |
| once every 3 months | 7 (14%)               | 4 (8%)                |
| once every 6 months | 2 (4%)                | 5 (10%)               |
| once a year         | 0 (0%)                | 1 (2%)                |

**TABLE 2**

#### **DURATION OF ARI IN PRESCHOOL CHILDREN WITH CHRONIC INFLAMMATORY DISEASES OF THE TONSILS AND ADENOIDS**

| Group            | Group 1<br>(n=50) [1] | Group 2<br>(n=50) [2] |
|------------------|-----------------------|-----------------------|
| Duration of ARIs | n (%)                 | n (%)                 |
| 3–4 days         | 1 (2%)                | 4 (8%)                |
| 5–7 days         | 12 (24%)              | 21 (42%)              |
| 7–10 days        | 16 (32%)              | 14 (28%)              |
| 10–14 days       | 12 (24%)              | 9 (18%)               |
| ≥ 14 days        | 9 (18%)               | 2 (4%)                |

of all respondents reported scores between 1 and 3 inclusive. After treatment, only 1 participant (0.67 %) rated sleep disturbance severity as 4 or higher. The most significant improvements were observed in Group 2 ( $p_{1-2} = 0.0114$ ).

Before any medical and/or surgical intervention, 63 % of all individuals examined had a prolonged runny nose (74 % in Group 1 and 52 % in Group 2). There were no significant differences in the severity of the clinical manifestations between the groups ( $p > 0.05$ ). After treatment, there was a significant change in the proportion of patients with a score of 0 for rhinorrhea in Group 1, from 26 % before treatment to 82 % after treatment ( $p < 0.0001$ ).

Before hospitalization and treatment, coughing was observed as a symptom in 51 % of all individuals examined. It was most frequently rated by patients as 1–3 points in 26 % of Group 1 and 34 % of Group 2. The number of patients who reported complete absence of this symptom (score of 0) significantly increased after surgical intervention in both groups. In Group 1, it increased

from 60 % to 92 % ( $p = 0.0002$ ). In Group 2, it increased from 56 % to 92 % ( $p < 0.0001$ ).

In addition to the above-mentioned symptoms, prior to treatment initiation, 35 % of all patients exhibited bad breath, and 28 % reported hearing loss. After 30 days of treatment, the number of patients experiencing bad breath significantly decreased by 30 % in Group 1 ( $p = 0.0001$ ) and by 36 % in Group 2 ( $p < 0.0001$ ), while the number of patients reporting hearing loss significantly decreased by 24 % in Group 1 ( $p = 0.0005$ ) and by 28 % in Group 2 ( $p = 0.0003$ ).

## DISCUSSION

### Summary of the Main Results

The study examined the most prevalent clinical symptoms of chronic inflammatory conditions of the palatine tonsils and adenoids, observed in more than  $\frac{3}{4}$  of cases, including nasal breathing difficulty, snoring, nasal voice, and sleep disturbances. More than half of the patients experienced prolonged runny nose and coughing. The most severe manifestations, rated 9–10 points on a severity scale, included snoring and nasal breathing difficulty. Almost all children who underwent removal of hypertrophied pharyngeal tonsils showed improvement in their clinical condition. However, when it comes to symptoms such as decreased severity of nasal breathing difficulties and frequency of sleep disturbances, higher therapeutic effectiveness was observed when the *Bifidobacterium longum* MC-42 strain was included in the diet after adenotomy.

### Discussion of the Main Result

The data from the survey conducted after the medical procedures support the effectiveness of adenotomy in reducing clinical symptoms, both in the absence of probiotic correction to the nasopharyngeal microbiome (Group 1) and in the presence of nasopharyngeal irrigation with a probiotic solution during the postoperative period (Group 2). Based on our findings, the most

efficient treatment and rehabilitation approach for restoring nasal breathing and sleep quality was the administration of the probiotic agent after adenotomy (Group 2).

It has been established that surgical treatment of adenoidal hypertrophy, when performed according to appropriate indications, has a positive impact on the quality of life for children [10]. The literature on improving the management of chronic adenoidal inflammation indicates that adenotomy is the most commonly performed surgical procedure in children at otorhinolaryngological departments [11]. In certain clinical cases, partial adenotomy may be necessary, as adenoidal tissue plays an important role in inducing mucosal and systemic immune responses [10]. Furthermore, an analysis of 22 randomized controlled trials that compared the effectiveness of complete versus partial removal of the palatine tonsils and adenoidal tissues in children revealed that tonsillotomy results in faster recovery within 4 days and a reduction in postoperative complications that require medical intervention within the first week after surgery [12]. Due to the lack of clear criteria for defining adenoid and palatine tonsil regrowth (recurrence), it is impossible to assess its prevalence in the general population. However, up to 25.7 % and up to 11.9 % of operated children undergo repeat adenotomy or tonsillotomy / tonsillectomy, respectively [13]. This emphasizes the importance of monitoring clinical symptoms and providing long-term follow-up for children after surgical intervention. Additionally, it suggests the need for further research into optimal methods of postoperative rehabilitation.

Conservative treatment methods for adenoiditis, such as irrigation therapy and topical antimicrobial agents, have been proven to be effective and are included in Russian clinical guidelines with a level of evidence A [14]. The use of bacterial lysates, immunomodulators, and naturopathic and homeopathic agents has been discussed in the medical community [15]. In this study, we analyzed the effectiveness of probiotic agents

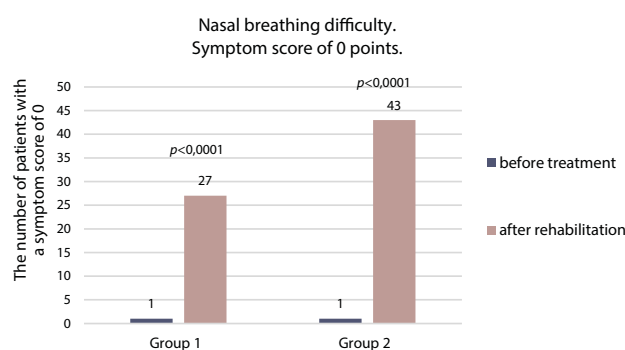


FIG. 1.

Assessment of nasal breathing difficulty by 0 points before and after the treatment.  $p$  is the reliability of the differences between the analyzed parameters before and after treatment

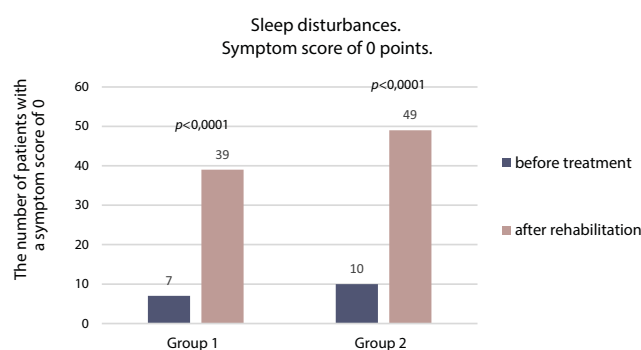


FIG. 2.

Assessment of sleep disorders by 0 points before and after the treatment.  $p$  is the reliability of the differences between the analyzed parameters before and after treatment

in correcting the microecological state of the nasopharynx. The results obtained reliably confirm a reduction in the severity of clinical manifestations after nasopharyngeal irrigation with a probiotic solution, whether used in conjunction with standard medication or after surgical intervention. Oral commensals have a positive effect on recurrent tonsillitis, in particular *S. salivarius*, has previously been shown to be effective when combined with traditional conservative treatment and oral probiotics. In such cases, examined children exhibited faster positive dynamics in both objective local and general manifestations of the disease, as well as subjective complaints such as a foreign body sensation and throat pain. The mechanism of action of this bacterium is based on its ability to produce salivaricin A, a bacteriocin that inhibits the most significant pathogen of recurrent tonsillitis, *S. pyogenes* [8]. There is also evidence to support the effectiveness of probiotic agents for respiratory infections, which are more common in patients with chronic inflammatory conditions of the nasopharynx. For example, a study involving 70 children who took probiotics containing *Bifidobacterium* and *Lactobacillus acidophilus* for 2 weeks showed a reduction in recurrent episodes of infectious rhinitis, otitis media, and bronchiolitis [16]. Maya-Barrios A. et al. (2021) [17] demonstrated that the use of *Limosilactobacillus reuteri* strains is also effective in reducing the symptoms of pharyngitis or tonsillitis in children. A study conducted in Russia reported that the administration of a probiotic containing *Bifidobacterium bifidum* reduces the frequency of respiratory diseases and alleviates their course [18]. The use of oral probiotics for chronic tonsil and adenoid diseases is justified due to the fact that viral infections can trigger exacerbations of these conditions. Based on this information, it can be concluded that probiotic supplementation in conjunction with surgical intervention can significantly improve the quality of life for patients with chronic upper respiratory tract diseases, not only by alleviating clinical symptoms but also by reducing the severity of respiratory infections.

#### Limitations

The limitations of the study include the small number of patients and the short observation period (1 month) after surgical and therapeutic interventions.

#### CONCLUSION

Therefore, the results of the study suggest that the applied treatment methods have a beneficial effect on the dynamics of clinical symptoms, confirming their high effectiveness. Furthermore, a more significant positive impact on the alleviation of difficulty in nasal breathing and sleep disturbances was observed when the probiotic treatment was administered after adenotomy.

#### Conflicts of interest

The authors declare no conflicts of interest.

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## HEREDITARY HYPOFIBRINOGENEMIA IN PRIMARY CARE PEDIATRICS: A CASE REPORT

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### ABSTRACT

**Introduction.** Hypofibrinogenemia is a rare coagulation disorder characterized by reduced plasma fibrinogen levels and an increased risk of hemorrhagic complications. Inherited forms, caused by mutations in the FGA, FGB, or FGG genes, have an estimated prevalence of 1:500,000 and often remain undiagnosed due to an asymptomatic course.

**Aim.** To present a rare clinical case of hereditary hypofibrinogenemia with a paucisymptomatic course in a child, in order to address challenges in diagnosis, long-term follow-up, and determining indications for prophylactic therapy to prevent hemorrhagic complications.

**Materials and Methods.** We report a clinical case of hereditary hypofibrinogenemia in an 8-year-old child. The diagnosis was suspected incidentally during a routine pediatric examination. The diagnosis was subsequently confirmed by a hematologist based on persistently low fibrinogen levels, a positive family history (maternal hereditary hypofibrinogenemia), and genetic testing. Differential diagnosis with acquired forms of hypofibrinogenemia was performed based on clinical and laboratory evaluation, including complete blood count, coagulogram, aggregatogram, biochemical parameters, and instrumental studies.

**Results.** The key feature of this case was the mild clinical presentation, which included a neonatal cephalohematoma, a single episode of blood in stool, and rare epistaxis. This aligns with literature data on the highly variable phenotypic expressivity of hypofibrinogenemia. The importance of a detailed family history and genetic testing in diagnosing inherited coagulopathies, even in the absence of overt bleeding, is emphasized.

**Conclusion.** This case highlights the need for physician vigilance regarding inherited coagulation disorders in patients with abnormal coagulation test results, even in the absence of significant clinical symptoms. The development and implementation of strategies for long-term patient monitoring and the prevention of potential hemostasis-related risks are of paramount importance.

**Keywords:** hereditary hypofibrinogenemia, coagulopathy, hemostasis, fibrinogen, children

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## НАСЛЕДСТВЕННАЯ ГИПОФИБРИНОГЕНЕМИЯ В АМБУЛАТОРНО-ПОЛИКЛИНИЧЕСКОЙ ПРАКТИКЕ ВРАЧА-ПЕДИАТРА (КЛИНИЧЕСКИЙ СЛУЧАЙ)

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### РЕЗЮМЕ

**Введение.** Гипофибриногенемия является достаточно редкой коагулопатией, характеризующейся снижением уровня фибриногена и повышением риска геморрагических осложнений. Наследственные формы, обусловленные мутациями в генах FGA, FGB, FGG, встречаются с частотой 1:500 000 и часто остаются недиагностированными из-за бессимптомного течения.

**Цель исследования.** Представить редкий клинический случай малосимптомного течения наследственной гипофибриногенемии у ребёнка для освещения вопросов диагностики, динамического наблюдения и определения показаний к профилактической терапии для предотвращения геморрагических осложнений.

**Материалы и методы.** В статье представлен клинический случай наследственной гипофибриногенемии у ребенка 8 лет. Диагноз был заподозрен случайно при плановом обследовании у педиатра, а затем при углубленном обследовании у гематолога установлен на основании стойкого снижения уровня фибриногена, подтвержденного генеалогического анамнеза (наследственная гипофибриногенемия у матери, генетического исследования). Проведен дифференциальный диагноз с приобретенными формами гипофибриногенемии на основании клинико-лабораторного обследования: общего анализа крови, коагулограммы, агрегатограммы, биохимических показателей, инструментальных исследований.

**Результаты.** Ключевая характеристика данного клинического наблюдения заключается в малосимптомном течении заболевания, проявляющемся неонатальной кефалогематомой, единичным эпизодом крови в стуле и редкими носовыми кровотечениями, что соответствует данным литературы о вариабельности степени тяжести клинической картины при гипофибриногенемии. Важен тщательный сбор семейного анамнеза в диагностике наследственных коагулопатий, несмотря на отсутствие выраженных геморрагических проявлений, а также генетическое исследование.

**Заключение.** Представленный клинический случай демонстрирует необходимость настороженности врачей в отношении наследственных коагулопатий у пациентов с изменениями коагулограммы даже при отсутствии клинической симптоматики. Особое значение приобретают разработка и реализация подходов к дальнейшему наблюдению за пациентами, а также профилактике потенциальных рисков, связанных с нарушениями системы гемостаза.

**Ключевые слова:** наследственная гипофибриногенемия, врожденные коагулопатии, гемостаз, фибриноген, дети

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## INTRODUCTION

Hypofibrinogenemia is a coagulation disorder characterized by a reduced plasma fibrinogen concentration [1, 2]. According to the World Federation of Hemophilia and national registries, fibrinogen deficiency accounts for approximately 8.0 % of all rare coagulopathies [3]. The prevalence of this condition is approximately 1 case per 500,000 population [4]. Despite its low prevalence, timely detection of hypofibrinogenemia is critically important for the prevention of potential hemorrhagic complications.

According to the classification of hypofibrinogenemias, hereditary (congenital) and acquired forms are distinguished [1]. Hereditary hypofibrinogenemia often remains unnoticed, but may have clinical consequences, including bleeding and an increased risk of thrombosis [3, 5]. In contrast to acquired forms, which are often caused by liver disease or disseminated intravascular coagulation (DIC), hereditary hypofibrinogenemia is characterized by a persistently low fibrinogen level throughout life and can be confirmed by a positive family history [1].

Historically, hereditary forms of fibrinogen deficiency were first described in the early 20<sup>th</sup> century, and in 1999, mutations in the genes responsible for its synthesis were identified [6]. Hereditary hypofibrinogenemia, associated with the genes encoding the fibrinogen chains (FGA, FGB, FGG), can lead to hemostatic abnormalities [7, 8]. Fibrinogen plays a key role in the blood coagulation process, being converted to fibrin by thrombin and forming a fibrin clot [1, 3, 9].

In clinical practice, two types are distinguished: hypofibrinogenemia and afibrinogenemia, in which plasma fibrinogen levels are below 1.8 g/L and 0.1 g/L, respectively [10]. In most cases, patients have an asymptomatic course or minimal bleeding from mucous membranes, with bleeding after trauma or surgical interventions usually of the microcirculatory type [3].

Diagnostic evaluation of hereditary hypofibrinogenemia is based on plasma fibrinogen levels, other baseline coagulogram parameters, and molecular genetic analysis to identify mutations in the relevant genes [11, 12]. In severe fibrinogen deficiency, prolongation of activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT) is observed, which is due to the absence of the necessary substrate for the conversion of fibrinogen to fibrin by thrombin [3].

Diagnosis of the hereditary form requires high accuracy and reliability, which underscores the need for timely detection and differential diagnosis with acquired forms of hypofibrinogenemia [13]. Early diagnosis (particularly of severe hypofibrinogenemia and afibrinogenemia) facilitates preventive measures and the prescription of replacement therapy when necessary to reduce the risk of complications associated with hemostatic abnormalities.

This clinical case of a child with hereditary hypofibrinogenemia illustrates the importance of a timely comprehensive approach to diagnosis and follow-up of patients. The study protocol was approved by the Ethics Committee of the Ivano–Matreninskaya City Children’s Clinical Hospital (Protocol No. 10 dated February 10, 2025) and complied with the principles of the Declaration of Helsinki.

## AIM

To present a rare clinical case of hereditary hypofibrinogenemia with a paucisymptomatic course in a child, in order to address challenges in diagnosis, long-term follow-up, and determining indications for prophylactic therapy to prevent hemorrhagic complications.

## MATERIALS AND METHODS

### History

An 8-year-old female patient, accompanied by her mother, presented to a hematologist at the outpatient department of the Ivano–Matreninskaya City Children’s Clinical Hospital in February 2025 regarding abnormal coagulogram findings from January 15, 2024: decreased fibrinogen to 1.17 g/L, prolonged APTT to 44 sec, prolonged TT to 29 sec, and decreased PT to 61.0 %. The examination was performed while the patient was in full clinical health and had no complaints.

The child was born from the first pregnancy, 1<sup>st</sup> delivery. According to the mother, the pregnancy was uncomplicated. Delivery was spontaneous at term (38 weeks). Birth weight was 3850 g; length 54 cm. Apgar score was 7–9. The infant cried immediately and was breastfed immediately after birth. Breastfeeding continued until 9 months of age. Growth and development were age-appropriate. Vaccinations were administered according to the national immunization schedule.

During collection of a detailed bleeding history, a family history was revealed: the mother had been observed for several years to have decreased fibrinogen to an average of 0.87 g/L, prolonged APTT to 44 sec, prolonged TT to 29 sec, prothrombin time to 13.2 sec, and decreased PT to 57.9 %, based on which a hematologist had diagnosed hypofibrinogenemia, and she was under dispensary follow-up. Genetic analysis confirmed the presence of a heterozygous mutation in the FGB G455A gene in the mother. Further family history did not reveal any other cases of significant bleeding or established coagulopathies in close maternal relatives (grandmother, grandfather).

From the medical history, it is known that the child suffered from a cephalohematoma at birth. In 2016, at the second week of their life, blood was found

in their stool, prompting the child to be examined at the Irkutsk Regional Infectious Diseases Clinical Hospital, the Irkutsk Regional Children's Clinical Hospital, and the Ivano-Matreninskaya City Children's Clinical Hospital, with a diagnosis of diarrhea of unspecified etiology. At 8 months old, the patient was diagnosed with hemangioma on the left side of the trunk and underwent surgical treatment. Rare episodes of epistaxis have also been noted. However, previous monitoring of complete blood count and coagulation tests were not performed.

### Physical examination

General condition was satisfactory. Well-being was not affected. Position was active. Axillary body temperature was 36.2°C. Blood pressure was 106/68 mmHg. Respiratory rate was 22/min. Heart rate was 85/min. Height was 130 cm; body weight was 33 kg. SDS BMI = 1.65. Skin was of normal color and clear. Visible mucous membranes were of normal color and clear. Subcutaneous adipose tissue was moderately developed. Peripheral lymph nodes were not palpable. Musculoskeletal system showed no deformities, joint mobility was full, and palpation was painless. The chest was of normal shape and size, symmetrical, painless on palpation, with clear pulmonary sound on percussion. Auscultation revealed vesicular breathing, conducted to all areas, no wheezing. The borders of relative and absolute cardiac dullness were within age-related norms. On auscultation, heart sounds were clear, rhythmic, with no murmur. The abdomen was of normal shape and size on inspection, soft and painless on palpation. The liver and spleen were not palpable. Intoxication, anemic, hemorrhagic, lymphoproliferative, hyperplastic, and ossalgic syndromes were absent.

### Results of laboratory and instrumental studies

Complete blood count (January 14, 2025): hemoglobin – 139.0 g/L, erythrocytes –  $5.07 \times 10^{12}/L$ , reticulocytes – 1.0 %, hematocrit – 41.8 %, platelets –  $272.0 \times 10^9/L$ , leukocytes –  $8.84 \times 10^9/L$ , segmented neutrophils – 56.0 %, monocytes – 5.9 %, lymphocytes – 36.1 %, eosinophils – 2.0 %, and ESR – 2 mm/h.

Biochemical blood test (January 14, 2025): total bilirubin – 10.2  $\mu\text{mol}/L$ , indirect bilirubin – 4.7  $\mu\text{mol}/L$ , direct bilirubin – 5.5  $\mu\text{mol}/L$ , ALT – 16.0 U/L, AST – 22.0 U/L, cholesterol – 3.1 mmol/L, total protein – 62.0 g/L, albumin – 41.0 g/L, urea – 3.2 mmol/L, creatinine – 53.0  $\mu\text{mol}/L$ , GFR – 124 mL/min/1.73m<sup>2</sup>, alkaline phosphatase – 125.0 U/L, LDH – 223.0 U/L, glucose – 4.3 mmol/L, CRP – 3 mg/L, RF – 1.2 IU/mL, ASO – 120 IU/mL, ferritin – 52.0 ng/mL, serum iron – 15.0  $\mu\text{mol}/L$ , transferrin saturation – 34.0 %, total iron-binding capacity – 73.2  $\mu\text{mol}/L$ , vitamin B12 – 250.0 pg/mL, and folic acid – 15 ng/mL.

Coagulogram (three measurements):

January 15, 2024: APTT – 44 sec, PT – 61 %, INR – 1.1, TT – 29 sec, fibrinogen – 1.17 g/L, and D-dimer – 0.2  $\mu\text{g}/\text{mL}$ .

February 14, 2025: APTT – 43.3 sec, PT – 61.5 %, INR – 1.12, TT – 22.9 sec, fibrinogen – 1.2 g/L, and D-dimer – 0.1  $\mu\text{g}/\text{mL}$ .

February 28, 2025: APTT – 44.5 sec, PT – 59.2 %, INR – 1.1, TT – 23.8 sec, fibrinogen – 1.1 g/L, and D-dimer – 0.2  $\mu\text{g}/\text{mL}$ .

Fibrinogen concentration was determined using the Clauss clotting method. Activated APTT, PT, TT, and other coagulogram parameters were measured on an automated analyzer. Platelet aggregation was studied using standard inducers (ADP, epinephrine, collagen, ristocetin, arachidonic acid) on a laser aggregometer.

Platelet aggregation with inducers (January 15, 2024): ADP – 71.4 %, epinephrine – 40.4 %, collagen – 77.1 %, ristocetin – 76.3 %, and arachidonic acid – 75 %.

Dysfibrinogenemia testing (March 20, 2025): ancistron test – 19 sec (normal 16–22 sec).

Antinuclear antibodies (February 14, 2025): less than 1:40.

Anti-dsDNA antibodies (February 14, 2025): 15 IU/mL – negative.

Anti-cardiolipin IgM and IgG antibodies (February 14, 2025): negative.

Lupus anticoagulant (February 14, 2025): not detected.

Urinalysis (January 14, 2025): light yellow, clear, pH 5.0, density 1020, glucose – not detected, ketones – not detected, bilirubin – not detected, squamous epithelium – 1 cell/ $\mu\text{L}$ , leukocytes – 1–2 cells/ $\mu\text{L}$ , erythrocytes – 1 cell/ $\mu\text{L}$ , protein – not detected, bacteria – not detected, mucus – trace, and salts – not detected.

24-hour proteinuria (January 15, 2025): 0.05 g/day.

Zimnitsky test (January 20, 2025): 6.00–9.00 – specific gravity 1018, 9.00–12.00 – 1022, 12.00–15.00 – 1020, 15.00–18.00 – 1019, 18.00–21.00 – 1017, 21.00–24.00 – 1019, 24.00–03.00 – no urine, 03.00–06.00 – 1010; 24-hour diuresis – 1350 mL, and day/night diuresis ratio – 3:1.

ELISA for helminths (January 21, 2025): anti-Toxocara canis – negative, *Opisthorchis felineus* – negative, *Echinococcus* spp. – negative, *Trichinella spiralis* – negative, and *Ascaris lumbricoides* – negative.

ELISA for TORCH infections (January 21, 2025): anti-CMV IgM, IgG – negative, anti-Toxo IgM, IgG – negative, anti-HSV ½ IgM, IgG – negative, anti-Rubella IgM, IgG – negative, anti-Herpes IgM, and IgG – negative.

ELISA for Epstein-Barr virus (January 21, 2025): anti-Epstein Barr IgM, IgG – negative.

ELISA for hepatitis (January 14, 2025): HBsAg, anti-HBcore – not detected, anti-HCV – not detected, and anti-HAV IgM – not detected.

ELISA for HIV (January 14, 2025): negative.

Mantoux test with 2 TU (May 14, 2024): negative.

ENT (January 14, 2025): nasal breathing free, nasopharyngeal mucosa pink, clear; palatine tonsils of normal size, no plaques, diagnosis – healthy.

Throat and nasopharyngeal swab for flora (January 14, 2025): no growth of pathogenic microflora, single colonies of *Streptococcus viridans* 10<sup>3</sup> CFU/mL.

Dentist (January 14, 2025): superficial caries of tooth 3.5.

Gynecologist (January 15, 2025): healthy.

Electrocardiography (January 15, 2024): sinus rhythm, HR 94–103/min, electrical axis of the heart in vertical position.

Comprehensive ultrasound of the abdominal organs, retroperitoneal space, peripheral lymph nodes, pelvis, and thyroid gland (January 15, 2024): no pathology detected.

Genetic testing of fibrinogen genes *FGB* and *FGG* (Sanger sequencing) (April 15, 2025): a heterozygous mutation *FGB*: -455G>A was identified in the *FGB* gene.

## DISCUSSION

The presented case of hereditary hypofibrinogenemia in an 8-year-old child demonstrates the typical features of the medical history (biological, genealogical), clinical, laboratory and instrumental diagnosis of hemostatic disorders, as well as the search for acquired causes that may lead to this condition, and the treatment of this rare inherited coagulation disorder.

The use of a comprehensive range of clinical and laboratory-instrumental studies in our patient made it possible to exclude pathology that could cause acquired secondary hypofibrinogenemia, such as infectious diseases, liver pathology, DIC, drug effects, hemolysis, kidney disease, hematological malignancies, etc.

Based on the data, including the family history (a lifelong persistent hypofibrinogenemia in the child's mother), the paucisymptomatic course of the disease (minimal hemorrhagic manifestations during the 8-year period of the child's life), stable hypofibrinogenemia confirmed by three hemostasis tests, prolonged thrombin time, exclusion of secondary causes of hypofibrinogenemia, and genetic analysis of fibrinogen genes, the patient was diagnosed with hereditary hypofibrinogenemia.

Hereditary hypofibrinogenemia is a well-known condition. In previous reports, there was not always a clear distinction made between hereditary and acquired forms of factor I deficiency [8]. This distinction is crucial for diagnosis and management.

Considering the prevalence of this pathology, it should be noted that the epidemiological characteristics of hypofibrinogenemia have now been adequately studied. Specifically, in a study conducted by Paraboschi E.M. et al. (2017), it was demonstrated that among 342 registered cases of congenital fibrinogen disorders, 58 % were hypofibrinogenemias, 28 % were dysfibrinogenemias, and only 14 % were afibrinogenemias. Additionally, the authors noted significant

variation in clinical manifestations based on the type of mutation [4].

In a study conducted by Yakovleva E.V. et al. (2022), data from 47 patients with hereditary fibrinogen disorders were analyzed. The study found that 38 % of the patients exhibited an asymptomatic course [10]. The authors emphasize that, even in the absence of clinical symptoms, these patients remain at a high risk of hemorrhagic complications during invasive procedures [10].

Of particular interest are the findings of Neerman-Arbez M. et al. (2016) that hereditary hypofibrinogenemia often presents in childhood and is frequently detected incidentally during routine examinations, as presented in our clinical case. In some instances, a family history may not be available due to the variable nature of the mutations [11].

The severity of hemorrhagic manifestations in patients with hereditary hypofibrinogenemia directly correlates with the degree of decrease in plasma fibrinogen levels [8]. Patients with severe hypofibrinogenemia (0.2–0.3 g/L) may experience spontaneous bleeding, whereas those with milder forms (1.0–1.5 g/L) usually only bleed or develop hematomas after trauma or invasive procedures [3, 8]. Our patient has a paucisymptomatic course, with a mean fibrinogen level of 1.1–1.2 g/L, comparable to those of her mother, who also has a mean fibrinogen level of 0.87–0.97 g/L.

Despite the typical laboratory signs, genetic testing is a method used to diagnose hereditary hypofibrinogenemia [6, 11, 12].

Long-term follow-up is recommended for the child, including monitoring of the coagulogram profile and other diagnostic tests, due to their hereditary nature and the child's female sex. If necessary, consideration may be given to replacement therapy, particularly in cases involving surgery or other invasive procedures [13].

## CONCLUSION

All physicians should be aware that hereditary hypofibrinogenemia may present with a frequently latent or paucisymptomatic course, and may not be accompanied by bleeding for many years, or may present with rare bleeding, usually of the microcirculatory type. It is also important to identify this pathology in patients with a positive family history, as early diagnosis and adequate follow-up can help to implement preventive measures and reduce the risk of hemorrhagic complications in such cases.

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### Conflict of interest

The authors declare no conflict of interest.

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## TYROSINEMIA TYPE 1: A CASE REPORT

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**ABSTRACT**

**Background.** Hereditary Tyrosinemia Type 1 (HT1) is an orphan disease belonging to the group of amino acid metabolism disorders, with an autosomal recessive pattern of inheritance. It is caused by a mutation in the FAH gene, which encodes the enzyme fumarylacetoacetate hydrolase. The clinical presentation is polymorphic. Diagnosis is based on the detection of specific metabolites in biological samples and molecular genetic testing. Early diagnosis determines the effectiveness of therapy and the prognosis of the disease.

**Clinical case description.** We present a clinical case of early detection of HT1 in a female newborn through expanded neonatal screening. The clinical picture was characterized by hepatomegaly, coagulopathy, and specific biomarkers, including elevated levels of succinylacetone in the blood. This finding served as the basis for immediate initiation of nitisinone therapy and an individually calculated low protein diet supplemented with a specialized amino acid mixture. Molecular genetic testing confirmed the diagnosis: a previously described pathogenic nucleotide sequence variant, chr15:80181069G>C in the FAH gene in a homo/hemizygous state, was identified. This variant leads to a missense substitution c.1090G>C, p.(Glu364Gln). During the follow up period, the patient has shown a favorable course of the disease.

**Conclusion.** Early diagnosis of HT1 based on specific succinylacetone screening and timely initiation of pathogenetic therapy combined with dietary management ensure a favorable prognosis for the disease.

**Keywords:** hereditary tyrosinemia type 1 (HT1), newborn infants, inherited metabolic disorders, clinical case

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## ТИРОЗИНЕМИЯ 1 ТИПА: ОПИСАНИЕ КЛИНИЧЕСКОГО СЛУЧАЯ

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### РЕЗЮМЕ

*Наследственная тирозинемия 1 типа (НТ1) — орфанное заболевание из группы нарушений метаболизма аминокислот с аутосомно-рецессивным типом наследования, обусловленная мутацией в гене FAH, который кодирует фермент фумарилацетоацетатгидролазу. Клиническая картина полиморфна. Диагностика заболевания основывается на выявлении специфических метаболитов в биологическом материале и молекулярно-генетическом исследовании. Ранняя диагностика определяет эффективность терапии и прогноз заболевания.*

**Описание клинического случая.** Представлен клинический случай раннего выявления НТ1 у девочки в рамках расширенного неонатального скрининга, клиническая картина характеризовалась гепатомегалией, коагулопатией и специфическими маркерами – повышение сукцинилацетона в крови, что послужило основанием для начала терапии нитизиномом и индивидуально рассчитанной низкобелковой диеты со специализированной аминокислотной смесью. Молекулярно-генетическое исследование подтвердило диагноз: выявлен описанный ранее как патогенный вариант нуклеотидной последовательности chr15:80181069G>C в гене FAH в гомо-/гемизиготном состоянии, приводящий к миссенс-замене с.1090G>C, p.(Glu364Gln). За время наблюдения за пациентом отмечается благоприятное течение заболевания. **Заключение.** Ранняя диагностика НТ1 на основе специфического скрининга сукцинилацетона и своевременное начало патогенетической терапии в сочетании с диетотерапией обеспечивают благоприятный прогноз заболевания.

**Ключевые слова:** наследственная тирозинемия 1 типа, новорожденные дети, наследственные болезни обмена веществ, клинический случай

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Hereditary tyrosinemia type 1, also known as hepatorenal tyrosinemia (HT1) (OMIM 276700), is an orphan disease belonging to the group of amino acid metabolic disorders with an autosomal recessive mode of inheritance. Its prevalence averages 1 per 100,000–200,000 live births, with significant variability reflecting demographic history and population genetics. The highest prevalence worldwide is observed in Quebec, Canada (1:1,846 live births). In Russia, marked regional heterogeneity has been identified with the highest incidence in the Chechen Republic (1:16,020) [1]. Meanwhile, the epidemiological profile of East Asia remains poorly characterized in literature.

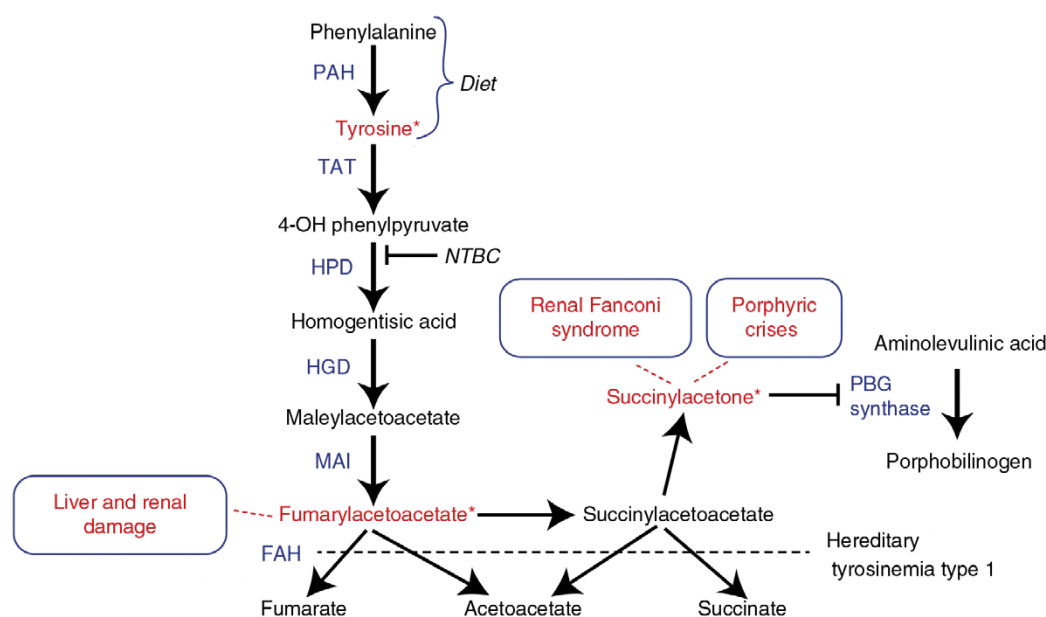
The *FAH* gene responsible for the development of this disorder is mapped to the chromosomal locus 15q23–q25. Its genomic organization spans a region of 30–35 kb and includes 14 coding exons. To date, more than 100 pathogenic variants of the *FAH* gene associated with HT1 have been described [2]. The global literature shows a clear geographical distribution of key pathogenic alleles. The splicing variant c.1062+5G>A is predominant in Northern Europe and the US, while c.554-1G>T mutation is characteristic of Central and Eastern Europe [3]. In East Asia, variants c.1100G>A and c.192G>T are more common. Studies on the Russian cohort revealed pronounced ethnic stratification of pathogenic variants. The c.1025C>T allele is prevalent among Chechen patients, while missense substitution c.1090G>C (p.E364Q) is frequent in Buryats, Nents, and Yakuts [4, 5].

The pathogenesis of the disease is based on a deficiency of the enzyme fumarylacetoacetate hydrolase (FAH), encoded by the *FAH* gene (15q25.1), leading to the accumulation of the abnormal metabolite succinylacetone in the body [6]. FAH deficiency causes accumulation of maleylacetoacetate, which with preserved maleylacetoacetate isomerase activity undergoes non-enzymatic

reduction to succinylacetone, exhibiting pronounced hepatotoxicity and nephrotoxicity. The key mechanism for its pathological action is potent inhibition of  $\delta$ -aminolevulinic acid dehydratase, leading to the accumulation of  $\delta$ -aminolevulinic acid, causing porphyria-like crises, manifested clinically as neurological disorders and jaundice. At the same time, intracellular transport is disrupted and the activity of tyrosine catabolic enzymes is modulated, exacerbating multi-organ involvement (Figure).

HT1 is characterized by high mortality in the absence of timely therapy: without treatment, up to 90 % of patients with the acute form die before the age of 10 from liver failure or hepatocellular carcinoma. There are two forms of the disease: acute and chronic. They differ in onset age and rate of progression. Acute form is the most common (75 %) and presents in neonatal period or first months of life with constellation of symptoms including hepatopathy (jaundice, hepatomegaly, coagulopathy), metabolic decompensation (vomiting, dehydration, failure to thrive), neurological disorders (hypotonia), and a characteristic (cabbage-like) odor. Intercurrent infections increase risk of acute hepatic crisis with ascites and bleeding. Without treatment prognosis is fatal due to liver cirrhosis or hepatocellular carcinoma before 1 year age. The chronic form is characterized by a delayed onset, an indolent course, and the predominance of neurological (polyneuropathy) and cardiac (hypertrophic cardiomyopathy) manifestations. The onset occurs in older children and adolescents [8].

The gold standard for early diagnosis of hereditary tyrosinemia type 1 is the measurement of succinylacetone in dried blood spots using tandem mass spectrometry (TMS/MS-MS). Succinylacetone is a highly specific and pathognomonic metabolite absent in healthy newborns. It accumulates exclusively in patients with fumarylacetoacetate



**FIG.**  
Tyrosine metabolism disorders observed in tyrosinemia type I [7]

hydrolase deficiency. Its detection in blood collected on days 3–5 of life provides diagnostic sensitivity and specificity exceeding 95 %. This allows verification of the diagnosis before the development of clinical manifestations of hepatic and renal involvement [9, 10].

The mainstay of treatment is dietary therapy, with restriction of tyrosine and phenylalanine intake. Currently, nitisinone (Nitisinon (Orfadin®) (NTBC, 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione) is used to treat the disease. It can prevent acute hyperbilirubinemic crises and slow down the progression of liver cirrhosis and Fanconi syndrome.

In domestic literature, the main focus is on diagnostic algorithms and clinical markers for suspicion of hereditary tyrosinemia type 1 during the disease manifestation stage [1, 11]. The implementation of expanded newborn screening has changed the clinical paradigm fundamentally: in most cases, diagnosis is verified before symptoms appear. Issues related to when to start pathogenetic therapy and its long-term effect on prognosis have become more important. The question about whether preventive treatment modifies clinical outcomes and the dynamics of biochemical markers compared to therapy initiated during a metabolic crisis remains unanswered.

## AIM

To describe the clinical and laboratory presentation of a patient with hereditary tyrosinemia type 1, detected at an early stage.

**Informed consent.** Written informed consent was obtained from the legal representative of the patient for the use of their medical data for scientific purposes.

## RESULTS

Patient D., a female, was born on February 6, 2024, at the age of two years, as a result of the fourth pregnancy, which was not remarkable. The first pregnancy ended in delivery, and the sibling was healthy. The second and third pregnancies resulted in medical abortions. The parents were of Buryat ethnicity and were not consanguineous. They were somatically healthy, and their delivery was spontaneous, unassisted, and took place at 38 weeks. The birth weight was 4170 g, the length was 53 cm, and Apgar scores were 7/8. At birth, the condition was satisfactory, and the infant cried immediately after birth. Breastfeeding began on day 1 and the infant actively fed. On day 2, a sample was taken for newborn screening. On day five, the infant was discharged with a diagnosis of cerebral ischemia, grade 1 patent foramen ovale, patent ductus arteriosus, and congenital anomaly of the urinary system (left-sided pyeloectasia).

As part of the expanded newborn screening program, tandem mass spectrometry was performed at Competence Laboratory III A in Irkutsk. This revealed an elevated concentration of succinylacetone (SA) in the dried blood

spot – 3.5  $\mu\text{M}/\text{mL}$ , which is above the normal range of 0–2  $\mu\text{M}/\text{mL}$ . A presumptive diagnosis of tyrosinemia type 1 was made. To confirm the diagnosis, biological material was sent for DNA testing to the Bochkov Medical Genetics Research Center.

Prior to receiving the results of DNA testing, the infant was admitted to hospital on day 6 after birth. On admission, the objective findings were: weight – 3890 g (-280 g from birth weight), condition of moderate severity, skin clear and pink. On pulmonary auscultation, vesicular breathing; heart sounds rhythmic; abdomen distended; liver +1.5 cm below the costal margin, the edge smooth and elastic; spleen not palpable. Neurological status: muscle hypotonia with symmetrical and decreased reflexes, no pathological reflexes. Hemodynamics stable: heart rate 158 bpm, and saturation 99 %.

Laboratory findings on admission: the hemogram showed leukocytosis ( $39.7 \times 10^9/\text{L}$ ; normal range  $9.0\text{--}25.0 \times 10^9/\text{L}$ ) without a pronounced left shift.

Biochemical analysis on admission revealed hyperbilirubinemia with a predominance of direct fraction (total bilirubin – 180.3  $\mu\text{mol}/\text{L}$ ; normal range 0–17.0  $\mu\text{mol}/\text{L}$ , direct – 11.7  $\mu\text{mol}/\text{L}$ ; normal range 0–5.1  $\mu\text{mol}/\text{L}$ ) and cholestasis syndrome: alkaline phosphatase – 2110.0 U/L (normal range 0–1076.0 U/L); CRP – 1.4 mg/L (normal range 0.2–9.0 mg/L); coagulogram showed prolongation of prothrombin time (PT – 14.5 sec; normal range 9.9–11.8 sec), decreased prothrombin index (PTI – 45.0 %; normal range 70–130 %), prolongation of activated partial thromboplastin time (APTT – 32.8 sec; normal range 22.1–28.1 sec), and elevated international normalized ratio (INR – 1.51; normal range 0.85–1.15).

Alpha-fetoprotein level – 56,000 ng/mL (normal for children aged 0 to 3 months: less than 1,000 ng/mL).

Tandem mass spectrometry: tyrosine – 307.4  $\mu\text{mol}/\text{L}$  (normal range 10–300  $\mu\text{mol}/\text{L}$ ); urinary succinylacetone measured by high-performance liquid chromatography with tandem mass spectrometry – 5.04 mM/mol creatinine (normal range 0–2.2 mM/mol creatinine).

In view of the identified laboratory abnormalities, the child was prescribed antibacterial therapy – ampicillin + sulbactam at a dose of 150 mg/kg/day; ursodeoxycholic acid – 30 mg/kg/day; vitamin K (1 mg intramuscularly, once); dietary therapy – a low-protein diet using a specialized amino acid formula without tyrosine and phenylalanine (Anamix Infant®) + breast milk, with a protein intake of 1.8 g/kg. The energy value of the diet is 95–145 kcal/kg/day. Pathogenetic therapy with nitisinone (NTBC, 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione) was started at a dose of 2 mg/kg/day until normalization of urine and blood succinylacetone levels, with subsequent dose adjustment based on expected body weight.

Sequencing performed at the Bochkov Medical Genetic Research Center identified a previously described pathogenic variant (HGMD\_ID CM1619683) in exon 13 of the FAH gene (NM\_000137.3). The nucleotide sequence variant chr15:80181069G>C in the FAH gene in a homo/hemizygous state leads to a missense substitution c.1090G>C,

p.(Glu364Gln), which is consistent with the diagnosis of hereditary tyrosinemia type 1 (ICD-10 code: E70.2).

During treatment, the child showed positive laboratory dynamics. The normalization of hemogram parameters was observed: leukocytes –  $8.1 \times 10^9/L$  (normal range  $6.6\text{--}11.2 \times 10^9/L$ ). Biochemical analysis showed mild residual hyperbilirubinemia with predominance of direct fraction (total bilirubin –  $18.4 \mu\text{mol/L}$ ; normal range  $0\text{--}17.0 \mu\text{mol/L}$ , direct –  $9.8 \mu\text{mol/L}$ ; normal range  $0\text{--}5.1 \mu\text{mol/L}$ ), alkaline phosphatase –  $819.0 \text{ U/L}$  (normal range  $0\text{--}1076.0 \text{ U/L}$ ). Coagulogram: PT – 9.9 sec (normal range 9.9–11.8 sec), PTI – 112.0 % (normal range 70–130 %), APTT – 31.1 sec (normal range 22.1–28.1 sec), INR – 0.96 (normal range 0.85–1.15). Tandem mass spectrometry: tyrosine –  $193.4 \mu\text{mol/L}$  (normal range  $10\text{--}300 \mu\text{mol/L}$ ), SA –  $0.5 \mu\text{mol/L}$  (normal range  $0\text{--}2 \mu\text{mol/L}$ ); urinary succinylacetone measured by high-performance liquid chromatography with tandem mass spectrometry –  $0.6 \text{ mM/mol creatinine}$  (normal range  $0\text{--}2.2 \text{ mM/mol creatinine}$ ).

The child was discharged from the hospital at the age of 1.5 months for outpatient follow-up. The weight gain at discharge was +1720 g, and the length increased by 11 cm. Following normalization of succinylacetone levels, the nitisinone dose was adjusted to 1 mg/kg/day, with recalculation based on the expected body weight.

During outpatient follow-ups, blood tyrosine and succinylethane concentrations are monitored every 3 months according to clinical guidelines. Succinylacetone remains within reference values, while tyrosine shows moderate elevation ( $362\text{--}465 \mu\text{mol/L}$ ; normal range:  $10\text{--}300 \mu\text{mol/L}$ ).

Currently, the child is 2 years old, weight – 14,260 g, height – 92 cm. The child's growth and development are appropriate for their age. Somatic and neurological status is unremarkable. The blood succinylacetone concentration is  $0.2 \mu\text{mol/L}$ , urinary succinylacetone is  $0.44 \text{ mM/mol creatinine}$ , alpha-fetoprotein is  $2.3 \text{ IU/mL}$  (less than  $10.0 \text{ IU/mL}$ ), and liver function tests are within reference ranges. Ultrasound of liver and kidneys – no pathology. Serum nitisinone concentration –  $17.547 \mu\text{g/mL}$  (therapeutic range  $13.17\text{--}19.73 \mu\text{g/mL}$ ). Currently, the child is receiving nitisinone (NTBC, 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione) at a dose of 1 mg/kg/day. Dietary therapy – a low-protein elimination diet based on amino acid formula without tyrosine and phenylalanine (Nutrigen®20). Phenylalanine + tyrosine – 60 mg/kg/day. Energy value of the diet – 900–1800 kcal/day.

## DISCUSSION

The presented case demonstrates a classic manifestation of the acute form of HT1 in the neonatal period. Key diagnostic “red flags” included:

- elevated succinylacetone levels in blood and urine;
- a combination of unconjugated and conjugated hyperbilirubinemia, with coagulopathy;
- a significant elevation of alpha-fetoprotein levels, exceeding the normal range.

Early detection of succinylacetone, a specific marker of HT1, allowed verification of the diagnosis before the development of irreversible liver changes and the timely initiation of nitisinone therapy, in accordance with clinical guidelines, which recommend that nitisinone initiation should not be delayed pending genetic confirmation when the clinical and biochemical picture is consistent with a positive succinylacetone test.

Dietary therapy remains an essential component of comprehensive management of HT1 patients, acting synergistically with pathogenetic nitisinone therapy. Current international and Russian clinical guidelines recommend individualized calculated restriction of natural protein intake to 20–30 % of the age-specific norm, supplemented with essential amino acids, vitamins, and trace elements using specialized formulas that do not contain phenylalanine and tyrosine [1, 7]. The key principle is not complete protein exclusion, but rather precise dosing of the “natural” protein intake, followed by supplementation with amino acid formulations. Regular monitoring every 3 months, depending on age and stability of parameters, allows for timely dietary adjustments, preventing both metabolic decompensations and iatrogenic protein-energy malnutrition. Restriction of traditional sources of complete protein increases the risk of deficiencies in calcium, iron, zinc, and vitamins B12 and D, necessitating mandatory nutraceutical support, regular monitoring of biochemical markers, and the involvement of a clinical dietician.

In the pathogenetic therapy of HT1, nitisinone (trade name Orfadin®) plays a key role. It was registered in the Russian Federation in 2009 (registration number LSR-007750/09). The pharmacological action of the drug is based on the inhibition of an enzyme involved in tyrosine catabolism, thereby blocking the formation and accumulation of toxic metabolites that damage hepatocytes and nephrons.

Clinical observations demonstrate that the initiation of nitisinone therapy at early stages of the disease prevents liver failure, reduces the incidence of renal involvement, and significantly decreases the risk of hepatocellular carcinoma [12–14]. International experience with nitisinone (NTBC, 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione) demonstrates a high prophylactic efficacy against hepatocellular carcinoma in patients with hereditary tyrosinemia type 1. When therapy is started before 2 months of age, a marked reduction in complications is observed, from 40 % in later initiation to 5 % in early initiation [15].

The prognosis for life with HT1 depends on several factors, including the timeliness of therapy initiation at the preclinical stage and adherence to treatment. This is achievable either through population-based newborn screening or through prenatal diagnosis in families with a positive history.

It is important to understand that metabolic decompensation can occur even during compliant treatment, triggered by respiratory or bacterial infections, stress, surgical interventions, fasting, or excessive physical exertion.

Therefore, caregivers should be informed about the risk of acute crises, precipitating factors, and symptoms of impending metabolic decompensation [1]. The management of HT1 patients requires a multidisciplinary approach involving pediatricians, geneticists, dietitians, and hepatologists, as well as lifelong monitoring of biochemical and instrumental parameters.

## CONCLUSION

The implementation of preventive therapy from the first days of life is made possible by identifying of specific laboratory markers of the disease before the development of clinical symptoms. This is ensured by a newborn screening program. In this regard, further refinement of management algorithms for patients with hereditary tyrosinemia type 1, including validation of target biochemical parameter ranges to monitor pathogenetic treatment effectiveness and long-term surveillance, is required.

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The study was conducted within the framework of the research topic: "Development of individualized approaches to the treatment of childhood orphan diseases: fundamental and clinical aspects".

### Conflict of interest

The authors declare no conflict of interest.

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## PREVENTIVE MEDICINE

### LEUKOCYTES AND PHAGOCYTOSIS IN MEN OF EXTREME PROFESSIONS IN THE SUBARCTIC REGION

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#### ABSTRACT

*Men over the age of 45 are a socially significant group, most of whom have reached professional maturity and make a significant contribution to the country's economy and their families. In the Arkhangelsk Region, the health status of 40–45-year-old men (57 %) is characterized by cardiovascular pathology, 14.7 % – by gastrointestinal pathology, 47.4 % – by bronchopulmonary pathology, and 17.0 % – by musculoskeletal disorders. Male specialists from the Hydrographic Service in Arkhangelsk carry out planned and special hydrographic works during the navigation period on a rotational basis, making short-term frequent trips of 16 days in the Northern Seas.*

**The aim.** To analyze the features of leukocyte content and phagocytosis in hydrographers of the Arkhangelsk region of the Hydrographic Service of the Russian Federation, taking into account their age and work experience in the northern seas.

**Materials and methods.** Thirty-six men working in the hydrographic service of the Federation Council of the Russian Federation were examined with short 16-day shifts: 15 men aged 30–40 with 10 years of work experience and 21 men aged 50–65 with 20 years of work experience. The leukocytes count and phagocytosis in peripheral venous blood were determined, taking into account age and work experience. For statistical processing, Microsoft Excel 2010 and SPSS 20.0 for Windows were used as software packages.

**Results.** It has been established that hydrographers of the younger age group with 10 years of work experience have a 1.5–2 times higher incidence of leukocyte, lymphocyte, and monocyte deficiency, which is associated with low phagocytosis activity. In the older age group of individuals with 20 years of work experience, leukocytosis, lymphocytosis, and monocytosis are recorded 1.5 times more frequently against the background of increased phagocytosis activity.

**Conclusion.** The phagocytic activity of neutrophils is higher in the older age group with 20 years of experience in extreme conditions.

**Keywords:** leukocytes, lymphocytes, phagocytosis, hydrographic service, subarctic region

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## СОДЕРЖАНИЕ ЛЕЙКОЦИТОВ И ФАГОЦИТОЗ У МУЖЧИН ЭКСТРЕМАЛЬНЫХ ПРОФЕССИЙ В УСЛОВИЯХ СУБАРКТИЧЕСКОГО РЕГИОНА

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### РЕЗЮМЕ

*Мужчины старше 45 лет являются социально значимой группой, большинство из которых достигло профессиональной зрелости, вносит значительный вклад в экономику страны, своей семьи. В Архангельской области состояние здоровья 40–45-летних мужчин (57 %) характеризуется сердечно-сосудистой патологией, 14,7 % – патологией желудочно-кишечного тракта, 47,4 % – бронхолегочной патологией и 17,0 % – заболеваниями опорно-двигательного аппарата. Специалисты гидрографической службы г. Архангельска мужского пола выполняют плановые и специальные гидрографические работы вахтовым методом в течение навигационного периода, совершая кратковременные частые рейсы длительностью 16 суток в Северных морях.*

**Цель исследования.** Проанализировать особенности содержания лейкоцитов и фагоцитоза у гидрографов Архангельского района гидрографической службы СФРФ с учетом возраста и стажа работы в условиях северных морей.

**Материалы и методы.** Обследовано 36 мужчин, работающих в гидрографической службе СФРФ, короткими вахтами по 16 суток: 15 человек в возрасте 30–40 лет со стажем работы 10 лет и 21 человек в возрасте 50–65 лет со стажем работы 20 лет. Определяли содержание лейкоцитов и фагоцитоз в периферической венозной крови с учетом возраста и стажа работы. Для статистической обработки применен пакет прикладных программ Microsoft Excel 2010 и SPSS 20.0 для Windows.

**Результаты.** Установлено, что у гидрографов младшей возрастной группы со стажем работы 10 лет в 1,5–2 раза чаще встречается дефицит лейкоцитов, лимфоцитов и моноцитов, взаимосвязанный с низкой активностью фагоцитоза. У старшей возрастной группы лиц со стажем работы 20 лет лейкоцитоз, лимфоцитоз и моноцитоз регистрируются в 1,5 раза чаще на фоне повышенной активности процессов фагоцитоза.

**Заключение.** Фагоцитарная активность нейтрофилов выше у старшей возрастной группы со стажем работы 20 лет в экстремальных условиях.

**Ключевые слова:** лейкоциты, лимфоциты, фагоцитоз, гидрографическая служба, субарктический регион

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## INTRODUCTION

The Arkhangelsk region is located in a variety of climatic and geographic zones, including arctic, subarctic, and temperate regions. Approximately 40 % of the region lies beyond the Arctic Circle and within the permafrost zone, where it is influenced by moist air masses from the Atlantic Ocean and the cold waters of the White Sea and Arctic Ocean. This combination of factors results in unfavorable climate and geographical conditions, characterized by low air temperatures, high humidity, increased air mass mobility, dramatic seasonal photoperiodicity, significant barometric pressure fluctuations, and elevated geomagnetic activity [1–3].

In the Arkhangelsk region, mortality among working-age individuals remains high, despite an annual decline. For example, in 2014 the proportion of deaths among the working-age population was 27.3 % of all deaths (32.0 % in 2010). The mortality rate for men is higher than for women in all age groups, especially during the working age period. It has been noted that men over the age of 45 form a socially significant group, as the majority have reached professional maturity and contribute significantly to the national economy and their families [4–6]. In the Arkhangelsk region, the health of 40-year-old men is characterized by cardiovascular pathology (57 %), gastrointestinal pathology (14.7 %), bronchopulmonary pathology (47.4 %), and musculoskeletal disorders (17.0 %) [4, 5, 7].

The Hydrographic Service of Russia is a structural unit within the Ministry of Defense of the Russian Federation. Its main functions include providing navigational, hydrographic, hydrometeorological, topographical, geodetic and marine navigation support to Russian maritime activities in accordance with international obligations related to the safety of life at sea in the inland waters within the jurisdiction of the Russian Federation.

As a rule, the personnel are male employees of working age who are physically fit for duty and undergo an annual medical examination in accordance with Regulation No. 302-N at the facility of the 1<sup>st</sup> branch of the Federal State Institution “1469 Naval Clinical Hospital of the Northern Fleet”. Male specialists from the Hydrographic Service based in Arkhangelsk perform both planned and specialized hydrographic work throughout the entire navigational period, undertaking short, frequent voyages of up to 16 days, as well as voyages lasting 265 days, to maintain light-houses. It was of interest to study the leukocyte count and phagocytosis in the immune response of individuals who work short 16-day shifts in the Northern seas.

It is known that neutrophils constitute the most abundant population of peripheral blood cells, accounting for up to 70 % of total leukocyte count. However, in a routine analysis, only approximately 50 % of leukocytes, the circulating pool, can be assessed. These cells do not have a specialized function and can differentiate into antigen-presenting cells depending on the microenvironment. The remaining 50 % are marginated neutrophils that are ready to leave the blood vessels and enter tissues

to perform phagocytosis [8]. According to a study conducted by the Institute of Environmental Physiology of the Ural Branch of the Russian Academy of Sciences in Arkhangelsk among practically healthy residents aged 20–77 years with non-extreme occupations during the period 2014–2024 [9], neutrophil apoptosis was accompanied by an increase in the number of band neutrophils, without affecting their phagocytic activity. The level of necrosis in northern populations is significantly lower than that of programmed neutrophil death, which helps to maintain cellular homeostasis without the need for an inflammatory response [10, 11].

The relevance of this study is supported by its scientific originality, as it represents the first in-depth investigation of the association between leukocyte count and phagocytosis in the immune responses of working-age adults, considering work experience, age, and shift duration in extreme climatic and occupational conditions of the Arctic seas. A correlation between leukocyte count and various immune imbalances has been identified. The data obtained during the study on the collaboration of immunocompetent cells enhance our current comprehension of the physiological processes underlying adaptive immune responses in individuals who work on a shift schedule in the Arctic regions. These results will contribute to developing strategies for preventing secondary immunodeficiency disorders associated with environmental factors and occupational activities in the Arctic area of the European North of Russia. The results enhance our comprehension of individual resilience and contribute significantly to the field of environmental physiology. The identified correlations between innate and acquired immune responses will enable future investigations to elucidate the mechanisms of activation at each phase of the acquired immune response, which can be utilized for health prediction and to justify remedial measures for workers operating in the Arctic coastal region.

## AIM

To analyze the features of leukocyte count and phagocytosis in hydrographers employed by the Arkhangelsk branch of the Hydrographic Service of the Russian Federation, considering their age and experience working in the Northern seas.

## MATERIALS AND METHODS

The study was conducted at the Laboratory of Immune Cell Physiology, Institute of Environmental Physiology, N. Laverov Federal Center for Integrated Arctic Research, Ural Branch of the Russian Academy of Sciences in Arkhangelsk, Russia.

A total of 36 male participants working in the Arkhangelsk branch of the Hydrographic Service of the Northern Fleet of the Russian Federation on short-term shifts (16-day shifts) were examined. Of these participants, 15 were

aged 30–40 years and had 10 years of work experience, while 21 were aged 50–65 years and had 20 years of work experience.

The study was conducted in accordance with the ethical principles outlined in the Helsinki Declaration, as revised and amended in 2013 by the World Medical Association. Approval for the study was granted by the Ethics Committee of N. Laverov Federal Center for Integrated Arctic Research (Protocol No. 001-20/01 dated January 20, 2025), under state assignment No. 125021902582-1 “Physiological significance of the features of adaptive and innate immune responses in the development of immune homeostasis reserves among Arctic residents, taking into account age, sex, and occupational status”.

The immunological examination of the subjects involved the assessment of peripheral blood leukocytes, lymphocytes, monocytes, eosinophils, and neutrophils, as well as the neutrophil phagocytic activity. The determination of neutrophil and lymphocyte counts in the peripheral blood was performed using a Pentra 60 ABX hematology analyzer (absolute values). The phagocytic activity and cell counts were determined by incubating the blood with latex particles for 30 minutes at 37°C, followed by Romanowsky–Giemsa staining. The cell counting was performed using an immersion microscope (Nikon Eclipse 50i). Statistical analysis was performed using Microsoft Excel 2010 and SPSS 20.0 for Windows. The differences between the compared parameters were assessed using Student’s *t*-test (for normal distribution) and considered significant at  $p < 0.05$ – $0.01$ .

## RESULTS AND DISCUSSION

The study conducted by Boscolo P. et al. demonstrated that the type of professional activity affects the activity of T-lymphocytes (CD5<sup>+</sup>) and natural killer (NK) cells. It was shown that high levels of work-related stress in factory and construction workers are correlated with decreased activity of NK lymphocytes and T cells (CD5<sup>+</sup>), whereas office workers and artists have increased NK cell activity due to lower levels of stress [12, 13]. Long-term studies on the immune status of northern populations conducted by Balashova S.N. et al. found that activation of granulocytes with the CD95<sup>+</sup> antigenic determinant in individuals with non-extreme working conditions is strongly correlated with an increase in the concentration of band forms of leukocytes and, simultaneously, with a significant decrease in the number of mature differentiated cells. It has been suggested that neutrophil apoptosis may not affect phagocytic response. However, it has been noted that a decrease in its intensity is associated with a lower phagocytic activity [14, 15].

The unique working conditions associated with shift-based employment in the Northern seas of the subarctic region can lead to physiological stress due to professional activity, lifestyle, and shift duration. This stress can affect the health of hydrographic professionals

by disrupting various bodily systems, including the immune system [1]. The prolonged exposure to extreme occupational and environmental factors can contribute to a decrease in immune homeostasis, leading to the development of secondary immunodeficiencies that are dependent on environmental factors. These deficiencies can manifest as a tendency towards chronic pathology [16].

Professional activity in the challenging conditions of the northern region, including military service, hydrographic work, outdoor activities, and others, requires intense physical energy expenditure. Prolonged exposure to extreme environmental conditions further weakens the immune system [17–19].

It should be noted that the total leukocyte count in the peripheral blood of the examined individuals was within the generally accepted physiological range. However, it is worth noting that the levels of neutrophils, eosinophils, and monocytes were slightly elevated (Table 1).

It was found that the total leukocyte count among men with 10 years of work experience was  $6.63 \pm 0.50 \times 10^9$  cells/L, and among men with 20 years of work experience it was  $7.02 \pm 0.54 \times 10^9$  cells/L. Leukopenia was not observed in either group. Leukocytosis was noted in 6.7 % and 14.3 % of cases, respectively.

The mean lymphocyte count in the study groups was  $2.27 \pm 0.23 \times 10^9$  cells/L and  $2.88 \pm 0.32 \times 10^9$  cells/L, respectively. Analysis of the frequency of abnormalities revealed that lymphopenia was present in 13.3 % and 4.8 % of cases, whereas elevated lymphocyte levels were seen in 6.7 % and 14.3 % of the examined individuals, respectively.

The total monocyte count was  $0.44 \pm 0.06 \times 10^9$  cells/L and  $0.43 \pm 0.05 \times 10^9$  cells/L, respectively. No significant decrease in this parameter was recorded among men with 10 years of work experience, whereas in the group of men with 20 years of experience, a deficiency of this parameter was observed in 4.8 % of cases (Table 2). Conversely, elevated monocyte levels were more frequent in the group with 20-year work experience (6.7 % and 14.3 %, respectively).

The total eosinophil count for both groups was  $0.20 \pm 0.03 \times 10^9$  cells/L. Elevated values were observed in 20 % and 19 % of cases, respectively, without significant difference based on age or work experience.

The total neutrophil count among men with 10 years of work experience was  $3.73 \pm 0.36 \times 10^9$  cells/L, and among men with 20 years of work experience it was  $3.52 \pm 0.36 \times 10^9$  cells/L. A slight neutrophil deficiency was observed twice as frequently in the older group.

Based on our data, the phagocytic index was  $6.04 \pm 0.13$  among men with 10 years of work experience and  $7.11 \pm 0.50$  among men with 20 years of work experience. The percentage of active phagocytes was  $67.57 \pm 2.84$  % and  $65.75 \pm 2.32$  %, respectively, without a significant difference by age or work experience. The mean level of neutrophil phagocytic activity in the peripheral blood of hydrographers working

**TABLE 1**

**THE AVERAGE LEUKOCYTE COUNT IN THE PERIPHERAL BLOOD OF MALE HYDROGRAPHERS IN THE ARKHANGELSK REGION OF THE HYDROGRAPHIC SERVICE OF THE FEDERATION COUNCIL OF THE RUSSIAN FEDERATION (M=+/-M, OF THE PHAGOCYTIC NUMBER AND PERCENTAGE OF ACTIVE PHAGOCYTES (%))**

| Parameter                                     | Sample size,<br>n=15                               | Sample size,<br>n=21                               | Reference<br>range | Student's<br>t-test |
|-----------------------------------------------|----------------------------------------------------|----------------------------------------------------|--------------------|---------------------|
|                                               | 10 years of work<br>experience,<br>age 30–40 years | 20 years of work<br>experience,<br>age 50–65 years |                    |                     |
| Total leukocyte count, $\times 10^9$ cells/L  | 6.63 $\pm$ 0.50                                    | 7.02 $\pm$ 0.54                                    | 4–8.8              | 0.526               |
| Total lymphocyte count, $\times 10^9$ cells/L | 2.27 $\pm$ 0.23                                    | 2.88 $\pm$ 0.32                                    | 1.5–3.5            | 0.080               |
| Total monocyte count, $\times 10^9$ cells/L   | 0.44 $\pm$ 0.06                                    | 0.43 $\pm$ 0.05                                    | 0.09–0.6           | 0.898               |
| Total eosinophil count, $\times 10^9$ cells/L | 0.20 $\pm$ 0.04                                    | 0.20 $\pm$ 0.03                                    | 0.02–0.3           | 0.950               |
| Total neutrophil count, $\times 10^9$ cells/L | 3.73 $\pm$ 0.36                                    | 3.52 $\pm$ 0.36                                    | 1.5–5.5            | 0.664               |
| Phagocytic number                             | 6.04 $\pm$ 0.38                                    | 7.11 $\pm$ 0.50                                    | 4–9                | 0.067               |
| Active phagocytes, %                          | 67.57 $\pm$ 2.84                                   | 65.75 $\pm$ 2.32                                   | >50                | 0.592               |

**TABLE 2**

**FREQUENCY OF OCCURRENCE OF IMMUNE IMBALANCES IN MALE HYDROGRAPHERS OF THE ARKHANGELSK DISTRICT OF THE HYDROGRAPHIC SERVICE OF THE FEDERATION COUNCIL OF THE RUSSIAN FEDERATION, (%)**

| Parameter                                | Sample size,<br>n=15                            | Sample size,<br>n=21                            |
|------------------------------------------|-------------------------------------------------|-------------------------------------------------|
|                                          | 10 years of work experience,<br>age 30–40 years | 20 years of work experience,<br>age 50–65 years |
| Leukocytes, $\times 10^9$ cells/L, <4.0  | no                                              | no                                              |
| Leukocytes, $\times 10^9$ cells/L, >8.8  | 6.7                                             | 14.3                                            |
| Lymphocytes, $\times 10^9$ cells/L, <1.5 | 13.3                                            | 4.8                                             |
| Lymphocytes, $\times 10^9$ cells/L, >3.5 | 6.7                                             | 14.3                                            |
| Monocytes, $\times 10^9$ cells/L, <0.09  | no                                              | 4.8                                             |
| Monocytes, $\times 10^9$ cells/L, >0.6   | 6.7                                             | 14.3                                            |
| Eosinophils, $\times 10^9$ cells/L, >0.3 | 20.0                                            | 19.0                                            |
| Neutrophils, $\times 10^9$ cells/L, <1.5 | 6.7                                             | 14.3                                            |
| Neutrophils, $\times 10^9$ cells/L, >5.5 | 6.7                                             | 4.8                                             |
| Phagocytic number >9                     | 6.7                                             | 38.1                                            |
| Decreased active phagocytes, %           | 6.7                                             | 4.8                                             |

in extreme professional conditions in the northern seas is near the upper limit of the physiological reference range.

Based on the results from long-term studies on the immune status of individuals engaged in extreme professions, such as hydrographers, in the Arctic regions, we strongly suggest including an assessment

of cellular and humoral immunity as part of the standard medical examination for shift workers. This would allow for the timely identification of immune imbalances and the correction of the immune system status. Based on these data, preventive measures can be implemented to prevent the development of environmentally-induced secondary immunodeficiency.

## CONCLUSION

Therefore, in younger men who have worked in the harsh conditions in the Northern seas (the Barents and the White Seas) in the Arkhangelsk branch of the Hydrographic Service of the Northern Fleet of the Russian Federation, deficiencies in leukocytes, lymphocytes, and monocytes, associated with low phagocytic activity, occur 1.5–2 times more often than in older workers with 20 years of work experience, who have leukocytosis, lymphocytosis, and monocytosis occurring 1.5 times more often, along with increased phagocytic activity. The neutrophil phagocytic activity is slightly higher in the older age group with 20 years of work experience in extreme conditions ( $p < 0.05$ ).

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### Conflicts of interest

The authors declare no conflicts of interest.

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## PHARMACOLOGY AND PHARMACY

### ANTI-INFLAMMATORY AND ANTINOCICEPTIVE ACTIVITY OF INDENOPYRROLES AND INDENOPYRIDAZINES IN THE EXPERIMENT

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#### ABSTRACT

**Background.** Currently, the main groups of anti-inflammatory drugs used in medical practice are steroidal (glucocorticoids) and non-steroidal agents. However, despite their widespread use, these groups of anti-inflammatory drugs have a fairly wide range of negative side effects, which makes the search for low-toxic substances with anti-inflammatory effects currently relevant.

**The aim.** To study anti-inflammatory, antinociceptive activity and acute toxicity of individual representatives of indenopyrroles and indenopyridazines.

**Methods.** The acute toxicity of the studied compounds was studied using the method of Prozorovsky VB. Anti-inflammatory activity was assessed in a carrageenan-induced rat paw edema model. The production of active oxygen radicals by leukocytes was evaluated using luminol-dependent chemiluminescence. The antinociceptive activity of the compounds was assessed using the "writhing" and "hot plate" tests in mice.

**Results.** The obtained results allowed the studied compounds to be classified as "practically nontoxic" (LD50 > 1000 mg/kg). Compounds 1b of the indenopyrrole series and indenopyridazines 2a-b were found to exhibit anti-inflammatory activity, reducing the severity of exudative inflammation. Compound 2a exhibited the greatest antiexudative activity, reducing edema by more than 60 % ( $p < 0.01$ ). Compounds 2a-b and compound 1b reduced the production of reactive oxygen species by more than 40 % ( $p < 0.05$ ) compared to the control. In the "hot plate" test, the studied compounds 1a, 1b, 2a-b exhibited an antinociceptive effect comparable to nimesulide; in the "acetic acid-induced writhing" test, the effect of compounds 1a, 2b, 2b was comparable to nimesulide, and the effect of compound 2a exceeded the comparison drug by 32.96 % ( $p < 0.05$ ).

**Conclusion.** The studied compounds of the indenopyrrole series and their indenopyridazine derivatives are virtually non-toxic compounds, exhibit an anti-inflammatory effect and reduce the severity of pain associated with inflammation.

**Key words:** indenopyrroles, indenopyridazines, anti-inflammatory activity, production of reactive oxygen species, analgesic activity, acute toxicity, mice, rats

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## ПРОТИВОВОСПАЛИТЕЛЬНАЯ И АНТИНОЦИЦЕПТИВНАЯ АКТИВНОСТЬ ИНДЕНОПИРРОЛОВ И ИНДЕНОПИРИДАЗИНОВ В ЭКСПЕРИМЕНТЕ

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### РЕЗЮМЕ

**Обоснование.** В настоящее время в медицинской практике в качестве основных групп противовоспалительных средств применяются средства стероидной (глюкокортикоиды) и нестероидной природы. Однако, не смотря на их широкое применение, указанные группы противовоспалительных средств обладают достаточно широким спектром негативных побочных эффектов, что делает поиск малотоксичных субстанций, обладающих противовоспалительным действием, в настоящее время актуальным.

**Цель исследования.** Изучение противовоспалительной, антиноцицептивной активности и острой токсичности отдельных представителей инденопирролов и инденопиридазинов.

**Методы.** Острую токсичность исследуемых соединений изучали по методу Прозоровского В.Б. Противовоспалительную активность оценивали в модели каррагенинового отека лапы крысы. С помощью люминолзависимой хемилюминисценции оценивали продукцию активных кислородных радикалов лейкоцитами. Антиноцицептивную активность исследуемых соединений изучали в тесте «корчей» и в тесте «горячая пластина» на мышцах.

**Результаты.** Полученные результаты позволили отнести исследуемые соединения к «практически нетоксичным» (ЛД<sub>50</sub> > 1000 мг/кг). Установлено, что соединение 1в ряда инденопирролов и инденопиридазины 2а-в проявляют противовоспалительную активность, уменьшая выраженность экссудативного воспаления. Наибольшую антиэкссудативную активность проявило соединение 2а, уменьшая отек более, чем на 60 % ( $p < 0,01$ ). Соединения 2а-в и соединение 1в уменьшали продукцию активных форм кислорода более чем на 40 % ( $p < 0,05$ ) по сравнению с контролем. В тесте «горячая пластина» исследуемые соединения 1а, 1в, 2а-в проявляли антиноцицептивный эффект сопоставимый с нимесулидом; в тесте «уксусные корчи» эффект соединений 1а, 2б, 2в был сопоставим с нимесулидом, а эффект соединения 2а превосходил препарат сравнения на 32,96 % ( $p < 0,05$ ).

**Заключение.** Исследуемые соединения ряда инденопирролов и их производные инденопиридазины являются практически нетоксичными соединениями, проявляют противовоспалительный эффект и уменьшают выраженность боли, связанной с воспалением.

**Ключевые слова:** инденопирролы, инденопиридазины, противовоспалительная активность, продукция активных форм кислорода, анальгетическая активность, острая токсичность, мышцы, крысы

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## INTRODUCTION

Inflammation, as a protective-adaptive reaction of the body, on the one hand, provides a non-specific defense against various pathogens and participates in the elimination of damaged host cells. On the other hand, it can accompany various pathological conditions such as trauma, infections, and autoimmune diseases. Under these circumstances, the endogenous mechanisms aimed at containing the local inflammatory process are often insufficient, leading to generalization of inflammation with the release of inflammatory mediators and pro-inflammatory cytokines into the systemic circulation [1]. Therefore, it is important for potential medications to effectively influence the course of inflammation at all stages of its development. This may be achieved through the multi-component mechanism of their anti-inflammatory action.

Currently, two main groups of anti-inflammatory medications are used in medical practice: steroid and non-steroidal drugs. However, despite their widespread use, these groups of anti-inflammatory drugs have a relatively broad range of adverse effects, making the search for low-toxic substances with anti-inflammatory activity highly significant [2, 3].

Indenopyrroles and their indenopyridazine derivatives, due to their high reactivity, show promise for the synthesis of a wide range of compounds, including those with biological activity. The indenopyrrole and indenopyridazine compounds studied here incorporate nitrogen-containing heterocyclic rings such as pyrrole and pyridazine into their structures. Previously, substances with anti-inflammatory activity have been identified within the class of pyrrole-containing compounds and their derivatives [4]. However, the impact of indenopyrroles and indenopyridazines on inflammatory processes has not yet been investigated.

## AIM

To study anti-inflammatory, antinociceptive activity and acute toxicity of selected representatives of indenopyrroles and indenopyridazines.

## MATERIALS AND METHODS

*In vivo* experiments were conducted using sexually mature white Wistar rats weighing 180–220 g and sexually mature white outbred CD-1 mice weighing 22–25 g.

All research procedures involving laboratory animals were performed in accordance with generally accepted ethical standards for animal care, as outlined in the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes [5] and recommendations from the Board of the Eurasian Economic Commission dated November 14, 2023 No. 33 "On the Guidelines for the Care and Use of Laboratory (Experimental) Animals in Preclinical (Non-clinical)

Studies" [6]. These studies were approved by the Bioethics Committee of the Perm State Pharmaceutical Academy of the Ministry of Health of the Russian Federation (Protocol No. 2-н-ж, dated January 16, 2024, Perm, Russia).

The compounds selected for this study were representatives of the indenopyrrole series (1a-c) and their indenopyridazine derivatives (2a-c), which differ in terms of the presence of various substituents within their molecular structures (Fig. 1). These compounds were synthesized at the Department of General and Organic Chemistry of the Perm State Pharmaceutical Academy of the Ministry of Health of the Russian Federation. The structure and purity of these indenopyrroles and indenopyridazines were confirmed through IR,  $^1\text{H}$  NMR, and  $^{13}\text{C}$  NMR spectroscopy [7].

The acute toxicity of the studied compounds was assessed using the method proposed by Prozorovsky V.B. [8]. The substances were administered via intraperitoneal injection in a 2 % starch mucilage at increasing doses of 500, 631, 794 and 1000 mg/kg. Based on the data obtained, the toxicity classification of the compounds was determined using the classification system proposed by Sidorov K.K. [9].

Acute inflammation was induced by administering 0.1 mL of a 1 % carrageenan solution to the plantar surface of the rat hind paw. Paw volume was measured using an oncometer before carrageenan injection (control) and then at 3 and 5 hours after carrageenan administration. The tested compounds were administered intraperitoneally at a dose of 50 mg/kg in a 2 % starch mucilage 30 minutes prior to carrageenan injection. The anti-inflammatory effect was assessed based on the reduction in paw edema [10].

Reactive oxygen species production was assessed using a luminol-dependent chemiluminescence (LDCL) assay. The reaction was carried out in 96-well flat-bottom plates (Greiner, Germany). Each well contained  $10^5$  cells (peritoneal leukocytes from mice) in 100  $\mu\text{L}$  of Hank's solution. Opsonized zymosan at a concentration of 150  $\mu\text{g/mL}$  was used as the LDCL inducer. Luminol at a concentration of  $10^{-5}$  M was used as a marker of the LDCL reaction intensity [9]. Results were recorded over 1 hour at 5-minute intervals using a luminometer (Tecan Trading AG, Switzerland).

The analgesic activity of the tested compounds was evaluated using the acetic acid-induced writhing test

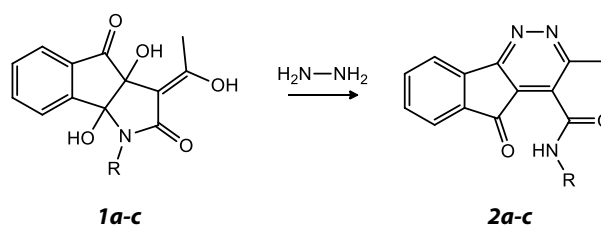


FIG. 1.

Structural formulas of the studied compounds

$\text{R} = 2\text{-ClC}_6\text{H}_4$  (1a, 2a),  $\text{R} = \text{H}$  (1b, 2b),  $\text{R} = 2\text{-CH}_3\text{OC}_6\text{H}_4$  (1c, 2c)

and the hot plate test in mice [10]. The tested compounds were administered intraperitoneally at a dose of 50 mg/kg in a 2 % starch mucilage. The control group of animals received an equal volume of a 2 % starch mucilage. Writhing was induced by intraperitoneal injection of a 0.75 % acetic acid solution (0.1 mL per 10 g of body weight) 30 minutes after administration of the tested compounds. The number of abdominal contractions (writhing episodes) was recorded for each animal over a 20-minute period following acetic acid administration. The criterion for analgesic activity was defined as a decrease in the number of writhing episodes relative to the control group.

In the hot plate test, animals were placed on a metal plate that was heated to 52°C for 60 minutes after administration of the tested compounds. The analgesic effect was assessed based on the latency time until a behavioral response to a thermal stimulus, which was manifested as licking, shaking of the hind paws, or jumping. The latency time was measured in seconds.

Nimesulide substance (Sigma-Aldrich) was administered intraperitoneally at a dose of 50 mg/kg in a 2 % starch mucilage and served as the reference drug in the study.

The data obtained were tested for normal distribution using the Kolmogorov–Smirnov test. The *p*-value from the Kolmogorov–Smirnov test was > 0.05, supporting the null hypothesis that the distribution is close to normal. Subsequently, the data were analyzed using an unpaired Student's *t*-test. Differences between groups were considered statistically significant at *p* < 0.05.

## RESULTS

As a result of the acute toxicity study following a single intraperitoneal administration of the indenopyrroles

and their indenopyridazine derivatives, the tested organic compounds were classified as belonging to class 5, “practically non-toxic compounds”, according to the classification of Sidorov K.K. [9].

All tested compounds, except for compound 1b, demonstrated a reduction in the severity of the inflammatory response when compared to the control group of animals, both at 3 hours and 5 hours after carrageenan administration. Compound 2a exhibited an effect that was comparable to that of the reference drug (Table 1).

The course of an inflammatory reaction is often accompanied by pain caused by irritation of nociceptors by inflammatory mediators such as exudates, including prostaglandins, kinins, and others. This has led to the investigation of the antinociceptive effects of certain compounds.

It was found that the studied indenopyridazines (2a-c) and indenopyrrole 1a reduced the number of involuntary contractions of abdominal muscles (writhing) when injected intraperitoneally with acetic acid (Table 2). An antinociceptive effect exceeding that of nimesulide was observed only for compound 2a.

In the hot plate test, which evaluates the effect of the studied compounds on the central mechanisms of pain perception, nearly all of the studied indenopyrroles and their indenopyridazine derivatives, with the exception of compound 1b, prolonged the time that animals spent on a heated plate (Table 3). The observation of antinociceptive activity in the hot plate assay suggests a potential impact on supraspinal mechanisms of pain regulation [10].

As is known, neutrophils, monocytes, and macrophages are the main players in the inflammatory response, as they are able to secrete not only inflammatory mediators, but also reactive oxygen species (ROS). These ROS can not only help eliminate pathogens, but also sustain the inflammatory response. In this context, the effect

TABLE 1

THE EFFECT OF INDENOPYRROLES (1A-C) AND INDENOPYRIDAZINES (2A-C) ON THE SEVERITY OF EXUDATIVE INFLAMMATION INDUCED BY CARRAGEENAN IN RATS

| Compound         | Increase in paw volume, % | Inhibition of paw edema, % | Increase in paw volume, % | Inhibition of paw edema, % |
|------------------|---------------------------|----------------------------|---------------------------|----------------------------|
|                  | at 3 h                    |                            | at 5 h                    |                            |
| Control (n=8)    | 42.89 ± 6.83              | -                          | 30.96 ± 4.18              | -                          |
| Nimesulide (n=8) | 11.39 ± 2.88*             | 73.42 ± 6.72               | 5.77 ± 1.64*              | 81.36 ± 5.29               |
| 1a (n=7)         | 19.47 ± 2.21*             | 54.59 ± 5.15               | 13.68 ± 1.70*             | 55.79 ± 5.50               |
| 1b (n=6)         | 25.02 ± 3.79              | 41.67 ± 8.85               | 15.99 ± 4.04              | 48.36 ± 13.05              |
| 1c (n=7)         | 24.40 ± 3.44*             | 43.09 ± 8.02               | 16.56 ± 3.37*             | 46.49 ± 10.88              |
| 2a (n=7)         | 15.26 ± 2.37*             | 64.43 ± 5.54               | 9.35 ± 2.06*              | 69.81 ± 6.64               |
| 2b (n=7)         | 20.86 ± 3.53*             | 51.35 ± 8.23               | 17.66 ± 3.03*             | 42.95 ± 9.79               |
| 2c (n=7)         | 16.24 ± 2.49*             | 62.12 ± 5.83               | 11.79 ± 2.57*             | 61.90 ± 8.31               |

**Note.** Data are presented as mean and standard error of the mean (M ± m). \* – *p* < 0.05 compared to the control group (Student's *t*-test). # – *p* < 0.05 compared to nimesulide (Student's *t*-test). *n* – number of animals per group.

of the studied compounds on ROS production by peritoneal leukocytes was evaluated using the LDCL assay.

As shown in Figure 2, indenopyrroles (1a-c) did not affect the spontaneous production of ROS by leukocytes in the LDCL assay. In the stimulated version of the assay, these compounds exhibited diverse effects on ROS production: compounds 1a and 1b enhanced ROS production, while compound 1c reduced ROS production from 5 to 30 minutes of observation (Fig. 2).

The effect of indenopyridazines (2a-c) on ROS production in the LDCL assay showed a similar trend in both the unstimulated and stimulated versions (Fig. 3). In unstimulated leukocytes, ROS production after treatment

with compounds 2a and 2b was lower compared to the control at 10–20 minutes after treatment, and at 35 minutes, a decrease in ROS production was seen for compounds 2a-c (Fig. 3A). In the stimulated version of the assay, ROS production following administration of compounds 2a-c decreased during the first 10 minutes but did not differ significantly from control values thereafter (Fig. 3B).

## DISCUSSION

The development of carrageenan-induced paw edema in rats at the evaluated time points (3 and 5 hours after

**TABLE 2**

**ANTINOCICEPTIVE ACTIVITY OF INDENOPYRROLES (1A-C) AND INDENOPYRIDAZINES (2A-C) IN THE VINEGAR WRITHE TEST IN MICE**

| Compound         | Number of writhes  |               |                           |                           | Total number              |
|------------------|--------------------|---------------|---------------------------|---------------------------|---------------------------|
|                  | Observation period |               |                           |                           |                           |
|                  | 0-5 min            | 5-10 min      | 10-15 min                 | 15-20 min                 |                           |
| Control (n=7)    | 9.28 ± 2.13        | 18.57 ± 2.21  | 17.86 ± 2.30              | 10.71 ± 0.52              | 56.43 ± 4.73              |
| Nimesulide (n=7) | 5.14 ± 1.08        | 12.43 ± 0.61* | 11.57 ± 1.02              | 8.14 ± 0.70*              | 37.29 ± 1.87*             |
| 1a (n=6)         | 4.0 ± 1.15         | 13.33 ± 1.76  | 11.83 ± 1.64              | 10.0 ± 1.86               | 39.17 ± 4.78*             |
| 1b (n=7)         | 8.71 ± 1.59        | 15.14 ± 0.96  | 13.14 ± 0.59              | 7.29 ± 0.47*              | 44.29 ± 1.96              |
| 1c (n=7)         | 8.14 ± 1.64        | 18.43 ± 3.55  | 13.00 ± 2.32              | 10.0 ± 1.90               | 49.57 ± 8.55              |
| 2a (n=6)         | 4.67 ± 1.48        | 11.67 ± 1.47* | 5.33 ± 0.92 <sup>*H</sup> | 3.33 ± 1.17 <sup>*H</sup> | 25.0 ± 3.25 <sup>*H</sup> |
| 2b (n=7)         | 6.29 ± 1.76        | 15.14 ± 1.89  | 12.00 ± 1.29              | 8.14 ± 1.39               | 41.57 ± 3.06*             |
| 2c (n=7)         | 8.00 ± 1.93        | 12.71 ± 1.96  | 9.57 ± 1.21*              | 7.71 ± 1.25               | 38.00 ± 5.17*             |

**Note.** Data are presented as mean and standard error of the mean (M ± m). n – number of animals per group. \* –  $p < 0.05$  compared to the control group (Student's t-test). H –  $p < 0.05$  compared to nimesulide (Student's t-test).

**TABLE 3**

**ANTINOCICEPTIVE ACTIVITY OF INDENOPYRROLES (1A-C) AND INDENOPYRIDAZINES (2A-C) IN MICE IN THE HOT PLATE TEST**

| No. | Compound         | Latency time, s | p vs. control |
|-----|------------------|-----------------|---------------|
| 1   | Control (n=7)    | 13.46 ± 0.75    | -             |
| 2   | Nimesulide (n=8) | 17.52 ± 1.02    | < 0.02        |
| 3   | 1a (n=8)         | 19.58 ± 1.53    | < 0.01        |
| 4   | 1b (n=8)         | 13.79 ± 1.58    | > 0.05        |
| 5   | 1c (n=7)         | 18.72 ± 1.14    | < 0.01        |
| 6   | 2a (n=8)         | 20.93 ± 2.05    | < 0.02        |
| 7   | 2b (n=8)         | 19.76 ± 1.24    | < 0.01        |
| 8   | 2c (n=8)         | 20.32 ± 2.10    | < 0.02        |

**Note.** Data are presented as mean and standard error of the mean (M ± m). H –  $p < 0.05$  compared to the reference drug nimesulide (Student's t-test). n – number of animals per group.

phlogogen administration) is associated with the active infiltration of the inflammatory focus by leukocytes, predominantly neutrophils and macrophages, which secrete pro-inflammatory cytokines as well as inflammatory mediators. Carrageenan can directly activate the migratory and secretory activity of macrophages through Toll-like receptor 4 (TLR-4). The stimulation of macrophage secretion leads to an increase in the synthesis of mediators, such as prostaglandins, leukotrienes, and pro-inflammatory cytokines (IL-1, IL-6, TNF- $\alpha$ ), which contribute to the development and maintenance of the inflammatory response [11]. Therefore, based on the results of our study, it can be hypothesized that the anti-inflammatory activity of the tested compounds is mediated by inhibition of leukocyte migration, the synthesis of pro-inflammatory cytokines and mediators, and also through an effect on TLR-4.

Involuntary contractions of the abdominal muscles during the acetic acid-induced writhing test are caused by the stimulation of nociceptors in serous membranes through acetic acid injection. This stimulation is also thought to lead to the release of biologically active substances, such as bradykinins, prostaglandins, and leukotrienes, during the early stage of primary hyperalgesia. Prostaglandins and leukotrienes are products of arachidonic

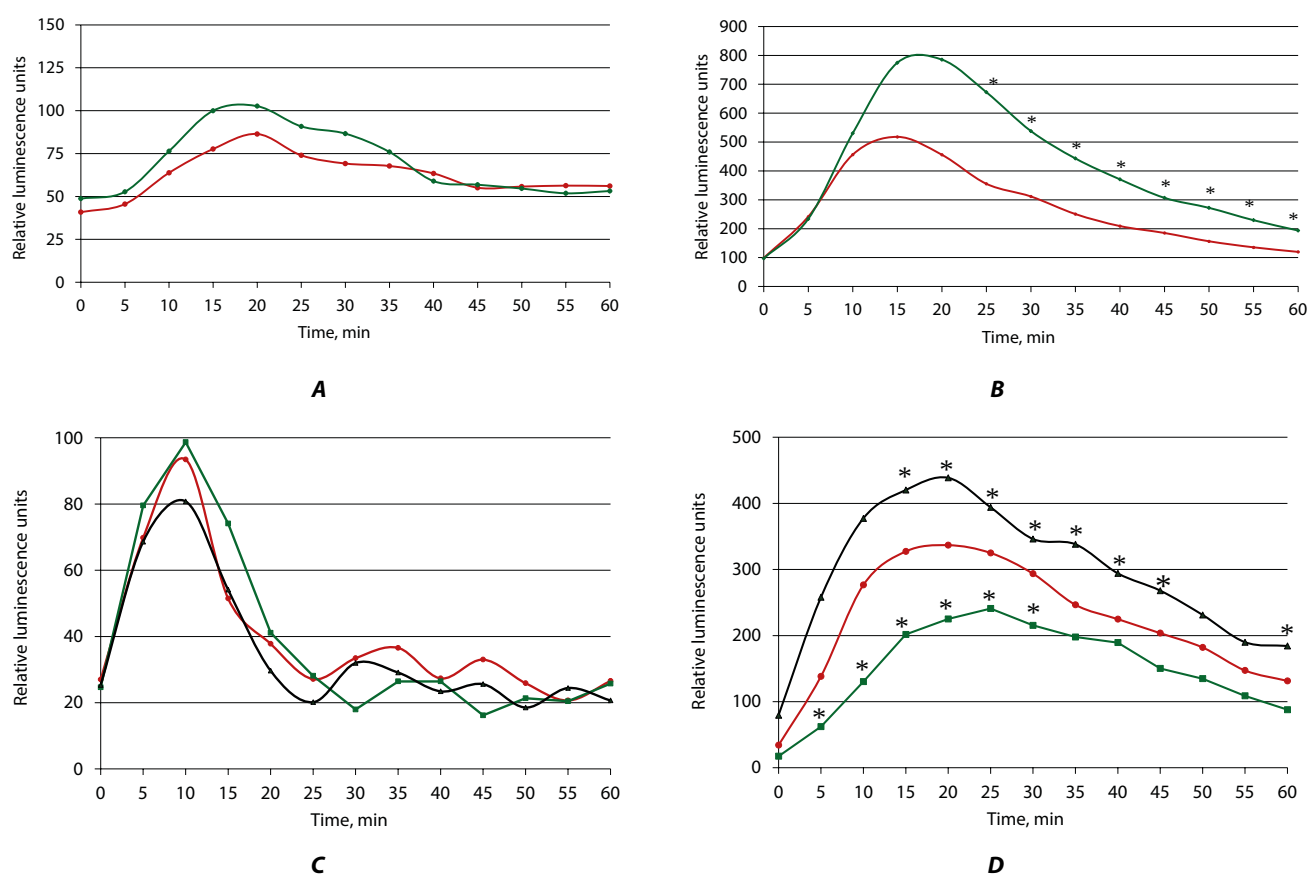
acid metabolism through the cyclooxygenase pathway. Previous studies have demonstrated the ability of compounds with a pyrrole ring structure to inhibit the expression and activity of COX-2 during inflammation [12]. This suggests that these compounds may also inhibit the expression or activity of the COX enzyme, thereby reducing the synthesis of prostaglandin E<sub>2</sub>, a primary sensitizer for nociceptors.

The results of the study on leukocyte-derived reactive radical production in the LDCL assay indicate that the mechanism by which indenopyridazine compounds exert their anti-inflammatory effects may be due to their inhibitory effect on radical production.

## CONCLUSION

Our data suggest that certain indenopyrrole and indenopyridazine derivatives exhibit anti-inflammatory and antinociceptive activity combined with low toxicity.

In the carrageenan-induced rat paw edema model, the studied indenopyridazines showed comparable anti-inflammatory effects to the reference drug, nimesulide. Additionally, they significantly reduced reactive oxygen species production in the LDCL assay by more than 40 %

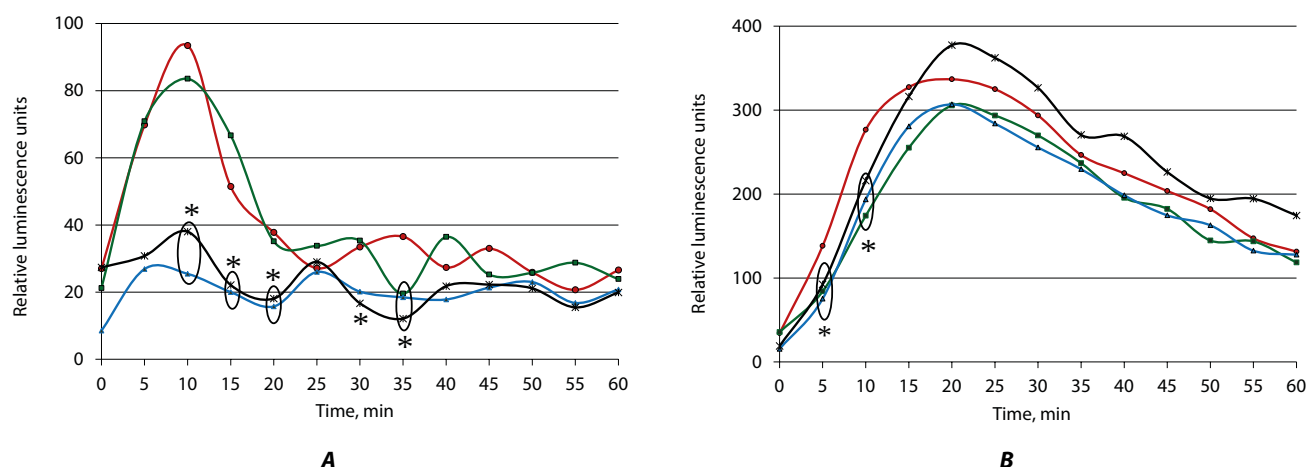


**FIG. 2.**

The effect of indenopyrroles on spontaneous (A, C) and stimulated (B, D) production of reactive oxygen species by mouse leukocytes

• – control, ▲ – compound 1a, ◆ – compound 1b, ■ – compound 1c;

\* –  $p < 0.05$  compared to control, number of animals in each group  $n=9$ .



**FIG. 3.**

The effect of indenopyridazines (2a-c) on spontaneous (A) and stimulated (B) production of reactive oxygen species by mouse leukocytes  
 • – control, × – compound 2a, ▲ – compound 2b, ■ – compound 2c, \* –  $p < 0.05$  compared to control, number of animals in each group  $n=9$ .

( $p < 0.01$ ) compared to the control group. Furthermore, compound 2a exceeded the reference drug by 32.96 % in the acetic acid-induced writhing assay, compared to the reference compound ( $p < 0.05$ ).

These results suggest that indenopyrroles and their derivatives may be promising candidates for further development as potential anti-inflammatory agents with analgesic properties.

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#### Conflicts of interest

The authors declare no conflicts of interest.

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## SURGERY

### TWO-STAGE SURGICAL TREATMENT OF A PATIENT WITH FLEXION-EXTENSION CONTRACTURE OF THE ELBOW JOINT USING HYDRAULIC MOBILIZATION

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#### ABSTRACT

**Background.** Numerous surgical techniques have been proposed for the treatment of patients with persistent elbow contractures that cause significant functional impairment. However, the treatment results are not always satisfactory for surgeons and patients due to the invasive nature of the procedure and the risk of contracture recurrence.

**Aim.** To demonstrate the feasibility of using hydraulic mobilization for the management of the soft tissue component of contracture to improve elbow function.

**Methods.** During the first stage of surgical treatment, hydraulic mobilization of the elbow joint and collateral ligaments was performed to increase the angle of extension. Saline solution was used to dilate the joint cavity, stretch the capsule, and release any scarring from the ligaments. Tunneling of the proximal metadiaphysis of the ulna and distal metadiaphysis of the humerus was also performed to improve blood flow to the bone and reduce intraosseous pressure. At the second stage of treatment (6 months after the first stage of surgery), arthrotomy of the left elbow joint, osteotomy of the mal-united fragment of the humeral capitellum, its resection, and plaster immobilization of the left upper limb were performed.

**Results.** After the first stage of treatment, the elbow extension angle was increased to full extension. After the second stage, elbow flexion function improved. The patient's quality of life improved, and they are now able to use their arm fully. No significant pain was reported.

**Conclusions.** In this case, the presented treatment strategy is justified and optimal, as it adheres to the principles of gentle tissue handling during surgery, early initiation of active motion, and avoidance of passive redressing manipulations that cause pain and microtrauma to the elbow joint and accelerate mesenchymal tissue maturation. Hydraulic mobilization requires no additional surgical approaches or special equipment, causes virtually no trauma to surrounding tissues, reduces surgical time, and eliminates the risks of open surgical techniques.

**Keywords:** elbow joint, contracture, hydraulic mobilization, osteotomy, results

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## ДВУХЭТАПНОЕ ХИРУРГИЧЕСКОЕ ЛЕЧЕНИЕ БОЛЬНОЙ СО СГИБАТЕЛЬНО-РАЗГИБАТЕЛЬНОЙ КОНТРАКТУРОЙ ЛОКТЕВОГО СУСТАВА С ПРИМЕНЕНИЕМ ГИДРАВЛИЧЕСКОЙ МОБИЛИЗАЦИИ

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### РЕЗЮМЕ

**Обоснование.** Для лечения больных со стойкими контрактурами локтевого сустава со значительными функциональными нарушениями предложено множество хирургических методик. Однако не всегда результаты лечения устраивают хирургов и пациентов в связи с травматичностью операции и рецидивом контрактуры.

**Цель.** Продемонстрировать возможность применения гидравлической мобилизации на этапе устранения мягкотканного компонента контрактуры для улучшения функции локтевого сустава.

**Методы.** На первом этапе оперативного лечения для увеличения угла разгибания произведены гидравлическая мобилизация локтевого сустава и коллатеральных связок с применением физиологического раствора с целью дилатации полости сустава, растяжения его капсулы, освобождения связок от возможных рубцов. Также была выполнена туннелизация проксимального метадиафиза локтевой кости и дистального метадиафиза плечевой для улучшения кровообращения в кости и снижения внутрикостного давления. На втором этапе лечения (через 6 месяцев после первого этапа) произведены артротомия левого локтевого сустава, остеотомия неправильно сросшегося фрагмента головчатого возвышения плечевой кости, его резекция, гипсовая иммобилизация левой верхней конечности.

**Результаты.** После первого этапа лечения угол разгибания увеличен до полного. После второго этапа — сгибательная функция локтевого сустава улучшена. Повысилось качество жизни пациента, обслуживает рукой себя полностью. Болевой синдром не выражен.

**Заключение.** В данном случае представленная тактика лечения является оправданной и оптимальной, так как соблюдаются принципы бережного отношения к тканям во время операции, раннего начала активных движений, отказ от редрессирующих пассивных манипуляций, вызывающих боль и микротравмы локтевого сустава, ускоряющих созревание мезенхимальной ткани. При гидравлической мобилизации не требуются дополнительных оперативных доступов, специального оборудования, практически не травмируются окружающие ткани, сокращается время операции, исключаются риски открытых оперативных методик.

**Ключевые слова:** локтевой сустав, контрактура, гидравлическая мобилизация, остеотомия, результаты

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## BACKGROUND

According to the scientific literature, 30 % to 60 % of patients following elbow injuries require various functional reconstructive procedures for persistent contractures and associated deformities [1, 2].

The management of patients with extra-articular elbow contractures (according to the classification of B.F. Morrey, 2000 [3]), i.e., those without bony obstruction to articular surface motion, often presents considerable challenges. As reported in an analytical review of the relevant literature [1], approximately half of the patients experience a substantial reduction in the range of motion in the operated elbow joint.

A wide array of surgical interventions has been proposed for the correction of severe elbow contractures with pronounced functional impairment, including redressation under anesthesia, arthroscopic debridement, arthrotomy with capsulectomy, among others [1, 3]. The application of a hinged external fixator in open arthrolisis has also been described. However, this technique may only yield a transient increase in the flexion-extension arc [4]. In cases where the range of motion deficit persists following surgery, mini-lateral and mini-medial incisions are employed [5]. Nevertheless, the outcomes are not consistently satisfactory for either surgeons or patients, owing to the invasiveness of the procedure and the risk of contracture recurrence.

## AIM

To demonstrate the feasibility of using hydraulic mobilization for the management of the soft tissue component of contracture to improve elbow joint function.

The patient provided written informed consent for the data publication for scientific purposes.

## MATERIALS AND METHODS

This study presents a case of minimally invasive surgical treatment in a patient with flexion-extension contracture of the elbow joint, utilizing hydraulic mobilization to address the soft tissue component at the first stage and resection of a malunited fragment of the humeral capitellum at the second stage. In view of concomitant osteoarthritis, the patient additionally underwent tunneling of the proximal metaphysis of the ulna and the distal metaphysis of the humerus. This approach is based on studies of tissue blood supply dynamics and intraosseous pressure, as reported by various researchers [6].

A working-age patient with post-traumatic combined flexion-extension contracture of the left elbow joint (age 45, dominant left upper extremity) was admitted for surgical intervention. Due to the impaired hand function, self-care was compromised and occupational

activity was limited (range of motion deficit of 100°, corresponding to severe contracture). Pain in the left elbow joint was elicited by sudden movements, and the injury occurred one year ago as a result of a fall, causing a fracture of the humeral capitellum, which was treated conservatively by immobilizing the left upper limb using a plaster cast.

Upon admission, the elbow flexion angle was 100° and the extension was 140°. Hand function was assessed by measuring the flexion angle (starting from 0°, with 40° considered the physiological norm) and the extension angle (maximum extension corresponding to 180°). Radiographs were taken in the following projections: anteroposterior in maximum possible extension, and lateral in maximum possible flexion and extension. The radiographic findings included evidence of osteoarthritis, a malunited fracture of the capitellum of the humeral condyle with anterior displacement of the fragment, abnormal bone density consistent with a chronic injury, and bone impingement during forearm flexion. The articular surface was centered. The olecranon fossa did not show any deformities (Fig. 1). The impairment of extension was attributed to desmogenic contracture caused by capsular remodeling and possible involvement of collateral ligaments.

At the first stage of surgical treatment, to increase the extension angle, hydraulic mobilization of the left elbow joint and collateral ligaments was performed, with the aim of dilating the joint cavity, stretching the capsule, and releasing the ligaments from potential adhesions. To treat osteoarthritis, further tunneling of the proximal metaphysis of the ulna and distal metaphysis of the humerus was performed. Subsequently, a plaster cast was applied to the patient's left upper limb, in a position of maximum extension.

The procedure involved the stepwise insertion of puncture needles into the ventrolateral (radiocapitellar joint) and dorsal (olecranon fossa) aspects of the elbow joint. Through these needles, the joint cavity was filled under syringe pressure with physiological saline (until tight filling, in this case 20 mL). The arm was then flexed and extended with moderate force 15 times. The joint cavity was lavaged with 100 mL of physiological saline.

Tunneling was performed by creating horizontal, end-to-end channels in the humerus and ulna bones (4 fan-shaped canals per bone). A sharpened 1.5 mm diameter K-wire for transosseous osteosynthesis was utilized.

During the inpatient stay (10 days) and outpatient follow-up (30 days), the patient completed elbow flexion and extension exercises twice daily, both actively and passively. During sleep and after exercise sessions, the patient's arm was immobilized using an anterior plaster splint in the maximum extension.

Full elbow extension was achieved 7 days after surgery (Fig. 2).

The second stage of surgery was performed 6 months after hydraulic mobilization. CT of the left

elbow joint (Fig. 3) revealed a malunited intra-articular fracture of the capitellum of the humeral condyle with anterior and proximal displacement of a bony fragment, creating a "hood-like" configuration.

An anterolateral arthrotomy of the left elbow joint, osteotomy of the malunited fragment of the humeral capitellum, its resection, and temporary plaster

splint immobilization of the left upper limb were performed.

A 6-centimeter skin incision was made along the anterolateral aspect of the lower third of the patient's arm to perform arthrotomy on the elbow joint. The humeral condyle was exposed through blunt and sharp dissection, and the trochlea was identified



**FIG. 1.**

Preoperative radiographs of the left elbow joint in a 45-year-old female patient Z. with flexion-extension contracture before treatment: A – anteroposterior projection; B – lateral projection in maximum extension; C – lateral projection in maximum flexion



**FIG. 2.**

Postoperative radiographs of the left elbow joint in a 45-year-old female patient Z. with flexion-extension contracture, obtained 7 days after the first stage of surgery: A – anteroposterior projection; B – lateral projection in maximum extension; C – lateral projection in maximum flexion



**FIG. 3.**

3D CT reconstruction images of the left elbow joint in a 45-year-old female patient Z. with flexion-extension contracture, obtained before the second stage of surgical treatment: A – ventral view; B – dorsal view; C – posterolateral view; D – medial view; E – lateral view

using a periosteal elevator. Osteotomy of the mal-united fragment of the capitellum and its resection were performed with an osteotome. The flexion angle in the elbow joint was increased to 55°, while extension reached 180°. The wound cavity was

irrigated with betadine solution. The wound was closed in layers, and dressings were applied. Hemostasis was maintained throughout the procedure using electrocautery.

During the first postoperative week, the patient performed elbow joint exercises at the hospital. To prevent the formation of heterotopic ossification, the patient was administered Mydocalm® 50 mg three times daily orally for 15 days. Additionally, indomethacin was prescribed in an increasing and then decreasing dose (25 mg daily on days 1–3, 50 mg in two doses on days 4–6, and 75 mg in three doses on days 7–9), followed by a three-day tapering regimen. Microwave therapy (150 GHz terahertz electromagnetic waves) was also administered.

## RESULTS

The treatment outcome was assessed as favorable. The flexion angle reached 70° (Fig. 4). Complete extension was achieved at the elbow joint.

At both short-term and long-term follow-up (one year after the second procedure), the patient's quality of life has substantially improved. The patient's ability to care for themselves has been restored, and the pain intensity has decreased markedly (from a score of 7 to 1 on the VAS scale). Additionally, the flexion-extension deficit has been reduced by half (Fig. 5).

## DISCUSSION

To improve blood circulation in the tissues of the elbow joint and reduce intraosseous pressure, subchondral tunneling of the metaphyses



**FIG. 4.**

Postoperative radiograph of the left elbow joint showing maximum flexion in a 45-year-old female patient Z. with flexion-extension contracture, taken 7 days following the second stage of surgical treatment

of the humerus and ulna was additionally performed on this patient. The clinical efficacy of tunneling the bones comprising the elbow joint has been previously demonstrated [7].

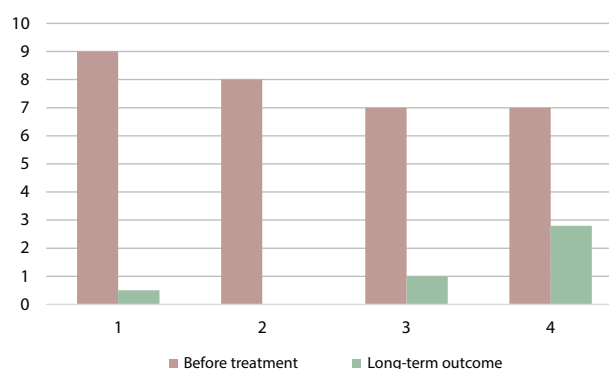
According to the fundamental works of B.F. Morrey, a limitation of extension by 30° or more significantly compromises upper extremity function, and this limitation is even less well tolerated by patients involved in gymnastics or throwing sports. In the present case, the patient reported significant functional limitations in daily activities due to a 40° extension deficit and a 60° flexion deficit. Therapeutic success was achieved through the use of a minimally invasive hydraulic mobilization technique at the first stage, which is indicated for treating desmogenic contractures. The chosen treatment strategy was appropriate and optimal, as it followed the principles of avoiding trauma to tissues, early functional loading, and avoiding forced manipulations that can cause pain and micro-trauma to joint tissues [2]. This approach helped prevent the development of arthrofibrosis, recurrence of contractures, and progression of osteoarthritis.

In contrast to arthroscopy [8, 9], hydraulic mobilization did not require any additional surgical approaches or specialized equipment. It caused minimal trauma to surrounding tissues, reduced operative time, and eliminated the risks associated with open surgical techniques.

The results achieved after the first stage (hydraulic mobilization) were found to be long-lasting. However, some researchers have reported that, despite comprehensive postoperative rehabilitation, the range of motion may decrease by up to 25 % over time [10]. The literature on the postoperative rehabilitation of the elbow joint is still limited, and most clinical studies do not fully describe rehabilitation protocols [11].

The second stage of the surgical treatment with arthrotomy was performed 6 months after the first procedure, in line with the principle of the “cooling-off period” and adequate postoperative rehabilitation [12]. Additionally, it has been suggested that conservative treatment should continue for at least 6 months prior to reconstructive surgery to prevent heterotopic ossification [13].

The resection of the malunited fragment of the humeral capitellum was justified based on its intra-articular location, as well as the presence of structural and morphological changes to the fragment, which were attributed to the chronic nature of the injury. Additionally, the existing elbow contracture and the possibility of a minimally invasive surgical approach were taken into consideration. Histomorphological studies [14] have demonstrated that two months or more following a fracture of the humeral capitellum, the hyaline cartilage on the articular surface demonstrates dystrophic changes and partial necrobiosis, while the cancellous bone in the distal fragment shows pronounced signs of avascular necrosis. As a result,



**FIG. 5.**

*Treatment outcome diagram showing quality of life, self-care ability, pain severity (VAS), and range of motion deficit before and after treatment: 1 – quality of life impairment; 2 – limited self-care ability; 3 – pain severity (VAS); 4 – range of motion deficit (10 points – 140° deficit, 0 points – no deficit)*

attempts at osteosynthesis at this stage may lead to unfavorable outcomes.

In the postoperative period, preventive measures were taken to reduce the risk of heterotopic ossification, including joint exercise with gradually increasing intensity, physiotherapy using various techniques such as magnetotherapy, terahertz electromagnetic waves, laser therapy, and phototherapy, and the use of pharmacological agents to enhance bone tissue microcirculation. Despite ongoing research into heterotopic ossification and its treatment, monitoring for this potential complication remains an essential part of patient management [15].

## CONCLUSION

This clinical observation demonstrates that complex reconstructive techniques are not always necessary for restoring elbow function. The development of less invasive, pathogenetically sound surgical approaches constitutes a promising area of research, and further study into their efficacy and refinement is justified. To determine the suitability of this approach for desmogenic and combined contractures, larger samples and more extensive statistical analyses are needed.

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### Conflicts of interest

The authors declare no conflicts of interest.

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## EXPERIMENTAL RESEARCHES

### EFFECT OF MELATONIN ON NEUROLOGICAL STATUS AND OXIDATIVE MODIFICATION OF PROTEINS IN EXPERIMENTAL CEREBRAL ISCHEMIA

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#### ABSTRACT

**Background.** Ischemic stroke (IS) remains one of the leading causes of death and disability worldwide. Due to the limitations of oral and parenteral routes of neuroprotectant administration in post-stroke patients, the use of rectal suppositories with melatonin (MT) is a promising direction for neuroprotective therapy.

**Aim.** To evaluate the effect of rectal suppositories containing MT on the dynamics of neurological status and products of oxidative protein modification in experimental acute cerebral ischemia (EACI).

**Materials and methods.** Male Wistar rats ( $n = 30$ ) were divided into 3 groups: I – sham-operated animals; II – animals with EACI according to the method of Chen S.T. et al.; III – animals with EACI that received rectal suppositories weighing 100 mg, containing 2.5 mg of MT. Neurological status was assessed on days 3 and 7 using the Garcia J.N. scale, the paw placement test, and the Bederson test. In the ischemic focus of the brain (IFB), the content of early and late products of protein oxidative modification (POM) was evaluated.

**Results.** In rats with EACI, neurological deficits developed on the corresponding day (decreased scores on the Garcia J.N. scale and the paw placement test, increased scores on the Bederson test) and an increase in the content of POM products in the IFB. Administration of MT suppositories contributed to the regression of these disorders: by day 7, there was an improvement in all neurological tests, and a decrease in POM products in the IFB.

**Conclusions.** The results obtained suggest that the neuroprotective effect of MT in the form of rectal suppositories in EACI is mediated through an antioxidant mechanism of action.

**Key words:** oxidative stress, melatonin, rectal suppositories, ischemic stroke

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## ВЛИЯНИЕ МЕЛАТОНИНА НА НЕВРОЛОГИЧЕСКИЙ СТАТУС И ОКИСЛИТЕЛЬНУЮ МОДИФИКАЦИЮ БЕЛКОВ ПРИ ЭКСПЕРИМЕНТАЛЬНОЙ ЦЕРЕБРАЛЬНОЙ ИШЕМИИ

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### РЕЗЮМЕ

**Обоснование.** Ишемический инсульт (ИИ) остается одной из ведущих причин смертности и инвалидизации населения в мире. В связи с ограничениями перорального и парентерального пути введения нейропротекторов у постинсультных пациентов, применение ректальных суппозиториев с мелатонином (МТ) представляет собой перспективное направление нейропротективной терапии.

**Цель.** Оценить воздействие ректальных суппозиториев, содержащих МТ, на динамику изменений неврологического статуса и продуктов окислительной модификации белков при экспериментальной острой церебральной ишемии (ЭОЦИ).

**Материалы и методы.** Крыс-самцов линии Wistar (30 особей) разделили на 3 группы: I – ложнооперированные животные; II – ЭОЦИ по методу Chen S.T. et al.; III – животные с ЭОЦИ, получавшие ректальные суппозитории массой 100 мг, содержащие 2,5 мг МТ. Неврологический статус изучали на 3-и и 7-е сутки по шкале Гарсиа Дж.Н., тесту с постановкой лапки, Bederson test; в очаге церебральной ишемии (ОЦИ) оценивали содержание продуктов окислительной модификации белков (ОМБ) (ранних и поздних).

**Результаты.** У крыс с ЭОЦИ на соответствующие сутки развивался неврологический дефицит (уменьшение баллов по Гарсиа Дж.Н. и тесту с постановкой лапки, рост — по Bederson test) и повышение содержания продуктов ОМБ в ОЦИ. Введение суппозиториев с МТ способствовало регрессу указанных нарушений: к 7 суткам фиксировалось улучшение показателей всех неврологических тестов и снижение продуктов ОМБ в ОЦИ.

Выявлена отрицательная корреляционная зависимость между показателями по шкале Гарсиа Дж.Н., тесту с постановкой лапки, Bederson test и продуктами ОМБ в ОЦИ.

**Заключение.** Полученные результаты позволяют предположить, что нейропротективное действие МТ в составе ректальных суппозиториев при ЭОИГМ реализуется через антиоксидантный механизм действия.

**Ключевые слова:** окислительный стресс, мелатонин, ректальные суппозитории, ишемический инсульт

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## INTRODUCTION

Ischemic stroke (IS) is a significant medical and social challenge in healthcare, with 12 million cases registered globally each year [1]. In Russia, this number reaches 500,000 per year, placing a significant socioeconomic burden on the healthcare system due to high disability rates and prolonged rehabilitation [2]. The key pathogenetic mechanisms leading to neuronal death in ischemic brain lesions during IS include neuroinflammation, glutamatergic excitotoxicity, and oxidative stress. These mechanisms interact to determine the size of the ischemic lesion and the severity of neurological deficits [3]. Current therapeutic strategies for IS have limited efficacy and safety, owing to a narrow therapeutic window, high risk of hemorrhagic transformation, poor permeability of the blood-brain barrier (BBB) to neuroprotective agents, limited knowledge of pharmacokinetics, and frequent adverse effects. These limitations emphasize the need for the development of novel therapeutic approaches focused on neuroprotection [4, 5].

Recent studies have confirmed the significant neuroprotective potential of melatonin (MT) in IS, attributed to its pleiotropic effects. One such effect is its strong antioxidant activity, which is exerted both by directly scavenging reactive oxygen species (ROS) and reactive nitrogen species (RNS) and by activating endogenous antioxidant systems, such as superoxide dismutase, catalase, and glutathione peroxidase. In addition to its potent antioxidant activity, MT also has other neuroprotective mechanisms. These include anti-inflammatory effects, such as inhibition of the NF- $\kappa$ B pathway and reduction in the synthesis of pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , and IL-6. It also has anti-apoptotic properties, such as caspase-3 suppression and stabilization of mitochondrial membranes. Furthermore, melatonin can reduce cerebral edema by regulating aquaporin-4 expression [6, 7, 8]. However, the clinical use of melatonin is limited by several factors. Oral formulations may not be suitable for patients with dysphagia, and parenteral administration has a short half-life (20–40 min), requiring frequent injections and potentially leading to local post-injection complications [9, 10].

In our previous study, we demonstrated the beneficial effects of melatonin incorporated into rectal suppositories on the neurological status, microcirculation, morphology, and morphometry of the ischemic brain lesion, as well as on lipid peroxidation parameters in ischemic brain tissue [11, 12]. This current study continues our research by comprehensively assessing the relationship between the neurological status and oxidative stress markers using melatonin-loaded rectal suppositories in experimental cerebral ischemia.

## AIM

To assess the effect of melatonin-containing rectal suppositories on the severity of neurological deficits

and protein oxidative modification products in experimental acute cerebral ischemia.

## MATERIALS AND METHODS

The study was conducted at the Department of Pathophysiology, Experimental Biological Clinic of South Ural State Medical University (SUSMU). A total of 32 adult Wistar male rats weighing  $240 \pm 20$  g were used in the study. The animals were housed in standard conditions in individual polypropylene cages (Velaz, type 3, Czech Republic) with corncob bedding and free access to food and water. They were fed a balanced complete feed (LBK) according to GOST R 50258–92. The facility maintained a day/night cycle, an air temperature of  $20 \pm 2^\circ\text{C}$ , and relative humidity of 60–80 %. The study was conducted in compliance with the guidelines of the European Convention on the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, and was approved by the Ethics Committee of SUSMU (Protocol No. 5, dated June 10, 2024).

The animals were randomly assigned to three groups: Group I ( $n = 10$ ) was a sham-operated group; Group II ( $n = 12$ ) consisted of animals with experimental acute cerebral ischemia (EACI); and Group III ( $n = 12$ ) was treated with an original rectal suppositories (100 mg) containing 2.5 mg of melatonin administered every 24 hours for 7 days [13]. The observation period lasted 7 days. Two animals (one in Group II and one in Group III) died within the first 6 hours after surgery and were excluded from the analysis. Consequently, the final sample sizes were: Group I ( $n = 10$ ), Group II ( $n = 11$ ), and Group III ( $n = 11$ ).

EACI was induced using a modified method developed by Chen S.T. et al., under combined Zoletil-xylazine anesthesia, in accordance with current guidelines for modeling cerebral ischemic stroke [14]. For Groups II and III, a 2-cm skin incision was made between the left ear and left eye. Soft tissues were dissected to the skull bones, and a 5-mm burr hole was drilled under continuous irrigation and cooling using a high-speed drill. Under a  $\times 10$  operating microscope, selective diathermocoagulation of cortical pial vessels was performed in the middle cerebral artery representation zone. Group I animals underwent the same surgical procedures, except for the selective diathermocoagulation of pial vessels.

On days 3 and 7 of the experiment, the neurological status of all animal groups was assessed using the Garcia J.H. scale, the Placing test, and the Bederson test. The results were expressed as scores [15, 16].

For the preparation of a 10 % cerebral ischemic lesion homogenate, animals were euthanized by decapitation, the brain was extracted, and the ischemic zones were identified based through visual inspection and anatomic landmarks [17, 18]. Collected ischemic brain tissue was then placed in ice-cold 0.1 M phosphate buffer (pH 7.4). Subsequently, approximately 100 mg of this tissue was then homogenized at a ratio of 1:10 (w/v) for 3 minutes at  $4^\circ\text{C}$ , yielding 1 mL of the homogenate.

Protein oxidative modification (POM) products in the cerebral ischemic lesion homogenate were determined by measuring the reactivity of protein carbonyl derivatives with 2,4-dinitrophenylhydrazine, both spontaneously and under metal-induced conditions. Subsequently, aldehyde dinitrophenylhydrazones (ADNPH) and ketone dinitrophenylhydrazones (KDNPH) were recorded using a spectrophotometer in the ultraviolet (UV) and visible spectral regions. Results were reported as optical density units per mg of protein (arbitrary units/mg), and the reserve-adaptive potential (RAP, %) was calculated as the ratio of spontaneously formed oxidation products to those formed under induced conditions [19, 20].

Statistical analysis was conducted using IBM SPSS Statistics 19 (SPSS: An IBM Company, USA). The normality of distribution was assessed using the Shapiro – Wilk test. As most parameters did not follow a normal distribution, the data are presented as median (Me) and interquartile range [Q1; Q3]. Non-parametric methods were used to analyze the differences between groups, including Kruskal – Wallis, Mann – Whitney, and Wald – Wolfowitz tests. Correlations between parameters were assessed using Spearman's rank correlation coefficient (R). Differences were considered statistically significant at  $p < 0.05$ .

## RESULTS

Modeling of experimental acute cerebral ischemia (EACI) resulted in the development of right-sided hemiparesis and impaired statolocomotor function in rats. On days 3 and 7, animals in Group II showed a significant decrease in scores on the Garcia J.H. scale and the Placing test, as well as an increase in scores on the Bederson test, compared to animals in Group I. On day 7, when compared to day 3, there was a decrease in scores on the Garcia J.H. scale ( $p = 0.042$ ) and the Placing test ( $p = 0.041$ ) and an increase on the Bederson test ( $p = 0.039$ ) (Table 1).

In animals of Group II, compared to Group I, there were increased levels of total protein oxidation modification

(POM) products in the homogenates from cerebral ischemic lesions on days 3 and 7 of the study under both spontaneous and induced conditions ( $p < 0.001$  for all comparisons at the respective time points). When analyzing the dynamics of spontaneous parameters in animals with EACI, a significant decrease in total POM products was observed by day 7 compared to day 3 ( $p = 0.042$ ). Similarly, the early ( $p = 0.041$ ) and late ( $p = 0.045$ ) POM fractions also showed a significant decrease. Under induced conditions, on days 3 and 7, Groups II also demonstrated higher levels of total POM products, as well as the early and late fractions, when compared to Group I ( $p < 0.001$ ). Furthermore, the dynamics from days 3 to 7 in EACI animals showed a statistically significant decrease in total POM ( $p = 0.038$ ), as well as in the early ( $p = 0.035$ ) and late ( $p = 0.043$ ) fractions.

The overall reserve-adaptive potential (RAP) of animals in Group II was significantly lower compared to that of Group I, both on day 3 ( $p = 0.003$ ) and day 7 ( $p = 0.002$ ). Within Group II, there was no significant difference in the overall RAP between days 3 and 7 ( $p = 0.287$ ) (Table 2).

The use of rectal suppositories containing MT contributed to a decrease in the severity of neurological deficits, as assessed on days 3 and 7. Compared to Group II, animals of Group III showed a significant increase in scores on the Garcia J.H. scale (day 3:  $p = 0.021$ ; day 7:  $p = 0.008$ ) and the Placing test (day 3:  $p = 0.015$ ; day 7:  $p = 0.009$ ), as well as a significant decrease on the Bederson test (day 3:  $p = 0.018$ ; day 7:  $p = 0.006$ ).

In the homogenates of cerebral ischemic lesions, animals in Group III compared to Group II showed a statistically significant decrease in the levels of total POM products on days 3 and 7, both under spontaneous and induced conditions. This reduction was observed in both the early and late fractions. On day 3, the differences were: for total POM products  $p = 0.012$ , for early fractions  $p = 0.015$ , for late fractions  $p = 0.018$ ; on day 7:  $p = 0.006$ ,  $p = 0.008$ , and  $p = 0.009$ , respectively. Analysis of the dynamics within Group III from days 3 to 7 revealed a significant decrease

**TABLE 1**  
**ASSESSMENT OF THE IMPACT OF MT THERAPY ON THE SEVERITY OF NEUROLOGICAL DEFICIT IN EACI (ME [Q1; Q3])**

| Parameter                 | Group I                 |                         | Group II              |                        | Group III                 |                            |
|---------------------------|-------------------------|-------------------------|-----------------------|------------------------|---------------------------|----------------------------|
|                           | Day 3<br>(n=5)          | Day 7<br>(n=5)          | Day 3<br>(n=6)        | Day 7<br>(n=5)         | Day 3<br>(n=6)            | Day 7<br>(n=5)             |
| Garcia J.H. scale, scores | 18.00<br>[18.00; 18.00] | 18.00<br>[18.00; 18.00] | 7.50<br>[7.00; 8.00]* | 4.50<br>[3.00; 6.00]*& | 11.50<br>[10.00; 13.00]** | 15.00<br>[14.00; 16.00]**& |
| Bederson test, scores     | 0.00<br>[0.00; 0.00]    | 0.00<br>[0.00; 0.00]    | 2.00<br>[2.00; 3.00]* | 4.00<br>[4.00; 4.00]*& | 1.00<br>[1.00; 2.00]**    | 0.00<br>[0.00; 1.00]**&    |
| Placing test, scores      | 9.50<br>[9.00; 10.00]   | 9.50<br>[9.00; 10.00]   | 4.00<br>[3.00; 4.00]* | 2.00<br>[1.00; 2.00]*& | 6.00<br>[6.00; 7.00]**    | 8.50<br>[8.00; 9.00]**&    |

**Note.** Significant differences according to the Mann – Whitney U-test are indicated as follows: \* – compared with Group I; # – compared with Group II; & – significant differences between day 3 and day 7 within the corresponding group ( $p < 0.05$ ).

in the concentrations of total POM products ( $p = 0.024$ ), total early ( $p = 0.021$ ) and total late ( $p = 0.027$ ) POM products. The overall RAP in rats from Group III was higher than in Group II on both day 3 ( $p = 0.031$ ) and day 7 ( $p = 0.017$ ) (Table 2).

A comparative study of the neurological status of animals in Group III compared to Group I showed a decrease in scores on the Garcia J.H. scale (day 3:  $p = 0.034$ ; day 7:  $p = 0.042$ ) and the Placing test (day 3:  $p = 0.027$ ; day 7:  $p = 0.038$ ), as well as an increase on the Bederson test (day 3:  $p = 0.029$ ; day 7:  $p = 0.031$ ) (Table 1).

When assessing POM products in the homogenates of cerebral ischemic lesions in animals of Group II compared with Group I on days 3 and 7, both under spontaneous and induced conditions, there was a statistically significant increase in total POM products as well

as early and late POM fractions ( $p < 0.001$  for all parameters at the respective time points). Overall RAP in animals from Group III compared to Group I was significantly reduced on days 3 and 7 ( $p = 0.034$  on day 3;  $p = 0.022$  on day 7) (Table 2).

Correlation analysis conducted on days 3 and 7 revealed a negative correlation between scores on the Garcia J.H. scale and the Placing test and lipid peroxidation (LPO) product levels in the ischemic lesion homogenate. Additionally, a correlation was observed between scores on the Garcia J.H. scale and the Placing test, and POM product levels in the ischemic lesion homogenate. The reduction in neurological deficit among animals with EACI treated with MT-containing rectal suppositories was associated with a decrease in both LPO and POM product levels in the ischemic lesion (Table 3).

**TABLE 2**  
**THE EFFECT OF MT ON THE CONTENT OF POM IN THE SITE OF CEREBRAL ISCHEMIA IN EACI (ME [Q1; Q3])**

| Parameter                                       | Group I                 |                         | Group II                 |                           | Group III                 |                            |
|-------------------------------------------------|-------------------------|-------------------------|--------------------------|---------------------------|---------------------------|----------------------------|
|                                                 | Day 3<br>(n=5)          | Day 7<br>(n=5)          | Day 3<br>(n=6)           | Day 7<br>(n=5)            | Day 3<br>(n=5)            | Day 7<br>(n=5)             |
| Total POM products (spontaneous), arb. units/mg | 65.0<br>[62.0; 68.0]    | 65.0<br>[62.0; 68.0]    | 220.0<br>[210; 230]*     | 200.0<br>[190; 210]*&     | 170.0<br>[160; 180]**     | 150.0<br>[140; 160]**&     |
| ADNPH (spontaneous), arb. units/mg              | 55.0<br>[52.0; 58.0]    | 55.0<br>[52.0; 58.0]    | 190.0<br>[180; 200]*     | 175.0<br>[165; 185]*&     | 150.0<br>[140; 160]*      | 130.0<br>[120; 140]**&     |
| KDNPH (spontaneous), arb. units/mg              | 11.0<br>[10.0; 12.0]    | 11.0<br>[10.0; 12.0]    | 25.0<br>[23.0; 27.0]*    | 22.0<br>[20.0; 24.0]*&    | 20.0<br>[18.0; 22.0]**    | 18.0<br>[16.0; 20.0]**&    |
| UV fraction (spontaneous), arb. units/mg        | 0.018<br>[0.016; 0.020] | 0.018<br>[0.016; 0.020] | 42.0<br>[40.0; 44.0]*    | 38.0<br>[36.00; 40.00]*&  | 30.0<br>[28.0; 32.0]**    | 25.0<br>[23.0; 27.0]**&    |
| Visible fraction (spontaneous), arb. units/mg   | 0.030<br>[0.028; 0.032] | 0.030<br>[0.028; 0.032] | 3.2<br>[3.0; 3.4]*       | 2.8<br>[2.6; 3.0]*&       | 2.2<br>[2.0; 2.4]**       | 1.8<br>[1.6; 2.0]**&       |
| Total POM products (induced), arb. units/mg     | 70.0<br>[67.0; 73.0]    | 70.0<br>[67.0; 73.0]    | 420.0<br>[400.0; 440.0]* | 380.0<br>[360.0; 400.0]*& | 300.0<br>[280.0; 320.0]** | 240.0<br>[220.0; 260.0]**& |
| ADNPH (induced), arb. units/mg                  | 20.0<br>[18.0; 22.0]    | 20.0<br>[18.0; 22.0]    | 250.0<br>[240.0; 260.0]* | 220.0<br>[210.0; 230.0]*& | 180.0<br>[170.0; 190.0]** | 150.0<br>[140.0; 160.0]**& |
| KDNPH (induced), arb. units/mg                  | 15.0<br>[14.0; 16.0]    | 15.0<br>[14.0; 16.0]    | 120.0<br>[115.0; 125.0]* | 100.0<br>[95.0; 105.0]*&  | 80.0<br>[75.0; 84.0]**    | 60.0<br>[55.0; 60.0]**&    |
| UV fraction (induced), arb. units/mg            | 10.0<br>[9.0; 11.0]     | 10.0<br>[9.0; 11.0]     | 30.0<br>[28.0; 31.0]*    | 25.0<br>[23.0; 27.0]*&    | 20.0<br>[18.0; 22.0]**    | 15.0<br>[14.0; 16.0]**&    |
| Visible fraction (induced), arb. units/mg       | 25.0<br>[23.0; 27.0]    | 25.0<br>[23.0; 27.0]    | 20.0<br>[19.0; 21.50]*   | 35.0<br>[33.0; 37.0]*&    | 20.0<br>[18.0; 22.0]**    | 15.0<br>[14.0; 16.0]**&    |
| RAP, %                                          | 92.9<br>[89.7; 96.0]    | 92.9<br>[89.7; 96.0]    | 52.4<br>[50.0; 54.8]*    | 52.6<br>[50.0; 55.3]*     | 56.7<br>[53.3; 60.0]**    | 62.5<br>[58.3; 66.7]**&    |

TABLE 3

**CORRELATIONS BETWEEN INDICATORS OF FREE RADICAL OXIDATION IN BRAIN TISSUE AND NEUROLOGICAL STATUS DURING MT THERAPY (RECTAL SUPPOSITORIES) IN EXPERIMENTAL CEREBRAL ISCHEMIA**

| Parameter                 | Total POM products (spontaneous),<br>arb. units/mg | Total POM products (induced),<br>arb. units/mg |
|---------------------------|----------------------------------------------------|------------------------------------------------|
| Garcia J.H. scale, scores | R= <u>-0.76</u><br>-0.71                           | R= <u>-0.81</u><br>-0.79                       |
| Placing test, scores      | R= <u>-0.66</u><br>-0.62                           | R= <u>-0.78</u><br>-0.74                       |

**Note.** R – Spearman's rank correlation coefficient; numerator – day 3, denominator – day 7.

## DISCUSSION

We hypothesize that simultaneous diathermocoagulation, damaging pial vessels, induced cerebral hypoperfusion. This reduction in cerebral blood flow, in turn, provoked mitochondrial respiratory chain dysfunction, accompanied by electron leakage and formation of reactive oxygen species (ROS), such as hydroxyl radical and peroxynitrite. These ROS exerted damaging effects on neuronal cell proteins and lipids [21]. Proteins in neuronal membranes and cytoskeletons are primary targets for ROS due to their presence of highly reactive amino acids, such as lysine, arginine, proline, cysteine, and methionine, leading to formation of carbonyl derivatives (ADNPH/KDNPH) [22]. These carbonyl derivatives, interacting with neuronal cytoskeletal proteins, such as  $\beta$ -actin and tubulin, disrupt axonal transport, cause loss of neuritic processes, and ultimately lead to cytoskeletal disintegration. Oxidation of cysteine residues in the active sites of ion-transporting proteins disrupts ion homeostasis, leading to membrane depolarization and increased intracellular  $\text{Ca}^{2+}$  concentration in neurons. This can activate excitotoxicity and lead to neuronal death [23]. Several previous studies have demonstrated that the stability of carbonyl derivatives, as opposed to rapidly metabolized LPO products, makes POM a reliable marker of oxidative damage in nervous tissue [24, 25].

The use of melatonin-containing rectal suppositories in animals with EACI has resulted in a partial reduction in neurological deficits, as measured by increased scores on the Garcia J.H. scale and the Placing test and decreased scores on the Bederson test. This may be due to the pleiotropic effects of melatonin, which is incorporated into rectal suppositories and has lipophilic properties that ensure its unhindered penetration through the intestinal mucosa and into the bloodstream [26]. The antioxidant effect of melatonin is achieved through its direct neutralization of ROS, forming non-toxic metabolites. By activating nuclear transcription factors that encode heme oxygenase-1 and thioredoxin-1, melatonin enhances the synthesis of antioxidant enzymes, such as heme oxygenase-1, quinone oxidoreductase-1, glutamate-cysteine ligase, superoxide dismutase,

and glutathione peroxidase. Furthermore, the primary melatonin metabolite, cyclic 3-hydroxymelatonin, exhibits a pronounced antioxidant activity that is superior to that of ascorbate and  $\alpha$ -tocopherol. The final metabolite, N1-acetyl-5-methoxykynuramine, is also a potent ROS scavenger and a cyclooxygenase inhibitor, further enhancing the antioxidant effects of melatonin [27]. Melatonin prevents programmed cell death in the penumbra via mitochondria-mediated and receptor-dependent mechanisms. It induces tyrosine kinase-dependent phosphorylation of STAT3, which dimerizes and translocates to the nucleus, enhancing transcription of the Bcl-2 family of genes (Bcl-2 and Bcl-xL), thereby inhibiting mitochondrial permeability, release of cytochrome c, and activation of caspase-9 and caspase-3, suppressing the intrinsic apoptotic pathway. Melatonin suppresses the expression of death receptors (Fas/CD95) on cell surfaces and reduces the sensitivity of neurons to the Fas ligand, thereby inhibiting the apoptotic pathway dependent on these receptors [28].

## CONCLUSION

The results of the study demonstrated the development of a marked neurological deficit in rats with EACI on days 3 and 7. This was objectively recorded using the Garcia J.H. scale, the Placing test, and the Bederson test. At the same time, a significant increase in the levels of all studied fractions of POM products (early and late) was observed in the cerebral ischemic lesion homogenate under both spontaneous and induced conditions in both groups of animals.

The use of melatonin-containing rectal suppositories contributed to a significant improvement in neurological function, as demonstrated by improved scores on all neurological tests. At the same time, a statistically significant reduction in the concentration of POM products was observed in the area of cerebral ischemia, although levels still did not reach those seen in the sham-operated group. Correlation analysis confirmed a strong relationship between the reduction in neurological deficit and the decrease in POM product levels in the cerebral ischemic lesion.

## Conflicts of interest

The authors declare no conflicts of interest.

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## DOSE- AND TIME-DEPENDENT EFFECTS OF THE DUAL GIP/GLP-1 RECEPTOR AGONIST TIRZEPATIDE ON EMOTIONAL OVEREATING IN A RAT MODEL OF INTRACRANIAL SELF-STIMULATION

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### ABSTRACT

**Background.** Despite the increasing prevalence of emotional overeating and obesity, the specific neurohormonal mechanisms involved in the pathogenesis of these pathological processes remain poorly understood. The incretin system may serve as an important therapeutic target for the correction of these eating disorders.

**Aim.** To study the effects of the dual GIP/GLP-1 receptor agonist tirzepatide on feeding behavior parameters in a rat model of emotional overeating induced by intracranial self-stimulation (ICSS).

**Methods.** Experiments were performed on male Wistar rats with electrodes implanted in the ventral tegmental area. Tirzepatide was administered subcutaneously at doses of 0.05, 0.10, and 0.30 mg/kg for 7 days. Food intake (number of seeds consumed) was assessed at 1 minute, 30 minutes, and on days 3 and 7 after administration.

**Results.** No statistically significant changes in feeding activity were observed 30 minutes after tirzepatide administration. On the third day, a decrease in food intake was observed only at the maximum dose (0.30 mg/kg). On the seventh day, a significant anorexigenic effect was observed at doses of 0.10 and 0.30 mg/kg, indicating a dose- and time-dependent effect.

**Conclusion.** Tirzepatide does not exert a significant acute effect on feeding activity in the emotional overeating model at 30 minutes after administration. Repeated administration of the drug revealed a dose- and time-dependent anorexigenic effect, manifested by a decrease in food intake on days 3 and 7 of the experiment. The most pronounced decrease in feeding activity was observed at doses of 0.10 and 0.30 mg/kg on day 7.

**Keywords:** tirzepatide, incretin, GLP-1, GIP, emotional overeating, brain stimulation, VTA, feeding behavior

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## ДОЗО- И ВРЕМЯЗАВИСИМОЕ ВЛИЯНИЕ ДВОЙНОГО АГОНИСТА РЕЦЕПТОРОВ GIP/GLP-1 ТИРЗЕПАТИДА НА ЭМОЦИОНАЛЬНОЕ ПЕРЕЕДАНИЕ В МОДЕЛИ ЭЛЕКТРИЧЕСКОЙ САМОСТИМУЛЯЦИИ МОЗГА У КРЫС

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### РЕЗЮМЕ

**Обоснование.** Несмотря на растущую распространенность эмоционального переедания и ожирения, остаются недостаточно изученными конкретные нейрогормональные механизмы в патогенезе данных патологических процессов. Инкретиновая система может служить важной терапевтической мишенью для коррекции этих расстройств пищевого поведения.

**Цель исследования.** Изучить влияние двойного агониста рецепторов GIP и GLP-1 тирзепатида на показатели пищевого поведения при экспериментальной моделировании эмоционального переедания, вызванного электрической самостимуляцией мозга у крыс.

**Методы.** Эксперименты выполнены на крысах-самцах линии Вистар с имплантированными электродами в вентральную область покрышки. Тирзепатид вводили подкожно в дозах 0,05; 0,10 и 0,30 мг/кг курсом в течение 7 суток. Оценивали количество семян, потреблённых животными за 1 минуту, 30 минут, а также на 3-и и 7-е сутки после введения препарата.

**Результаты.** Показано, что на 30-й минуте после введения тирзепатида статистически значимых изменений пищевой активности не выявлялось. На третьи сутки снижение потребления пищи отмечалось только при максимальной дозе (0,30 мг/кг). На седьмые сутки достоверное анорексигенное действие наблюдалось при дозах 0,10 и 0,30 мг/кг, что свидетельствует о дозо- и времязависимом характере эффекта.

**Заключение.** Тирзепатид не оказывает выраженного острого влияния на пищевую активность в модели эмоционального переедания на 30-й минуте после введения. При повторном введении препарата выявляется дозо- и времязависимый анорексигенный эффект, проявляющийся снижением потребления пищи на 3-и и 7-е сутки эксперимента. Наиболее выраженное снижение пищевой активности наблюдается при дозах 0,10 и 0,30 мг/кг на 7-е сутки введения.

**Ключевые слова:** тирзепатид, инкретин, GLP-1, GIP, эмоциональное переедание, самостимуляция мозга, VTA, пищевое поведение

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## BACKGROUND

Pharmacological intervention strategies involving simultaneous targeting of multiple regulatory signaling pathways are being extensively explored in modern medicine for the treatment of polyetiological chronic diseases, including metabolic disorders, obesity, and type 2 diabetes mellitus. The concept of polypharmacology involves the development of agents that act on multiple biological targets to enhance therapeutic efficacy and broaden the spectrum of action compared to monotargeted drugs [1, 2].

The underlying rationale of such approaches is to improve treatment outcomes through synergy arising from the concurrent activation of complementary mechanisms. Traditionally, this has been achieved via polypharmacotherapy, where patients receive a combination of several drugs [2]. However, advances in peptide engineering have enabled the transition to the creation of “multifunctional” molecules capable of acting on multiple pharmacological targets simultaneously within a single compound [2].

Most such hybrid peptides target G protein-coupled receptors (GPCRs). In recent years, a number of multi-receptor agonists have entered clinical trials as potential therapies for type 2 diabetes, obesity, and non-alcoholic steatohepatitis. The most advanced representative of this class to date is the dual GIP and GLP-1 receptor agonist – tirzepatide [3].

Initial clinical trials of tirzepatide have demonstrated unprecedented efficacy in lowering blood glucose levels and body weight in patients with type 2 diabetes [4]. Approximately one-third of patients receiving the maximum dose (15 mg) achieved normoglycemia, and about 25 % of participants lost at least 15 % of their baseline body weight within 26 weeks of therapy [5, 6]. Additionally, a marked reduction in fasting triglyceride levels was observed, and analysis of the insulin resistance index (HOMA2-IR) indicated improved tissue insulin sensitivity, which was only partially explained by weight loss [7, 8].

Such comprehensive metabolic improvement is consistent with the hypothesis that adding GIP activity to classical GLP-1 agonist therapy enhances glycemic control through several mechanisms: stimulation of insulin secretion by pancreatic  $\beta$ -cells, normalization of white adipose tissue function, and a pronounced anorexigenic effect mediated through integration of signals from both receptors at the level of the central nervous system [6, 8]. It is hypothesized that the high clinical efficacy of tirzepatide is largely determined by its specific pharmacological properties at each receptor.

The incretin system plays a key role in the regulation of energy homeostasis and feeding behavior. The major incretin hormones are glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), secreted by enteroendocrine cells of the intestine in response to food intake. GLP-1 receptors have been shown to be expressed in brain centers

regulating behavior and reward, including the ventral tegmental area and the nucleus accumbens, which may influence motivation for food and other rewards [4]. Activation of GLP-1R in these structures leads to reduced food intake and modulation of dopaminergic transmission, indicating the involvement of the incretin system in the regulation of the hedonic component of feeding [5]. GIP receptors are also expressed in the CNS, and their co-activation with GLP-1R is considered a promising mechanism for enhancing metabolic and behavioral effects [8]. GLP-1 receptor agonists have been shown to reduce engagement of reward systems, decreasing addictive-like behavior in models of nicotine use disorders [5].

However, experimental data on the effects of dual GIP/GLP-1 agonists, such as tirzepatide, on behavior under conditions of hyperactivation of the reward system remain extremely limited. Tirzepatide has been shown to attenuate dopaminergic signals associated with alcohol and reduce reward-related behavioral manifestations, indicating an impact on central reward mechanisms [5].

Therefore, the relevance of this study lies in the experimental evaluation of the effects of tirzepatide on emotional overeating in a model induced by brain self-stimulation. Since this disorder is characterized by combined dysregulation of reward systems, food motivation, and energy homeostasis, it is fundamentally sensitive to drugs with a multimodal mechanism of action.

## AIM

To investigate the effects of the dual GIP/GLP-1 receptor agonist tirzepatide on feeding behavior parameters in an experimental model of emotional overeating induced by electrical self-stimulation of the brain in rats.

## METHODS

The study was conducted in accordance with the requirements of the European Convention for the care, maintenance, and euthanasia of laboratory animals and was approved by the local ethics committee of Saint Petersburg State Pediatric Medical University (Protocol No. 58/03, dated September 25, 2025).

**Animals.** A total of 24 male Wistar rats obtained from the Rappolovo laboratory animal breeding facility (Leningrad Region) were used in the study. Animals were housed in standard plastic cages, 5 per cage, with free access to water and food, under an inverted light cycle (8:00 AM – 8:00 PM) at a temperature of  $22 \pm 2^\circ\text{C}$ . All experiments were conducted during the autumn-winter period.

**Surgery.** Upon reaching a body weight of 250 g, rats underwent stereotaxic surgery. Rats were anesthetized with Zoletil® 100 at a dose of 50 mg/kg

(tiletamine, zolazepam; Virbac, France). Bipolar stainless steel electrodes (0.2 mm diameter) were implanted using a stereotaxic apparatus (Harvard Apparatus, USA) under aseptic conditions. Electrodes were implanted into the right ventral tegmental area (VTA) at the following coordinates relative to Bregma: AP = -5.3 mm, L = 0.8 mm, H = 8.2 mm.

**Self-stimulation response study.** In a Skinner box (29.2 × 30.5 × 24.1 cm), a lever (4.5 × 2.0 cm) was positioned 3.0 cm above the floor. The chamber was connected to a computer via an interface and controlled by software (Institute of Experimental Medicine). Each lever press delivered a train of rectangular pulses (500 ms duration, 100 Hz pulse frequency, 1 ms pulse duration). Animals were trained for 10 minutes daily over 5 days. During these sessions, current intensity was adjusted to achieve maximum lever-pressing rates without impairing motor function or inducing seizures. Animals that failed to press the lever more than 50 times during 10-minute sessions were excluded from the experiment. The remaining animals were tested during 30-minute sessions over 5 additional days to establish a stable baseline response rate [9].

**Self-stimulation and feeding behavior competition test.** After stable self-stimulation parameters were established, animals were placed on a food deprivation schedule. Rats were trained for 30 minutes daily to access a feeder containing abundant shelled sunflower seeds over 5 days in the self-stimulation testing chamber. The self-stimulation lever was disabled during this phase. Subsequent experiments were conducted under choice conditions between self-stimulation and food consumption: a feeder was placed in the Skinner box, and the lever was activated with a fixed current intensity. All animals were required to maintain stable body weight [9].

**Hypothalamic stimulation-induced behavior test.** Following the competition test, animals were returned to ad libitum access to standard pelleted chow and water. Each animal was placed in a specialized chamber for 5 minutes containing a feeder with sunflower seeds and several wooden chips. At the end of this habituation period, forced hypothalamic stimulation (10 periods of 30 seconds each) was applied at the current intensity used during the competition test. Food-directed responses or chewing induced by stimulation were recorded [10].

**Self-stimulation-induced emotional overeating test.** In the emotional overeating test in satiated rats, the threshold current intensity required for lever presses during self-stimulation was used. Threshold determination was performed by varying current intensity in 5  $\mu$ A increments. Following threshold determination, a feeder was placed in the Skinner box and the self-stimulation lever was activated. The number of approaches to the feeder, number of seeds consumed, number of lever presses at fixed threshold current intensity, and feeding behavior parameters were recorded over 10 minutes. On subsequent days, drugs were administered

20 minutes prior to testing, and then the rat was placed in the chamber to assess self-stimulation-induced overeating [10].

Initially, 28 animals were included in the study. Following postoperative recovery and the self-stimulation training phase, 4 animals failed to develop a stable behavioral response (did not press the lever) and were consequently excluded from further experimentation. Thus, 24 rats were included in the final analysis.

Randomization was performed using simple random sampling with a random number generator. Prior to group allocation, animals were stratified by body weight (250 g) to ensure group comparability at baseline. Each animal was then assigned an individual number, and group allocation was performed by an independent investigator not involved in subsequent behavioral testing.

**Drugs.** Tirzepatide, a synthetic peptide, an unbalanced dual agonist of the glucose-dependent insulinotropic polypeptide (GIPR) and glucagon-like peptide-1 (GLP-1R) receptors, was used in the study. Tirzepatide (PROMOMED JSC, Russian Federation) was administered subcutaneously in the scruff region at doses of 0.05, 0.1, and 0.3 mg/kg body weight in a volume of 1 mL/kg, calculated individually based on animal weight, over a 7-day course. A 0.9 % sterile sodium chloride solution was used as a control solution administered subcutaneously. Rats were tested 20 minutes after the last administration.

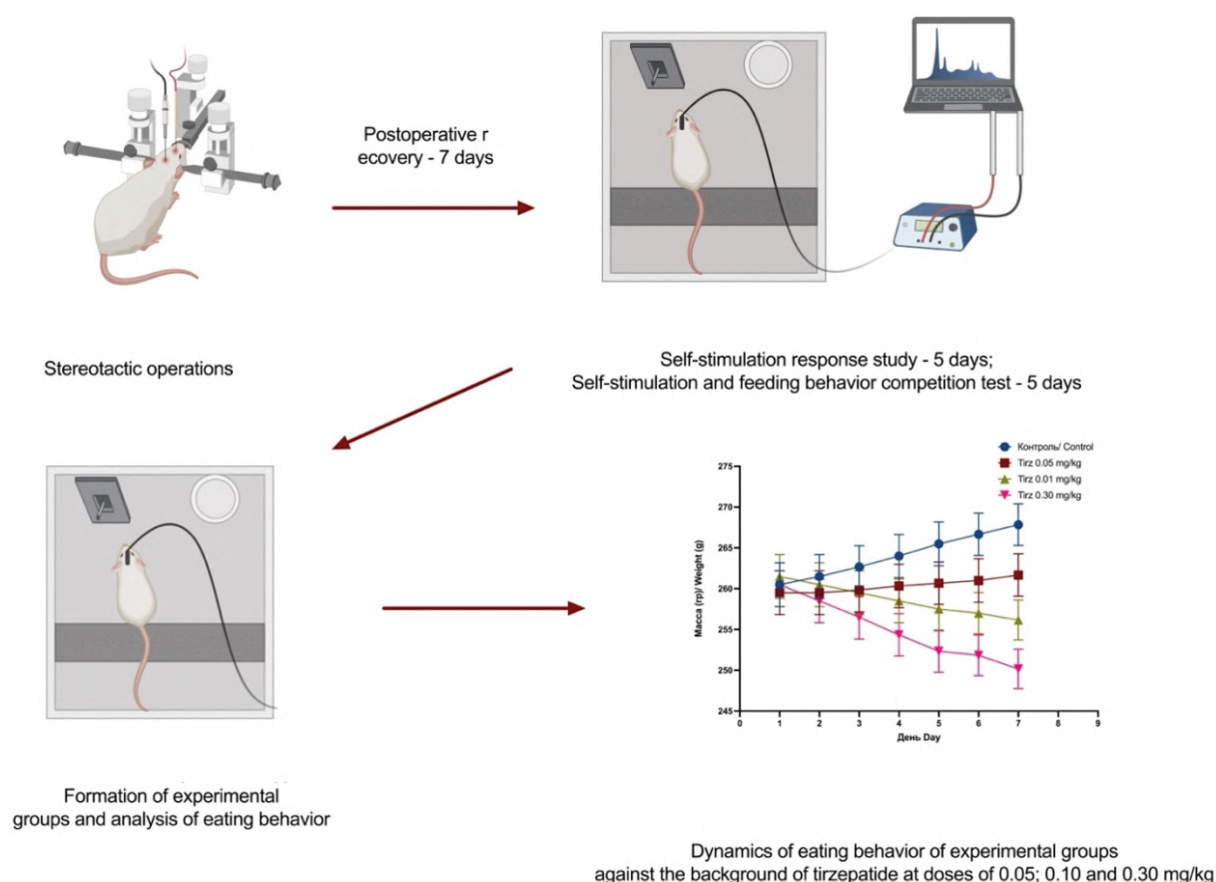
A schematic representation of the experimental design is provided in Figure 1.

**Statistical analysis.** Statistical significance was assessed using GraphPad Prism 10. Data were tested for normality (Shapiro – Wilk test). As distributions deviated from normality, the non-parametric Kruskal – Wallis test was used with subsequent Dunn's post-hoc test. For analysis of parameter dynamics over time, the Friedman test with Wilcoxon – Holm pairwise comparisons was applied. The significance level was set at  $p < 0.05$ .

## RESULTS

According to the Kruskal – Wallis test, differences between the four groups were non-significant at 30 minutes post-administration ( $H = 2.097$ ,  $p = 0.5525$ ), indicating no overall dose effect on the number of seeds consumed. Dunn's post-hoc analysis also revealed no significant differences between the control group and the groups receiving the compound at 0.05, 0.10, and 0.30 mg/kg ( $p > 0.05$ ) (Fig. 2).

The Kruskal – Wallis test revealed an overall dose effect on day 3 of administration ( $H = 9.641$ ,  $p = 0.0219$ ), indicating significant variability in median values between groups. Dunn's post-hoc analysis showed a significant difference only between the control group and the group receiving the compound at 0.30 mg/kg ( $p = 0.0265$ ). No significant differences were found



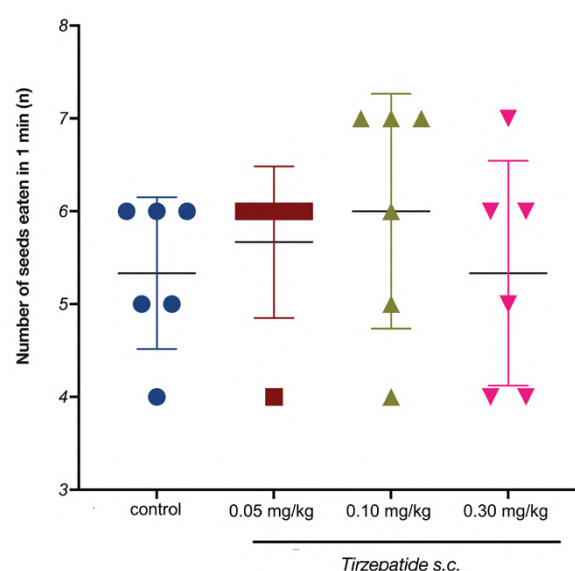
**FIG. 1.**  
Study design scheme

between the control and the 0.05 mg/kg and 0.10 mg/kg groups (Fig. 3).

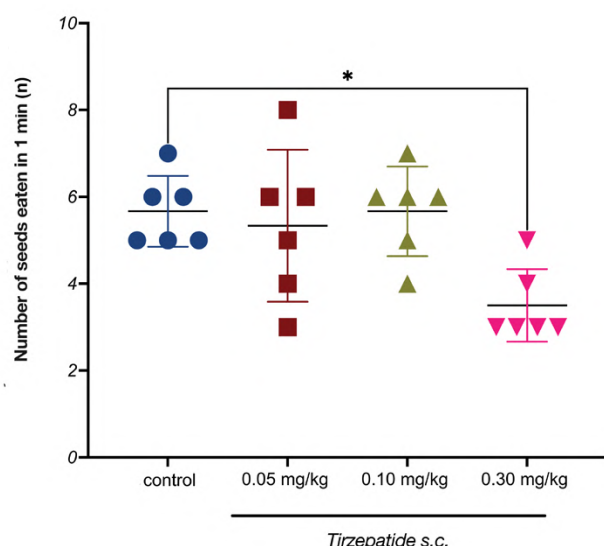
The Kruskal – Wallis test revealed an overall dose effect on day 7 of administration ( $H = 14.16, p = 0.0027$ ), indicating significant variability in median values between groups. Dunn's post-hoc analysis showed significant differences between the control group and the groups receiving the compound at 0.10 mg/kg and 0.30 mg/kg ( $p = 0.0442$ ). No differences were found between the control and the 0.05 mg/kg group (Fig. 4).

Analysis of body weight dynamics revealed differential changes depending on the tirzepatide dose. In the control group, physiological weight gain was observed throughout the observation period. In the group receiving tirzepatide at 0.05 mg/kg, weight gain was slower compared to controls. In the 0.10 mg/kg group, a trend toward gradual weight loss was observed, while at 0.30 mg/kg, pronounced weight loss was registered from day 2–3 of the experiment (Fig. 5A).

By day 7, intergroup differences in body weight were statistically significant. The Kruskal – Wallis test revealed an overall dose effect ( $H = 13.19, p < 0.01$ ). Post-hoc comparison showed significant weight loss in the tirzepatide 0.30 mg/kg group compared to controls ( $p < 0.01$ ).

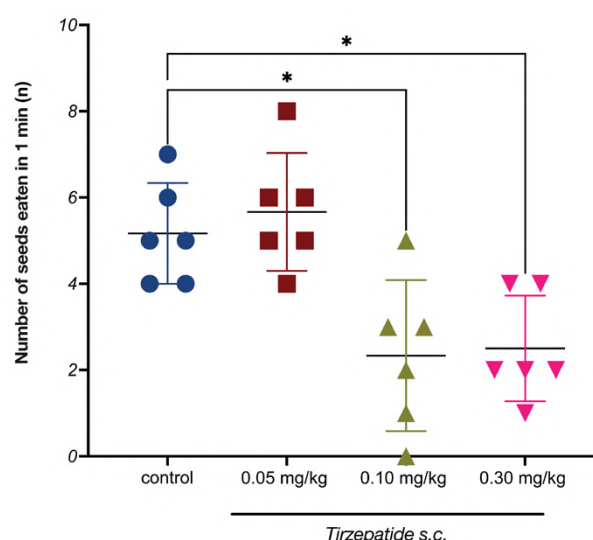


**FIG. 2.**  
Feeding activity, defined as the number of seeds consumed per minute, in the control group and in groups receiving the test compound at various doses, assessed at 30 minutes post-administration. Each dot represents an individual animal



**FIG. 3.**

Feeding activity, defined as the number of seeds consumed per minute, in the control group and in groups treated with the test compound at various doses on day 3 post-administration (Mean  $\pm$  SD). Each dot represents an individual animal. \* $p < 0.05$  (Kruskal-Wallis test followed by Dunn's post-hoc test)



**FIG. 4.**

Feeding activity, defined as the number of seeds consumed per minute, in the control group and in groups treated with the test compound at various doses on day 7 post-administration (Mean  $\pm$  SD). Each dot represents an individual animal. \* $p < 0.05$  (Kruskal – Wallis test followed by Dunn's post-hoc test)

The 0.10 mg/kg group also showed a statistically significant difference relative to controls ( $p < 0.05$ ), while the 0.05 mg/kg dose did not differ from controls (Fig. 5B).

Thus, tirzepatide administration was associated with dose-dependent weight loss, most pronounced at 0.30 mg/kg, confirming the pharmacological activity of the drug under repeated administration conditions.

## DISCUSSION

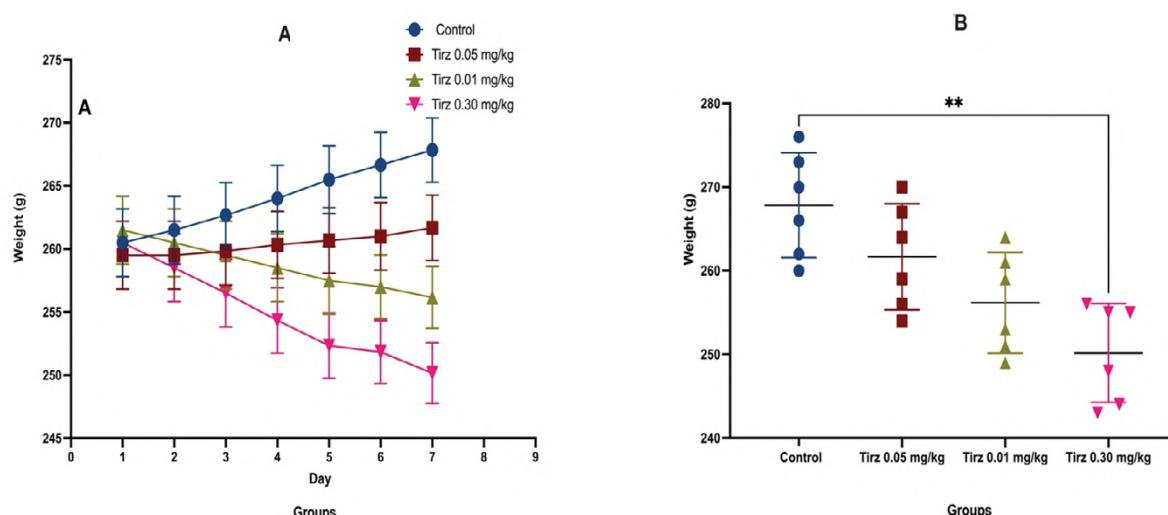
The present study demonstrated that tirzepatide exerts dose- and time-dependent effects on feeding behavior in a model of emotional overeating induced by electrical self-stimulation of the brain. The observed gradual development of the anorexigenic effect confirms that the influence of dual GIP/GLP-1 receptor activation on behavioral parameters requires repeated administration and is not immediate. This profile is consistent with clinical data on the gradual reduction of appetite and body weight during tirzepatide therapy [11, 12].

The absence of a statistically significant effect 30 minutes after a single drug administration may indicate that short-term incretin receptor activation does not lead to immediate behavioral changes under conditions of an activated reward system. This aligns with data showing that central GLP-1 receptor effects develop gradually and are associated with changes in hypothalamic and mesolimbic neuronal activity [13]. It has been shown that blockade of endogenous GLP-1R signaling in the ventral tegmental area (VTA) and nucleus

accumbens (NAc) led to a significant increase in food intake, establishing the physiological significance of GLP-1 signaling in the mesolimbic reward system [14]. Our hypothesis is supported by functional magnetic resonance imaging studies in patients receiving GLP-1 agonists, demonstrating reduced reward center reactivity to food stimuli after several weeks of therapy, but not in the first days of treatment [15–17].

The reduction in food intake observed on day 3 only at the maximum dose indicates the existence of a threshold level of receptor activation required to achieve the behavioral effect. Similar data on dose-dependency of incretin effects have been demonstrated in preclinical models for GLP-1R agonists. Activation of central GLP-1Rs has been shown to suppress reward and motivation for food intake, interacting with the mesolimbic system [17]. Concurrent activation of GIPR and GLP-1R has been shown to enhance metabolic and behavioral effects compared to isolated stimulation of a single receptor, supporting the concept of synergy of dual agonists [18].

The most pronounced reduction in feeding activity by day 7 of drug administration indicates the establishment of a stable pharmacological effect. Clinical studies demonstrate a similar dynamic of gradual intensification of tirzepatide's anorexigenic action during the first weeks of therapy [11, 12]. In patients with type 2 diabetes mellitus, tirzepatide was non-inferior to semaglutide and superior in terms of mean change in glycated hemoglobin and body weight from baseline at 40 weeks [19]. Furthermore, changes in eating behavior patterns in patients during long-term drug administration have been demonstrated [20].



**FIG. 5.**  
**A** – Body weight changes (Mean  $\pm$  SD) in the control group and in groups treated with tirzepatide at doses of 0.05, 0.10, and 0.30 mg/kg.  
**B** – Individual body weight values on day 7 of the experiment, with the group mean indicated by a horizontal line. Each dot represents an individual animal. \*\* $p < 0.01$  (Kruskal – Wallis test followed by Dunn’s post-hoc test).

From a neurobiological perspective, it is well established that GLP-1 receptors are expressed in structures involved in the regulation of both homeostatic and hedonic feeding, including the hypothalamic arcuate nucleus, ventral tegmental area, and nucleus accumbens [13, 14]. GLP-1R activation in the VTA reduces dopaminergic activity and diminishes the motivational salience of food stimuli. Central delivery of the GLP-1R agonist exendin-4 has been shown to suppress phasic dopamine release induced by intravenous cocaine administration. This effect was selective for dopamine signaling in the NAc [21].

The influence of GIP receptors on central mechanisms has been studied less extensively. However, it has been shown that their co-activation may enhance GLP-1 effects through alterations in intracellular signaling and potentiation of metabolic responses [18, 22]. The hypothesis that dual GIP/GLP-1 activation leads to more pronounced modulation of mesolimbic circuits compared to selective GLP-1R stimulation appears biologically plausible but requires direct neurochemical confirmation.

The present study demonstrates the effect of tirzepatide on feeding behavior in a model of artificial hyperactivation of the reward system. In contrast to most preclinical studies evaluating hyperphagia in diet-induced obesity models, this model allows investigation of the hedonic component of feeding associated with activation of the mesolimbic dopaminergic system. Therefore, this work expands the mechanistic understanding of the clinical effects of tirzepatide.

The data obtained suggest that tirzepatide may be considered a potential tool for correcting not only

metabolic disorders but also conditions characterized by pathologically enhanced food motivation. Clinical data indicate the presence of a pronounced hedonic component of feeding and elements of compulsive behavior in a subset of patients with obesity [23–25]. If the hypothesis of central modulation of the reward system is confirmed, this may expand the therapeutic potential of dual incretin agonists in patients with emotional overeating and comorbid impulse control disorders [26].

Therefore, the present study confirms the dose- and time-dependent nature of tirzepatide’s anorexigenic effect in an experimental model of emotional overeating, demonstrates the potential involvement of central mechanisms of motivational regulation, and establishes a foundation for further translational research.

## CONCLUSION

The obtained data confirm that tirzepatide, upon repeated administration, reduces feeding activity in a model of emotional overeating and exhibits a dose- and time-dependent effect. However, to demonstrate a primarily central (motivational) mechanism of action, additional studies with control of locomotion/somatic status and an extended panel of behavioral and neurobiological markers of the reward system are required. The potential role of dual incretin activation in the correction of feeding behavior relevant to clinical forms of obesity with a pronounced hedonic component is substantiated, while simultaneously indicating the limitations of interpretation necessary for clinical translation.

# Conflict of interest

The authors declare no conflict of interest.

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## A NEW LOOK AT THE ALIMENTARY ROUTE OF TUBERCULOSIS TRANSMISSION USING A RABBIT MODEL (EXPERIMENTAL STUDY)

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### ABSTRACT

**Background.** The increase in the number of cases of generalized tuberculosis infection with intestinal damage, especially in HIV-positive patients, underscores the need for further study of this problem. Tuberculosis is transmitted by airborne droplets; however, there are known cases of intestinal tuberculosis in which infection occurred through the alimentary route. Since such cases are extremely rare, there are likely specific immunological and epidemiological conditions that allow *Mycobacterium tuberculosis* to penetrate the intestinal wall and develop primary inflammation.

**The aim.** To study the possibility of an alimentary route of tuberculosis infection in immunocompetent and immunocompromised individuals using a reproducible rabbit model.

**Materials and Methods.** The experiment involved rabbits, divided into three groups. Animals were orally administered a suspension of a standard laboratory isolate *Mycobacterium tuberculosis* H37Rv. The first group included immunocompetent rabbits immunosuppressed with infliximab, the second group had animals reinfected against the background of immunodeficiency, and the third group included rabbits with a combination of drug-induced immunosuppression and chemically induced enterocolitis. To assess the results of infection, clinical observation, detection of MTB DNA in feces, skin test with ATR, pathoanatomical, histological and molecular genetic examination of organs were used.

**Results.** Despite the temporary shedding of MTB DNA in feces (60–70 %), no signs of tuberculous inflammation in the organs were present in animals in groups 1 and 2. In group 3, macro- and microscopic signs of tuberculosis were detected in the lungs of 83.3 % of the animals and in the mesenteric/intrathoracic lymph nodes of 58.3 %. At the same time, no specific granulomatous intestinal lesions were found in any of the groups.

**Conclusion.** The development of tuberculous enterocolitis in rabbits following oral infection is unlikely. The alimentary route of infection can lead to generalized tuberculosis only when two key predisposing factors are present: systemic immunosuppression and previous damage to the intestinal barrier.

**Keywords:** experimental tuberculosis, alimentary route of infection, animal model, rabbit, model of tuberculous enterocolitis, erosions and ulcers of the intestine, dissemination

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## НОВЫЙ ВЗГЛЯД НА АЛИМЕНТАРНЫЙ ПУТЬ ЗАРАЖЕНИЯ ТУБЕРКУЛЕЗОМ С ИСПОЛЬЗОВАНИЕМ КРОЛИЧЬЕЙ МОДЕЛИ (ЭКСПЕРИМЕНТАЛЬНОЕ ИССЛЕДОВАНИЕ)

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### РЕЗЮМЕ

**Обоснование.** Увеличение числа случаев генерализованной туберкулезной инфекции с поражением кишечника, особенно у ВИЧ-позитивных пациентов диктует необходимость дальнейшего изучения данной проблемы. Туберкулез передается воздушно-капельным путем, однако известны случаи туберкулеза кишечника, при которых инфицирование произошло алиментарным путем. Поскольку такие случаи крайне редки, вероятно, существуют особые иммунологические и эпидемиологические условия для проникновения *Mycobacterium tuberculosis* в стенку кишки и развития в ней первичного воспаления.

**Целью** настоящего исследования является изучение возможности алиментарного пути заражения туберкулезной инфекцией у иммунокомпетентных и иммунокомпрометированных особей на воспроизводимой кроличьей модели.

**Материалы и методы.** В эксперименте были использованы кролики, распределенные на три группы. Животным перорально вводили суспензию культуры стандартного лабораторного штамма *Mycobacterium tuberculosis* H37Rv. Первая группа включала иммунокомпетентных и иммуносупрессированных инфликсимабом кроликов, вторая – животных с повторным заражением на фоне иммунодефицита, третья – кроликов с комбинацией медикаментозной иммуносупрессии и химически индуцированного энтероколита. Для оценки результатов заражения использовали клиническое наблюдение, детекцию ДНК МБТ в кале, кожную пробу с АТР, патологоанатомическое, гистологическое и молекулярно-генетическое исследование органов.

**Результаты.** У животных 1-й и 2-й групп, несмотря на временное выделение ДНК МБТ с калом (60–70 %), признаки туберкулезного воспаления в органах отсутствовали. В 3-й группе макро- и микроскопические признаки туберкулеза выявлены в легких у 83,3 % животных и в мезентериальных/внутригрудных лимфатических узлах у 58,3 %. При этом специфического гранулематозного поражения кишечника не обнаружено ни в одной из групп.

**Заключение.** Развитие туберкулезного энтероколита у кроликов при пероральном заражении маловероятно. Алиментарный путь инфицирования может приводить к генерализованному туберкулезу только при сочетании двух ключевых предрасполагающих факторов: системной иммуносупрессии и предшествующего повреждения кишечного барьера.

**Ключевые слова:** экспериментальный туберкулез, алиментарный путь заражения, животная модель, кролик, модель туберкулезного энтероколита, эрозия и язвы кишечника, диссеминация

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## INTRODUCTION

Despite significant progress in the fight against tuberculosis (TB), it continues to be a global infectious threat to the world's population. The HIV pandemic, the use of genetically engineered biological drugs (interleukin inhibitors) and glucocorticoids, as well as comorbid conditions such as diabetes mellitus, autoimmune and oncological diseases have created a significant number of immunocompromised patients. In these individuals, there have been an increasing number of cases of generalized and extrapulmonary TB, including abdominal involvement [1]. Among all forms of abdominal TB, enterocolitis is the most severe and can lead to complications such as perforation, bleeding, and intestinal obstruction [2]. Animal models can be used to study the routes of infection, disease pathogenesis, and test new therapeutic approaches.

Over the past several decades, extensive research has demonstrated that rabbits can be used as a suitable model to study pathological processes caused by *Mycobacterium tuberculosis* (MTB). This includes active TB and latent TB infection. The outcome of infection in rabbits is determined by the nature of the MTB strain that infects them, the inoculum dose, and any prior vaccination. Rabbits are also an excellent model to study extrapulmonary TB, as their clinical signs, disease progression patterns, and immunopathological reactions are similar to those seen in humans [3, 4]. Outbred rabbits have been shown to be relatively resistant to intravenous and aerosol infection with MTB cultures, and usually recover from infection within 4–6 months. After experimental aerosol infection, rabbits develop pulmonary granulomas and, in some cases, cavities that resemble those seen in humans [5].

In most cases, TB is transmitted via the airborne route. After the development of the primary focus in the lungs or mediastinal lymph nodes, the disease spreads to other organs and tissues through lymphohematogenous dissemination, when the immune system is weakened [6, 7]. However, there have been reports of cases of intestinal tuberculosis where infection occurred through the alimentary route, either through consumption of contaminated milk or ingestion of sputum [7]. These cases are extremely rare and specific immunological and epidemiological factors likely allow MTB to penetrate the intestinal wall and cause primary inflammation.

## AIM

To study the possibility of an alimentary transmission of tuberculosis in immunocompetent and immunocompromised individuals using a reproducible rabbit model.

## MATERIALS AND METHODS

The experimental study was conducted on 32 male Soviet Chinchilla rabbits, weighing 2540–3110 g [8]. All

animals were sourced from the Rappolovo Laboratory Animal Nursery at the Kurchatov Institute Research Center and were free from any visible signs of disease. Prior to the study, they underwent a two-week quarantine period at a certified animal facility located at the St. Petersburg Research Institute of Phthysiopulmonology under the Ministry of Health of the Russian Federation.

Rabbits were maintained under identical conditions, receiving a standard diet and water supply. All procedures involving animals were carried out in accordance with ethical principles outlined in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (CETS No. 170) and in compliance with GOST 33216-2014 Guidelines for Handling Laboratory Rodents and Rabbits [9]. The study was approved by the independent ethics committee of the St. Petersburg Research Institute of Phthysiopulmonology (Protocol No. 80.3, dated June 23, 2021), and fully complied with the decree of the chief sanitary physician of the Russian Federation, No. 4, dated January 28, 2021, which approved the sanitary regulations, SanPiN 3.3686-21, on Sanitary and Epidemiological Requirements for the Prevention of Infectious Diseases.

A suspension of the standard international virulent test strain *Mycobacterium tuberculosis* H37Rv was used as the infectious agent. This strain was obtained on August 7, 2013 from the Scientific Center for Expert Evaluation of Medicinal Products of the Ministry of Health of the Russian Federation. The inoculum dose used was  $10^6$  colony-forming units (CFU), which corresponds to 0.1 mg/0.5 mL in 2 mL of saline solution for rabbits. This mycobacterial suspension was administered orally using two enteric-coated gelatin capsules via a tablet dispenser (Neoterica Vetpro, Russia).

Depending on the experimental infection conditions, all animals were randomly divided into three groups. Group 1 consisted of 10 rabbits, which were divided into two subgroups: subgroup 1a ( $n = 5$ ) receiving oral infection without prior immunosuppressive treatment, and subgroup 1b ( $n = 5$ ) with prior drug-induced immunosuppression. Group 2 ( $n = 10$ ) consisted of rabbits with secondary drug-induced immunodeficiency, who received oral infection twice with a 30-day interval. Group 3 ( $n = 12$ ) included rabbits with both drug-induced immunodeficiency and chemical enterocolitis induction, before receiving MTB-containing capsules (Table 1).

To model drug-induced immunodeficiency, the levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) were reduced by administering the TNF- $\alpha$  inhibitor infliximab intravenously into the marginal ear vein. Infliximab is a chimeric murine-human monoclonal antibody produced by BIOCAD, a Russian company, and was administered at a dose of 16 mg/kg of body weight [10, 11].

Infliximab was selected as the immunosuppressive agent due to its ability to exhibit sufficient

cross-reactivity and neutralization of rabbit TNF- $\alpha$  *in vivo* despite differences in amino acid sequences between human and rabbit TNF- $\alpha$ . This has been confirmed in studies using animal models of various immune-mediated inflammatory diseases [12, 13].

To induce non-specific colitis, 0.1 % and 0.5 % dextran sulfate sodium solutions (DSS) (TdB Labs, Sweden, CAS No. 9011-18-1, molecular weight 40,000 Mw) were used, based on their safer concentrations and ability to induce catarrhal and erosive-ulcerative inflammation [14, 15]. The rabbits were given water containing the DSS solution via drinking bottles for 6 days (up to 1350 mL on average). Six animals received 0.1 % DSS solution, and the other six received 0.5 % DSS solution mixed with fennel tea to mask the unpleasant taste and odor of the solution.

During the course of the experimental study, animals were monitored on a daily basis. Body weights were recorded on days 20, 60 and 90 of the study. On day 10, samples of rabbit feces were analyzed for the presence of *Mycobacterium tuberculosis* DNA. On day 35, a skin test using recombinant tuberculosis allergen (RTA) was performed (Diaskintest, Russia). The test results were assessed 72 hours after the test.

Euthanasia was carried out on day 90 after infection by anesthetic overdose: propofol 70 mg and rocuronium bromide 30 mg were administered intravenously. During the necropsy, samples were collected for morphological and bacterial examination from the greater omentum, small and large intestinal fragments, mesenteric lymph nodes, heart, liver, spleen, kidneys,

and lungs. Samples were also taken from the intestines, lungs, lymph nodes, and omentum for molecular genetic analysis.

Biological material was labeled and fixed in a 10 % neutral buffered formalin solution. Microscopic analysis was performed using a Nikon Ci-S microscope (Nikon Corporation, Japan), with video-digital image processing to archive and analyze the material. Histological sections were stained using hematoxylin and eosin as well as the Ziehl-Neelsen technique. MTB DNA in the tissues was detected using polymerase chain reaction (PCR), employing the AmpliTub-RV reagent kit from Syntol LLC (Russia).

Data collection, correction, systematization, and visualization were performed using Microsoft Office Excel 2016. Statistical analysis was carried out using Python v. 3.12 from the Python Software Foundation<sup>1</sup>.

Qualitative data are presented in the form of absolute numbers with percentages (%). Nominal data were compared between groups using Pearson's  $\chi^2$  test or Fisher's exact test (when expected values were  $<10$ ). Quantitative data (body weight) are presented as an arithmetic mean (M)  $\pm$  standard deviation (SD). The Shapiro – Wilk test was used to assess the normality of the distribution. To evaluate body weight dynamics within Group 3 (comparing paired samples on days 2 and 10), a paired Student's *t*-test was used since the data distribution did not deviate from normality. Differences were considered statistically significant at  $p \leq 0.05$  and a two-tailed significance level was employed.

**TABLE 1**  
**DISTRIBUTION OF ANIMALS INTO GROUPS AND TIMEFRAMES OF VARIOUS STAGES OF THE EXPERIMENT**

| Groups            | DSS                 | Infliximab       | MTB              | Euthanasia |
|-------------------|---------------------|------------------|------------------|------------|
| Subgroup 1a (n=5) | -                   | -                | on day 1         | on day 90  |
| Subgroup 1b (n=5) | -                   | on day 1         | on day 2         | on day 90  |
| Group 2 (n=10)    | -                   | on days 1 and 29 | on days 2 and 30 | on day 90  |
| Group 3 (n=12)    | from day 1 to day 6 | on day 9         | on day 10        | on day 90  |

<sup>1</sup> <https://www.python.org/>

## RESULTS

During the entire observation period, no differences or distinctive behavioral features were noted in rabbits from Groups 1 and 2. Both groups exhibited active feeding and drinking behavior, and their average body weight increased by  $142 \pm 13.5$  g every 30 days. In contrast, all 12 animals in Group 3 showed behavioral changes from day 2 onwards. They exhibited lethargy, apathy, hypodynamia, and decreased appetite. These symptoms completely resolved by day 16 of the experiment. Additionally, 9 out of 12 rabbits in Group 3 developed digestive disturbances (frequent loose stools) on days 2–5 due to DSS administration, accompanied by a transient body weight loss averaging  $151 \pm 9.3$  g ( $p < 0.01$ , paired Student's *t*-test). By the end of the first month of the experiment, animals in Group 3 had begun to gain weight actively, and by day 30 their activity and feeding behavior did not differ from those in Groups 1 and 2. Detection of MTB DNA in fecal samples on days 7–10 showed similar positive test rates across groups: 6 out of 10 rabbits (60 %) in Group 1, 7 out of 10 (70 %) in Group 2, and 9 out of 12 (75 %) in Group 3 ( $p = 0.74$ ). Assessment of the skin test using recombinant tuberculosis allergen (RTA) showed erythema and papule formation only in 8 rabbits from Group 3 (66.7 %).

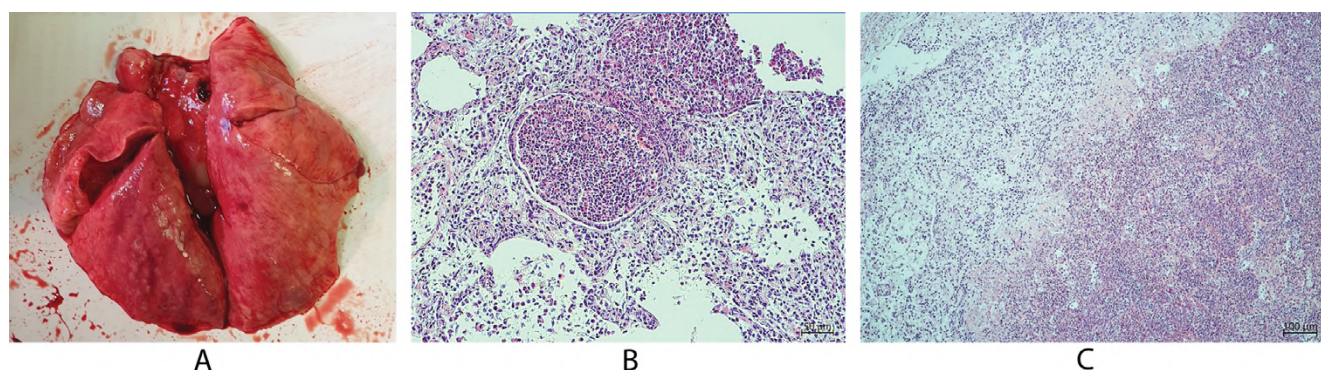
At the time of autopsy, there were no signs of tuberculous inflammation in the major organs and tissues of the animals in Groups 1 and 2. In the myocardium of most rabbits, there was moderate hydropic degeneration of cardiomyocytes, stromal edema, and venous congestion. In the liver, the most common findings (75 %) were moderate venous congestion, hepatocyte degeneration, mild focal lymphoid infiltration of individual portal tracts, or mild fibrosis. No pathological changes were observed in the kidneys. The serous membranes of the thoracic and abdominal cavities remained unchanged

in all animals. Signs of non-specific mild to moderate enteritis were present in four animals (20 %). Notably, rabbits that received the TNF- $\alpha$  inhibitor showed indirect signs of immunosuppression (in the spleen and lymph nodes), such as reduced follicle size, indistinct follicle borders, a weak macrophage response, and congestion in the splenic red pulp. In the lungs of 13 animals (65 %), signs of non-specific bronchitis, pneumonia, or lung abscess were observed (Fig. 1). Bacterioscopic and molecular genetic methods did not detect acid-fast bacilli (AFB) or MTB DNA in any of the examined organs and tissues of the animals in Groups 1 and 2, while specific signs of tuberculous inflammation were detected in the rabbits in Group 3.

The autopsy of rabbits from Group 3 showed macroscopic signs of complete tuberculous infection in the lungs in 10 animals (83.3 %), as well as enlargement of mesenteric and intrathoracic lymph nodes in 7 animals (58.3 %). The examination of small and large intestine loops revealed signs of serous edema, transient areas of visceral peritoneal hyperemia, and erosion or shallow ulceration on the mucous membrane in 75 % of cases ( $n = 9$ ).

The histological examination of intestinal tissue did not reveal any specific granulomatous reaction in any of the animals. Instead, the observed changes were consistent with a non-specific enterocolitis, characterized by submucosal edema, leukocytic infiltration, villous atrophy, and the presence of erosions (Fig. 2). AFB were not detected in the intestinal tissues using Ziehl – Neelsen staining, and MTB DNA was not detected in any of the samples analyzed using PCR.

In individual mediastinal and mesenteric lymph nodes ( $n = 7$ ; 58.3 %), disruption of the lymphoid tissue architecture was observed, due to the formation of epithelioid cell granulomas with caseous central masses. The lymphoid tissue was preserved in subcapsular regions. The granulomatous inflammation consisted primarily of activated macrophages with abundant



**FIG. 1.**

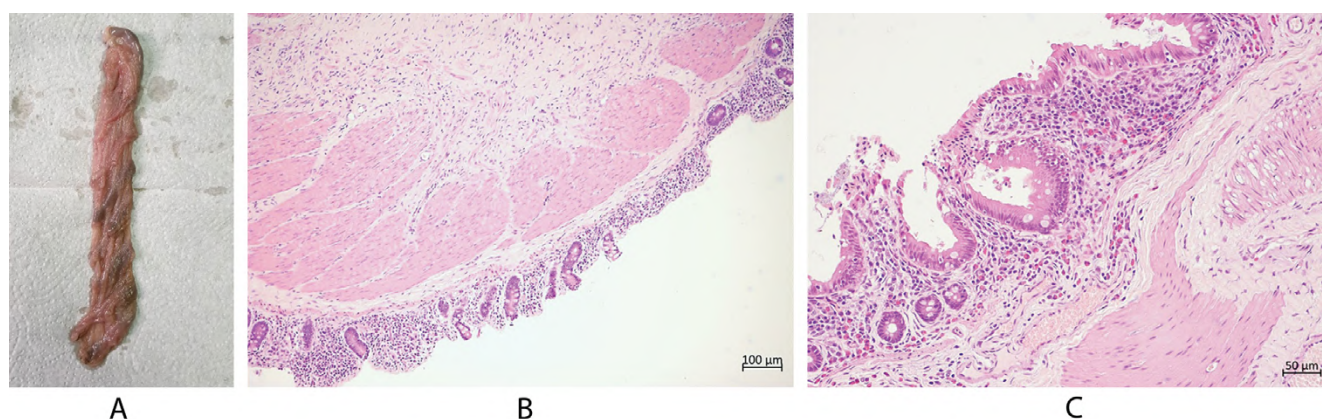
*A – gross specimen: rabbit lungs with signs of pneumonia; B – lung tissue with signs of nonspecific acute purulent endobronchitis, hematoxylin and eosin staining, scale bar 50 µm; C – acute abscess in rabbit lung tissue; hematoxylin and eosin staining, scale bar 100 µm*

cytoplasm, epithelioid cells, and occasionally Langhans giant cells. Sections stained with the Ziehl – Neelson method revealed numerous microcolonies of acid-fast bacilli (Fig. 3).

Detection of MTB DNA in lymph node tissue samples yielded positive results for 5 samples (41.7 %). The granulomatous inflammation was detected in the spleens of 3 rabbits (25 %) (Fig. 4).

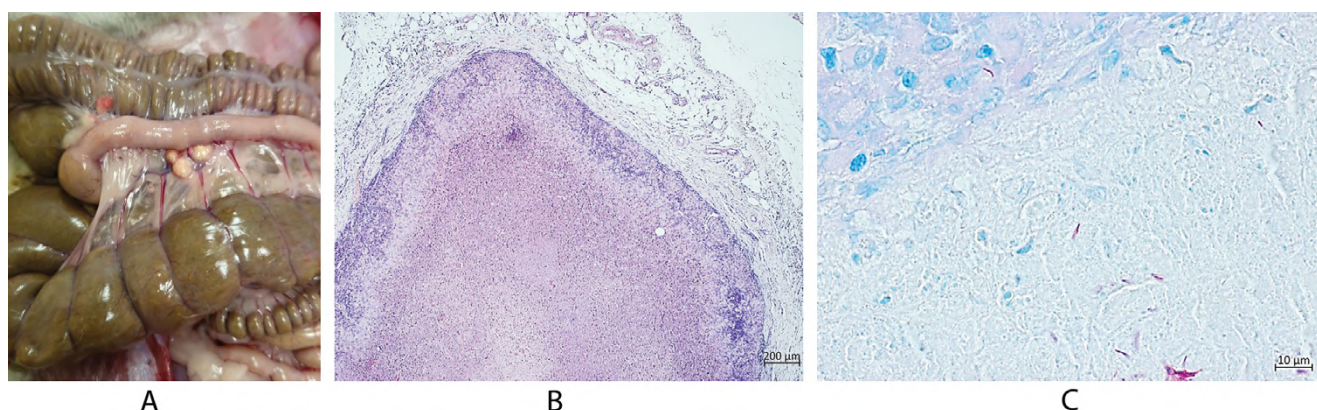
Examination of lung tissue from 10 animals (83.3 %) in Group 3 revealed tuberculous inflammation of varying severity. There was a predominance of destructive

forms and the formation of small cavities. Numerous dystelectases, as well as productive and alternative epithelioid cell granulomas with central caseous necrosis, were observed in the alveolar pattern (Fig. 5). There was also moderate interstitial inflammation with an exudative component in the surrounding tissues. Single AFB was detected in sections stained with the Ziehl – Neelsen method. The kidneys, liver, and myocardium of these rabbits also showed moderate signs of intoxication, including venous congestion, hemorrhage, degenerative changes, and stromal edema in the internal



**FIG. 2.**

*A – gross specimen: rabbit intestine (view of mucosa) with signs of enteritis; B – eroded intestinal mucosa with signs of active chronic inflammation; hematoxylin and eosin staining, scale bar 100 µm; C – intestinal mucosa with signs of chronic inflammation, superficial microerosions with translocation of microbial flora under the epithelium of the extrusion zone; hematoxylin and eosin staining, scale bar 100 µm*



**FIG. 3.**

*A – gross specimen: enlarged mesenteric lymph nodes of the rabbit small intestine; B – tuberculous mesenteric adenitis with subtotal involvement, formation of an extensive caseous-necrotic focus surrounded by an epithelioid cell wall with occasional Langhans-type multinucleated giant cells, stained with hematoxylin and eosin, scale bar 200 µm; C – acid-fast mycobacteria in the cytoplasm of epithelioid cells and freely present in necrotic masses of the affected lymph node, Ziehl – Neelsen staining, scale bar 10 µm*

organs. Reactive changes and blurred follicular structures were noted in the immune system organs (spleen and other lymph nodes).

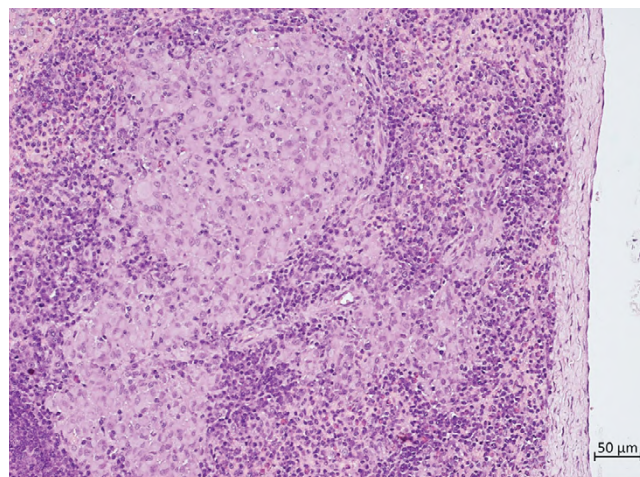
Therefore, in rabbits from Groups 1 and 2, even under conditions of immunosuppression and repeated oral exposure, no signs of tuberculous inflammation were detected. In contrast, the vast majority of animals in Group 3 exhibited pulmonary and lymph node foci of tuberculosis, with no granulomatous involvement of the intestines.

## DISCUSSION

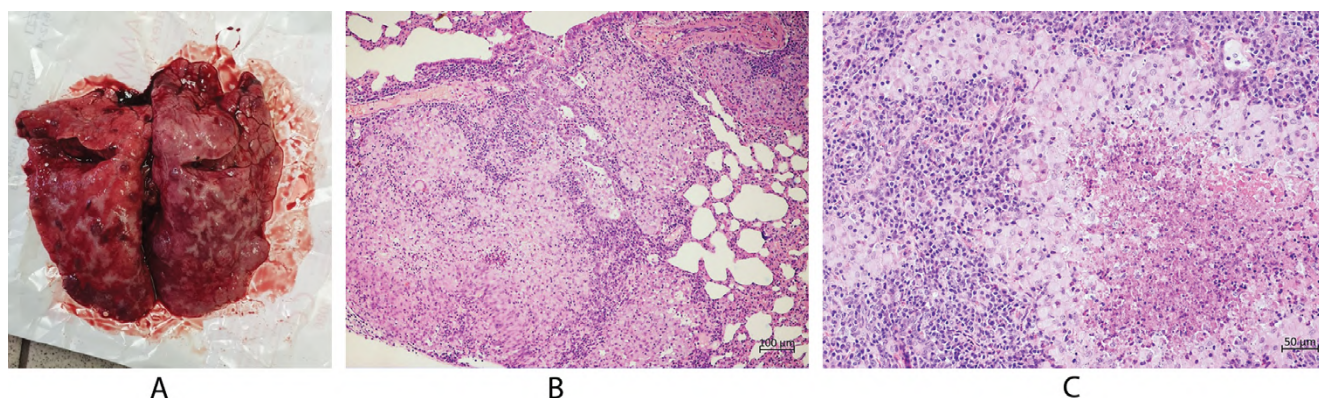
The alimentary route of tuberculosis transmission is considered in the literature as an additional route of infection [16, 17]. Additionally, the development of tuberculous enterocolitis has also been associated with the consumption of contaminated dairy products or the ingestion of infected sputum in the presence of a primary pulmonary tuberculosis focus [18]. Experimental evidence supporting these assertions was obtained over a century ago and was primarily descriptive. For instance, in 1868, J.A. Villemin added sputum from tuberculosis patients to the diet of several guinea pigs and euthanized the animals after three months. Upon autopsy, erosive changes were observed in the small intestine and cecum, although histological or culture confirmation of tuberculosis infection was not provided in this study [19]. Later, in 1933, M. McConkey and D.T. Smith conducted a series of similar experiments. Seventy-two adult guinea pigs were fed a diet mixed with infected sputum on a daily basis for four months. Half of the animals were maintained on a diet that was partially deficient in ascorbic acid.

Of these, 26 guinea pigs developed ulcerative intestinal tuberculosis. Among the remaining 35 animals, whose diet was supplemented with adequate vitamin C, only two were found to have intestinal tuberculosis with ulcers [20].

Commenting on the results of these experiments, one can note an undeniable methodological error in the choice of model animal. Guinea pigs are well known to be extremely sensitive and susceptible



**FIG. 4.** Tuberculous inflammation in the spleen tissue with the formation of irregularly shaped productive epithelioid-cell granulomas containing occasional Langhans-type multinucleated giant cells, stained with hematoxylin and eosin, scale bar 50 μm



**FIG. 5.** A – gross specimen: lungs of a rabbit with caseous pneumonia. B, C – Tuberculous inflammation in lung tissues: subpleural acinous-nodular confluent productive epithelioid-cell granulomas (B) with Langhans-type giant multinucleated cells and caseous-necrotic foci (C), stained with hematoxylin and eosin, scale bar 100 μm (B), 50 μm (C)

to *Mycobacterium tuberculosis* infection through any route of transmission, including the alimentary route [21]. The digestive tract of guinea pigs does not constitute a significant barrier to mycobacteria, but in addition to the intestine, the lungs and immune system organs are likely to be affected as well [22].

The most reliable information regarding the alimentary route of infection in guinea pigs was obtained from an experiment conducted by J. Gao et al. in 2018 [23]. In the experimental group, animals were administered 100 mL of drinking water containing  $2 \times 10^7$  CFU of the standard MTB strain H37Rv via drinking bottles for 8 hours. Euthanasia was performed at the end of the 4-month experiment. Granulomatous lesions were found in the lungs, spleen, and lymph nodes of the animals, and culture of homogenates from these tissues demonstrated the growth of MTB colonies on solid media after 1.5 months. At the same time, no specific changes indicative of tuberculous enterocolitis were detected in histological sections from various intestinal segments. It should be noted that the experimental animals were fed commercial feed that contained sufficient ascorbic acid. This may represent one of the factors in the immune defense and enteroprotective effects that prevent the development of inflammation in the intestinal wall [23].

Using the PubMed and Scopus databases, no publications were found on the alimentary infection of rabbits with MTB or on the modeling tuberculous enterocolitis in this species. The only exception is the study conducted by V.G. Shtefko (1941), who administered adrenaline intraperitoneally to rats and rabbits followed by intravenous infection with *M. tuberculosis* emulsion [24]. According to the author, this combination led to the development of ulcerative intestinal tuberculosis in 70–80 % of the surviving animals. However, most of the rabbits and rats died due to adrenaline shock. It is important to note that the reliability of these data is questionable, as the study lacks evidence that the ulcers were of a tuberculous origin. It is more likely that “stress-induced” adrenal ulcers of the intestine were described [25].

In the current study, we were also unable to replicate either infiltrative or erosively-ulcerative tubercular enterocolitis. However, we obtain reliable evidence of alimentary infection in laboratory animals, resulting in the development of generalized forms of tuberculosis. Despite the vulnerability of the rabbit’s intestine (due to its weak motility and physiological stasis), the immune system of these animals adequately responds to the presence of MTB in the food bolus [26, 27]. According to our observations, when immunocompetent rabbits are orally infected with mycobacterial suspensions, tuberculous inflammation does not develop due to the natural immune barrier provided by intestinal epithelial cells, immunocompetent agranulocytes, and immune proteins prevents it. Most likely, the majority of the pathogen is destroyed

in the intestine and undergoes temporary migration through the gastrointestinal tract, as seen in rabbits from Groups 1 and 2, where positive MTB DNA was detected in fecal samples. Drug-induced immunosuppression and repeated exposure to MTB suspension did not significantly contribute to the development of localized or systemic tuberculous inflammation in rabbits in Groups 1 and 2, as demonstrated by skin testing with RTA and post-mortem examination.

In contrast, data obtained from the autopsy of rabbits in Group 3 indicate that under conditions of systemic immunosuppression and disruption of the intestinal immune barrier (DSS-induced enterocolitis), MTB penetrates the intestinal wall. However, specific inflammation in the mucosal and submucosal layers does not develop, or it is abortive. Nevertheless, involvement of the mesenteric lymph nodes in approximately half of the cases (58 %;  $p = 0.001$ ) may indirectly indicate MTB penetration from the intestine to these nodes, with formation of a so-called primary tuberculous complex, and subsequent persistence of MTB in the lymphoid tissue. This conclusion is supported by a positive RTA skin testing in 76 % of cases. The autopsy findings of rabbits in Group 3 suggest that, against the background of immunodeficiency, generalization of the infection occurs with lymphohematogenous involvement of the spleen (25 %;  $p = 0.063$ ) and lungs (83 %;  $p < 0.001$ ). Therefore, it is only when two predisposing factors are present in combination (immunosuppression and inflammation-induced damage) that favorable conditions are created for the entry of *Mycobacterium tuberculosis* into the intestinal wall and subsequent circulation, contributing to the development of tuberculous lymphadenitis, splenitis, and pulmonary tuberculosis, without typical intestinal involvement.

## RESUME

Oral administration of *M. tuberculosis* to immunocompetent rabbits does not lead to the development of tuberculous enterocolitis or generalized tuberculosis, despite the temporary shedding of MTB DNA in the feces. This indicates a transient presence of mycobacteria in the intestine. Neither drug-induced immunosuppression nor repeated oral challenges are sufficient to overcome the intestinal barrier and cause specific tuberculous inflammation in the intestines or other organs.

The combined effect of immunosuppression and chemical damage to the intestinal mucosa (DSS-induced enterocolitis) creates conditions for MTB to penetrate into the systemic circulation, leading to the subsequent development of tuberculous lymphadenitis (58.3 %) and destructive pulmonary tuberculosis (83.3 %).

The absence of granulomatous inflammation in the intestine following alimentary infection,

in conjunction with lesions in the lungs and lymphatic system, suggests that the intestine acts as a barrier that is overcome by mycobacteria only when its protective properties are severely compromised, with subsequent infection progressing via lymphohematogenous dissemination.

The rabbit is an adequate model for studying the alimentary route of tuberculosis infection, particularly for analyzing the conditions that promote systemic dissemination of the infection without the formation of a primary intestinal focus.

Detection of MTB DNA or AFB in feces does not serve as reliable evidence of either tuberculous enterocolitis or tuberculosis infection in general.

## CONCLUSION

The present study demonstrates that the alimentary route of tuberculosis infection requires not only the presence of the pathogen in the intestinal lumen, but also a significant disruption of local and systemic immune defense mechanisms. These findings are significant for comprehending the pathogenesis of rare cases of abdominal tuberculosis in humans, and they confirm the role of the intestine as a potential route of entry for the infection under specific circumstances.

## Conflicts of interest

The authors declare no conflicts of interest.

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## DEVELOPMENT OF A NON-TERMINAL TECHNIQUE FOR SPERM COLLECTION IN WISTAR RATS TO STUDY THE INFLUENCE OF ENVIRONMENTAL FACTORS ON REPRODUCTION

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### ABSTRACT

**Background.** In more than 30 % of cases, infertility is attributed to male factors. The diagnosis of male infertility is based on semen analysis. The assessed semen parameters include sperm concentration, motility, and morphology. Laboratory animal models enable the study of various biological processes, including the effects of external and internal factors on reproduction processes. However, to date, there is no simple, safe, non-terminal method for sperm collection in animals.

**Aim.** To develop a non-terminal sperm collection technique using optimal electrical stimulation parameters in Wistar rats to study the influence of environmental factors on the reproductive function.

**Materials and methods.** The study was performed on 30 male Wistar rats weighing 280–300 g. For sperm collection, a rectal electrical stimulator was used to obtain ejaculate. A hemocytometer and two types of microscopes were used for sperm analysis and counting: a Micromed P-1 LED (Russia) and a Levenhuk D90L LCD digital microscope (China).

**Results.** All animals were divided into 6 groups of five individuals each, according to the electrical stimulation protocol. All stimulation protocols resulted in erection in all groups of animals. However, the safest and most cell-saving method was found to be rectal electrical stimulation of the seminal colliculus of rats at settings of 60 Hz and 4 V.

**Conclusion.** A method for sperm collection in Wistar rats with an optimized electrical stimulation protocol has been developed, enabling the study of various factors affecting male fertility.

**Keywords:** non-terminal ejaculate collection, rectal electrostimulator, optimal electrical stimulation parameters, sperm count and morphology, environmental factors, reproduction

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## РАЗРАБОТКА МЕТОДИКИ ПРИЖИЗНЕННОГО ПОЛУЧЕНИЯ СПЕРМАТОЗОИДОВ У КРЫС WISTAR ДЛЯ ИЗУЧЕНИЯ ВЛИЯНИЯ ФАКТОРОВ ВНЕШНЕЙ СРЕДЫ НА РЕПРОДУКЦИЮ

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### РЕЗЮМЕ

Более чем в 30 % случаев проблема фертильности обусловлена мужскими факторами бесплодия. Диагностика мужского бесплодия основана на анализе эякулята (спермы). К исследуемым параметрам спермы относятся: концентрация и подвижность сперматозоидов, а также их морфология. Модели лабораторных животных позволяют изучать различные биологические процессы, включая воздействие внешних и внутренних факторов на процессы репродукции. Однако до настоящего времени отсутствует простой, безопасный клеточно-сберегающий способ получения сперматозоидов у животных.

**Цель.** Разработать методику прижизненного получения сперматозоидов с использованием оптимальных параметров электростимуляции у крыс Wistar для изучения влияния факторов внешней среды на репродуктивную способность животных.

**Материалы и методы.** Исследование выполнено на 30 крысах-самцах Wistar массой 280–300 г.

Для получения сперматозоидов использовали ректальный электростимулятор для получения эякулята у животных. Для анализа и подсчета сперматозоидов использовали камеру Горяева и микроскопы 2 видов: «Микромед Р-1 LED», Россия; цифровой микроскоп «Levenhuk D90L LCD», Китай.

**Результаты.** Все животные были поделены на 6 групп по пять особей в каждой в зависимости от режима электростимуляции. Все используемые режимы электростимуляции позволяли добиться эрекции во всех группах животных. Однако было установлено, что наиболее безопасным и клеточно-сберегающим способом получения сперматозоидов является ректальная электростимуляция семенных бугорков крыс при показателях электрического тока 60 Гц и 4 В.

**Вывод.** Разработан метод получения сперматозоидов у крыс Wistar с обоснованным режимом электростимуляции, позволяющим изучать влияние различных факторов на мужской фактор фертильности.

**Ключевые слова:** прижизненное получение эякулята, ректальный электростимулятор, оптимальные параметры электростимуляции животных, количество и морфология сперматозоидов, факторы внешней среды, репродукция

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## INTRODUCTION

Currently, the problem of fertility impairment is a major medical and social issue that is prevalent in society. According to the latest statistics from the World Health Organization, 50 to 80 million people worldwide suffer from infertility [1, 2].

Infertility is defined as a reproductive system disorder characterized by the inability to achieve clinical pregnancy after 12 or more months of regular unprotected sexual intercourse [3]. It has been established that one in six individuals worldwide is affected by infertility [4]. Fertility problems are attributed to female factors in 50 % of cases, to male factors in more than 30 %, and to combined disorders in both partners in approximately 20 % of cases [5, 6].

Among the causes of male infertility, the most important include urogenital and endocrine disorders, immunological and genetic defects, lifestyle factors (smoking, alcohol and drug use), as well as adverse environmental factors [7–10].

The diagnosis of male infertility is based on ejaculate (semen) analysis. The assessed semen parameters include sperm concentration, motility, and morphology [11, 12]. Laboratory animal models enable the study of various biological processes, the reproduction of specific pathological conditions, the assessment of the safety of pharmaceutical agents on quantitative and qualitative sperm parameters, as well as the efficacy of novel drugs developed to enhance reproductive function [13].

Among the existing methods for obtaining ejaculate in animals, the following are used: testicular tissue dissection [14], sperm retrieval from the cauda epididymidis [15], and sperm extraction from testicular tissue [16]. The aforementioned techniques are applicable only to deceased animals or following euthanasia. These methods are not suitable for the non-terminal collection of germ cells and cannot be used to evaluate ejaculate parameters dynamically in response to environmental factors.

Among the promising methods for experimental animal studies when natural semen collection is not feasible, electroejaculation should be considered [17]. The advantages of electroejaculation include the collection of maximum ejaculate volume with minimal cell damage. Furthermore, this technique allows the study of the effects of various agents on the male reproductive system in rats dynamically, without the need for euthanasia. Despite the existence of numerous modifications of the seminal tubercle electrostimulation method [18, 19], to date, no standardized stimulation protocol (frequency, voltage, current intensity, and duration of exposure) has been developed, which justifies the relevance of developing a step-by-step method for sperm collection in male rats.

## AIM

To develop a non-terminal sperm collection technique using optimal electrical stimulation parameters

in Wistar rats to study the influence of environmental factors on the reproductive function.

## MATERIALS AND METHODS

The study was performed on 30 male Wistar rats weighing 280–300 g. All experiments were conducted in accordance with the Interstate Standard GOST 33215–2014 “Guidelines for the Care and Maintenance of Laboratory Animals. Rules for Equipping Premises and Organizing Procedures” and GOST 33216–2014 “Guidelines for the Care and Maintenance of Laboratory Animals. Rules for Conducting Procedures Using Animals”. All animals were housed at the Medical and Biological Center for Biomodeling of the Kirov State Medical University under identical standard conditions, in cages measuring 25 × 19 × 40 cm, one animal per cage, with free access to food and water, on a 12-hour light cycle (light on from 8:00 AM to 8:00 PM, and off from 8:00 PM to 8:00 AM). All rats underwent a two-week adaptation period prior to the experiment. Experimental manipulations were performed at the same time each day, at 9:00 AM. The experiment was approved by the local ethics committee of the Kirov State Medical University (Protocol No. 16/2022 dated October 24, 2022).

For sperm collection, a rectal electrical stimulator for animal ejaculate collection was used [20]. Male rats were divided into six groups of five animals each. Group allocation was based on the electrical stimulation parameters (frequency, current intensity, and voltage).

Prior to the procedure, animals were anesthetized by inhalation anesthesia using medical ether (Kuzbas-sorgkhim LLC, Russia). The animal was placed under a 3-L glass jar containing cotton wool soaked in ether and kept there until immobilization was achieved. The onset of anesthesia was determined by the loss of motor activity and decreased muscle tone. To confirm the absence of pain sensitivity and distress during electrical stimulation, light pressure was applied to the paws and skin: muscle relaxation, absence of protective reflexes, and lack of resistance indicated the animal was ready for the procedure. Following anesthesia, the animal was placed in the supine position and secured in a restrainer for safe manipulation. Fixation ensured a stable body position, prevented injury, and allowed accurate insertion of the rectal electrical stimulator. Next, the rectum was emptied by palpation. The anal orifice and the rectal probe were lubricated with vaseline oil and inserted into the rectum to a depth of 2.5–3 cm (Fig. 1).

After confirming that the probe was fully inserted into the animal's anal orifice, the electrical stimulator, pre-set to the required parameters, was switched on. Erection served as evidence of stimulation of the seminal colliculus. In its absence, the rectal probe was moved within the rectum, pressing against its anterior wall until erection was achieved. Electrical stimulation of male rats was continued until erection and ejaculate were obtained. The following parameters were recorded:

duration of electrical stimulation required to obtain ejaculate, severity of erection and muscle contraction, and the presence or absence of ejaculation.

The following visual criteria were used to assess erection quality: "+" – weak erection: partial filling of the corpora cavernosa, an increase in penile volume without pronounced rigidity or extrusion from the prepuce. "++" – moderate erection: characterized by full penile rigidity and extrusion from the preputial sac; however, the elevation angle relative to the animal's body axis is less than 90°. "+++" – pronounced erection: maximum rigidity, complete penile extrusion accompanied by characteristic color changes and a specific position facilitating ejaculate collection.

The degree of muscle response to electrical stimulation was assessed by the involvement of muscle groups: "+" – moderate contraction: localized muscle response limited to the pelvic girdle and hind limbs. "++" – pronounced contraction: generalized muscle response involving the back and forelimb muscles, with pronounced trunk arching requiring additional animal fixation to maintain the rectal probe in the correct position.

The obtained ejaculate was collected using a single-channel variable-volume pipette with 200 µL plastic tips (Thermo Scientific, USA) and mixed with Sil-Select Plus Sperm Washing medium (FertiPro, Belgium) in Eppendorf tubes. Microscopy of the obtained samples was then performed to assess sperm count and morphology.



**FIG. 1.**  
*Animal preparation for transrectal electrical stimulation*

Sperm concentration was counted using a Goryaev counting chamber. A 10-fold diluted ejaculate sample (10 µL) was introduced with a pipette into the chamber at the edge of the coverslip so that the ejaculate filled the entire chamber. Subsequently, at ×200 magnification (Micromed P-1 LED, Russia), cells were counted in 50 large squares of the chamber, while noting the presence or absence of sperm motility.

The following formula was used to calculate sperm concentration [21]:

$$C = \frac{a \times 250 \times 10}{50 \times 1000} \text{ million/mL,}$$

where

a – total number of cells counted in 50 large squares of the Goryaev chamber;

250 – volume of one large square of the Goryaev chamber;

10 – dilution factor of the original ejaculate;

1000 – conversion factor from 1 µL to 1 mL;

50 – number of large squares in which sperm were counted.

For sperm morphology assessment, microscopy was performed using a digital microscope with a ×40 objective (Levenhuk D90L LCD, China).

Sperm motility was evaluated in accordance with WHO guidelines, with three categories distinguished: progressively motile (PR) – actively moving linearly or in large circles; non-progressively motile (NP) – movement in place; and immotile (IM) – complete absence of movement.

Statistical analysis was performed using non-parametric methods. Quantitative data (sperm concentration) are presented as median and interquartile range – Me (25%–75%). Motility data are presented as mean ± standard error ( $M \pm m$ ). The Kruskal – Wallis test was used to detect differences between groups. Differences were considered statistically significant at  $p < 0.05$ .

## RESULTS

The current parameters used to obtain ejaculate samples from rats by transrectal electrical stimulation of the seminal tubercles are presented in Table 1.

During the study, erection was observed in all six experimental animal groups upon electrical stimulation, manifesting as an increase in penile volume and, in some cases, cyanosis due to blood engorgement (Fig. 2).

As a result of transrectal electrical stimulation of the seminal colliculus, ejaculate was obtained from animals in experimental groups No. 2, 3, 4, 5, and 6. In group No. 1, ejaculation did not occur even after 3 minutes of electrical stimulation (Table 2).

The data presented in Table 2 indicate that all stimulation protocols resulted in erection in all animal groups. The minimum stimulation time required to obtain ejaculate was observed in groups No. 3–6: up to 1 minute. In these same groups (No. 3–6), pronounced erection was observed. However, in groups No. 5 and 6, a more active response in the form of generalized muscle contractions of the entire body was observed at a frequency of 70 Hz, which was considered a negative factor. Despite the rigidity of the rectal probe (a metal probe was used for stimulation), intense muscle contractions caused physical displacement of the probe relative to the projection of the rectal nerve plexuses, making it difficult to maintain stable stimulation even with constant manual control of its position. At a frequency of 60 Hz, no excessive motor activity or generalized muscle contractions were observed, indicating adequate anesthetic depth and absence of pain sensitivity. This protocol ensured a stable neuromuscular status in the animals during the experiment, providing a physiological background for transrectal stimulation under anesthesia.

Ejaculate samples from animal groups No. 3–6 were collected for analysis. The results of sperm concentration and motility assessment are presented in Table 3.

Based on the obtained data, the highest mean sperm concentration was observed in experimental groups No. 3 and 4 – 8.70 (8.10; 9.40) million cells/mL and 7.45 (6.90; 8.15) million cells/mL, respectively. Motility analysis revealed that in group No. 3, progressively motile sperm predominated ( $PR = 62.4 \pm 5.1\%$ ). In group No. 4, a decrease in the proportion of progressively motile sperm to  $34.8 \pm 4.4\%$  was observed, accompanied by an increase in the number of non-progressively motile sperm. In groups No. 5 and 6, no motile cells were detected. To exclude the influence of external factors (temperature shock, waiting time), sample collection was performed according to a uniform protocol: analysis was performed no later than 5 minutes after ejaculate collection, and tubes were kept in a thermostat at 37°C. Given the presence of motile sperm in groups No. 3 and 4, the absence of activity in groups No. 5 and 6 is directly related to excessive high-frequency current

exposure (70 Hz) to the rat seminal colliculus tissue at the time of ejaculation.

In groups No. 5 and 6, the sperm counts were lower than in groups No. 3 and 4 – 6.05 (5.50; 6.70) million cells/mL and 4.90 (4.45; 5.40) million cells/mL, respectively. Morphological assessment of cells in these animal groups revealed morphological abnormalities, manifested as detachment of the sperm head from the tail (Fig. 3).



**FIG. 2.**  
*Erection induced in a male rat*

**TABLE 1**

**ELECTRICAL STIMULATION PARAMETERS FOR TRANSRECTAL EJACULATE COLLECTION FROM THE SEMINAL COLLICULUS IN RATS**

| Characteristics<br>of electroejaculation parameters | Animal group |         |         |         |         |         |
|-----------------------------------------------------|--------------|---------|---------|---------|---------|---------|
|                                                     | 1 (n=5)      | 2 (n=5) | 3 (n=5) | 4 (n=5) | 5 (n=5) | 6 (n=5) |
| Frequency, Hz                                       | 50           |         | 60      |         | 70      |         |
| Voltage, V                                          | 4            | 9       | 4       | 9       | 4       | 9       |
| Current intensity, A                                | ≤8           |         |         |         |         |         |

**TABLE 2**

**OUTCOMES OF TRANSRECTAL ELECTRICAL STIMULATION OF THE SEMINAL COLLICULUS IN MALE RATS**

| Parameters                                             | Characteristics of electroejaculation parameters |                |                |                |                |                |
|--------------------------------------------------------|--------------------------------------------------|----------------|----------------|----------------|----------------|----------------|
| Animal group                                           | 1 (n=5)                                          | 2 (n=5)        | 3 (n=5)        | 4 (n=5)        | 5 (n=5)        | 6 (n=5)        |
| Frequency, Hz                                          | 50                                               |                | 60             |                | 70             |                |
| Voltage, V                                             | 4                                                | 9              | 4              | 9              | 4              | 9              |
| Current intensity, A                                   | ≤8                                               |                |                |                |                |                |
| Duration of electrical stimulation to obtain ejaculate | Up to 3 min                                      | Up to 2 min    | Up to 1 min    | Up to 1 min    | Up to 1 min    | Up to 1 min    |
| Median time to ejaculation (Me (25%–75%), min)         | No ejaculate obtained                            | 1.7 (1.5; 2.1) | 0.5 (0.4; 0.6) | 0.6 (0.4; 0.7) | 0.7 (0.5; 0.8) | 0.6 (0.4; 0.7) |
| Muscle response severity                               | +                                                | +              | +              | +              | ++             | ++             |
| Erection quality                                       | +                                                | ++             | +++            | +++            | +++            | +++            |
| Ejaculation achieved                                   | -                                                | +              | +              | +              | +              | +              |
| Sperm present                                          | -                                                | +              | +              | +              | +              | +              |

**Notes.**

1. In group No. 1, no ejaculate was obtained within 10 minutes of stimulation.

2. Muscle contraction: “+” – moderate (local contractions of the pelvic girdle); “++” – pronounced (generalized contractions of the trunk musculature).

3. Erection: “+” – weak (partial filling of the corpora cavernosa); “++” – moderate (complete rigidity and extrusion from the prepuce); “+++” – pronounced (maximum rigidity with hyperemia).

**TABLE 3**

**ASSESSMENT OF SPERM CONCENTRATION, MOTILITY, AND MORPHOLOGY IN EJACULATE SAMPLES**

| Parameters                                    |                               | Characteristics of electroejaculation parameters |                      |                      |                      |
|-----------------------------------------------|-------------------------------|--------------------------------------------------|----------------------|----------------------|----------------------|
| Animal group                                  |                               | 3 (n=5)                                          | 4 (n=5)              | 5 (n=5)              | 6 (n=5)              |
| Frequency, Hz                                 |                               | 60                                               |                      | 70                   |                      |
| Voltage, V                                    |                               | 4                                                | 9                    | 4                    | 9                    |
| Current intensity, A                          |                               | ≤8                                               |                      |                      |                      |
| Median sperm count (Me (25%-75%), million/mL) |                               | 8.70<br>(8.10; 9.40)                             | 7.45<br>(6.90; 8.15) | 6.05<br>(5.50; 6.70) | 4.90<br>(4.45; 5.40) |
| Sperm motility, %                             | progressively motile (PR)     | 62.4±5.1                                         | 34.8±4.4             | 0                    | 0                    |
|                                               | non-progressively motile (NP) | 15.2±2.4                                         | 28.6±3.2             | 0                    | 0                    |
|                                               | immotile (IM)                 | 22.4±4.8                                         | 36.6±5.4             | 100                  | 100                  |

The analysis revealed statistically significant differences between the study groups in both sperm concentration ( $p < 0.05$ ) and motility parameters ( $p < 0.01$ ). The mean sperm concentration in group No. 3 (8.70 million cells/mL) was significantly higher than in groups No. 4, 5, and 6. Analysis of cell motility in these groups also revealed significant differences. Thus, in group No. 3, progressively motile sperm significantly predominated ( $PR = 62.4 \pm 5.1 \%$ ), which was significantly higher than in group No. 4 ( $PR = 34.8 \pm 4.4 \%$ ) ( $p < 0.05$ ) and groups No. 5 and 6, where motile cells were completely absent ( $p < 0.01$ ). In group No. 4, a statistically significant increase in the proportion of non-progressively motile sperm was observed compared to group No. 3:  $28.6 \pm 3.2 \%$  vs.  $15.2 \pm 2.4 \%$ .

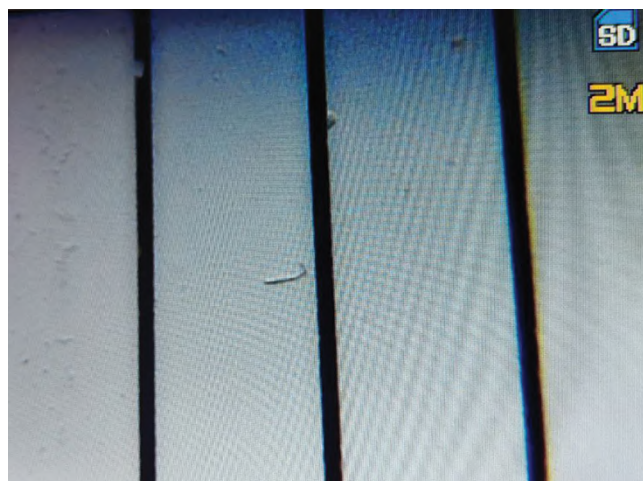
The low sperm concentration in groups No. 5 and 6 is explained by the fact that only intact cells are counted. Sperm destruction at 70 Hz results in a significant portion of the biological material being converted into cellular debris, which cannot be quantified. The presence of morphological abnormalities in cells indicates the traumatic effect of the 70 Hz stimulation protocol at both voltage settings. Given this fact, these electrical stimulation parameters should not be used for sperm collection to avoid compromising sperm morphology.

## DISCUSSION

The electrostimulation method is the most promising for obtaining sperm from male animals when studying their fertility level and analyzing the role of the male factor in reproduction. Its key advantage lies in the ability to obtain native ejaculate rather than epididymal suspension. Unlike work with suspension, ejaculate collection allows assessment of the functional status of cells in the presence of accessory sex gland secretions, ensuring maximum accuracy of semen parameters close to physiological norms. Furthermore, the use of this method complies with international bioethical principles. Non-terminal sample collection eliminates the need for animal euthanasia, enabling studies on the same individuals and increasing the human nature of the experiment. The ability to select male animals with equal sperm cell potential for experimental work allows the exclusion of the male infertility factor and assessment of the role of the female organism in reproduction under the influence of various external and internal factors.

Literature analysis revealed that various stimulation protocols have been used in experimental studies on transrectal electrical stimulation of the seminal colliculus (Table 4).

The identification of numerous modifications of the seminal colliculus electrostimulation method and the absence of a standardized stimulation protocol (frequency, voltage, current intensity, duration) justified the need to develop a step-by-step method for sperm



**FIG. 3.**  
*Sperm head in the Goryaev chamber, at  $\times 400$  magnification*

collection in male rats. To determine the most favorable stimulation protocol, based on the results of previous studies, two frequency settings (60 Hz and 70 Hz) and two voltage settings (4 V and 9 V) were applied at one current intensity level. The criteria for selecting the optimal stimulation protocol were minimal time required to obtain ejaculate. Compared to previously published methods, the proposed protocol significantly reduces the stimulation time required to induce erection and successfully collect material to 1 minute. This not only increases experimental productivity but also minimizes stress exposure to the animal, prevents muscle contractions, and preserves sperm morphological integrity.

The study established that the safest and most cell-sparing method for obtaining active, morphologically intact sperm is transrectal electrical stimulation of the rat seminal colliculus at 60 Hz and 4 V. Conversely, exceeding the frequency threshold above 60 Hz triggers a cascade of irreversible destructive processes in the cell structure. It is known that at 70 Hz, high-frequency microvibrations resonate with elements of the sperm cytoskeleton. Due to the inertial mass difference between the head and tail, excessive mechanical stress is generated in the vulnerable neck region, leading to massive decapitation of cells [22]. In addition to mechanical rupture, the intense electric field induces membrane electroporation and oxidative stress, disrupting ionic homeostasis and leading to rapid loss of motility [23]. An additional negative factor is local tissue heating, causing denaturation of axonemal proteins and the tail motor apparatus [24].

The use of optimal parameters of 60 Hz and 4 V allows the collection of a greater number of cells (8.7 (8.10; 9.40) million cells/mL) within 1 minute, not only compared to other stimulation protocols within this experimental study but also compared to previously published studies using various current parameters.

TABLE 4

## COMPARATIVE EVALUATION OF TRANSRECTAL ELECTRICAL STIMULATION METHODS FOR THE RAT SEMINAL COLLICULUS [17, 18, 19]

| Electrical stimulation protocol | Frequency, Hz            | Voltage, V | Current intensity, A     | Stimulation duration, min |
|---------------------------------|--------------------------|------------|--------------------------|---------------------------|
| 1                               | 60                       | 8.1        | No information available | 10.0                      |
| 2                               | 60                       | 5.8        | No information available | 5.5                       |
| 3                               | 60                       | 7.4        | No information available | 5.0                       |
| 4                               | 60                       | 3          | 0.5                      | until ejaculate obtained  |
| 5                               | No information available | 2–10       | No information available | until ejaculate obtained  |

## CONCLUSIONS

A non-terminal sperm collection technique has been developed for Wistar rats, with the key advantage being the optimization of electrical parameters – 60 Hz and 4 V. The selection of these parameters allowed a significant reduction in the duration of the procedure, which favorably distinguishes the proposed method from existing analogues and minimizes stress exposure to laboratory animals.

The scientifically validated stimulation protocol ensures the collection of morphologically intact and functionally active sperm, opening broad opportunities for the application of the obtained results: in studying the etiology and pathogenesis of various forms of male infertility; for analyzing the efficacy and safety of novel immunobiological agents aimed at improving male fertility; for developing and refining *in vitro* fertilization protocols in laboratory animals; and for investigating the effects of complex environmental and internal factors on reproductive potential.

The developed technique represents a reliable tool for standardizing andrological research, ensuring high reproducibility of results while strictly adhering to bioethical principles.

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## Conflicts of interest

The authors declare no conflicts of interest.

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## COMPARATIVE ANALYSIS OF MACROPHAGE INTERACTIONS WITH ANTIGENS OF TUBERCULOUS AND NON-TUBERCULOUS MYCOBACTERIA *IN VITRO*

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### ABSTRACT

**Background.** Ongoing research is investigating the interaction between mycobacteria and macrophages. Upon infection with mycobacteria, macrophages release cytokines. These include IFN $\gamma$ , TNF, IL-1, IL-6, and IL-12p40, among others. These substances play a critical role in the host response to tuberculosis and other mycobacterial infections.

**Aim.** To investigate and compare the cytokine patterns produced by human macrophages in response to interaction with antigens from both pathogenic and conditionally pathogenic mycobacterial strains *in vitro*.

**Methods.** Blood monocytes were cultured for five days in the presence of 10 % active human serum. The resulting macrophages were then cultured with mycobacterial antigens from *Mycobacterium tuberculosis*, *Mycobacterium avium*, and *Mycobacterium kansasii* for 24 hours. Changes in macrophage gene expression profiles and the production of pro-inflammatory and anti-inflammatory cytokines were then assessed. A control group of "naive" macrophages cultured without any mycobacterial antigen was also used.

**Results.** Stimulation of human macrophages with antigens from different mycobacterial species resulted in the formation of distinct cell phenotypes: protective (*M. tuberculosis*), characterized by the expression of IL6, IFN $\gamma$ , and TNF; pro-inflammatory (*M. kansasii*), with active expression of IL6, IL1 $\beta$ , and TNF; and pro-inflammatory (*M. avium*), expressing IL6 and IL12. The cytokine production findings were fully consistent with the gene expression data.

**Conclusion.** These findings on macrophage-mycobacterial antigen interactions provide insight into the mechanisms by which both tuberculous and nontuberculous mycobacterial infections establish chronic macrophage infection.

**Keywords:** macrophages, cytokines, chemokines, *M. tuberculosis*, *M. avium*, *M. kansasii*, *in vitro* research

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## СРАВНИТЕЛЬНЫЙ АНАЛИЗ ОСОБЕННОСТЕЙ ВЗАИМОДЕЙСТВИЯ МАКРОФАГОВ С АНТИГЕНАМИ ТУБЕРКУЛЕЗНЫХ И НЕТУБЕРКУЛЕЗНЫХ МИКОБАКТЕРИЙ *IN VITRO*

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### РЕЗЮМЕ

**Обоснование.** В настоящее время активно продолжают исследования, связанные с изучением взаимодействия микобактерий и инфицированных ими макрофагов человека и животных. В ответ на инфекцию макрофаги производят специфические молекулы, известные как цитокины, среди которых выделяются IFN $\gamma$ , TNF, IL-1, IL-6, IL-12p40 и IL-12p70. Эти вещества играют ключевую роль в патогенезе туберкулёза и других заболеваний, вызванных микобактериями. В этой связи, **целью нашей работы** было сравнительное исследование паттернов цитокинов, вырабатываемых макрофагами человека в результате взаимодействия с антигенами патогенных и слабопатогенных микобактерий *in vitro*.

**Методы.** Из моноцитов крови здоровых добровольцев получали культуру макрофагов. Макрофаги культивировали с микобактериальными антигенами *Mycobacterium tuberculosis*, *Mycobacterium avium* и *Mycobacterium kansasii*. Через 24 часа проводили исследование изменения профиля экспрессии генов макрофагов (экспрессионный анализ) и продукции этими клетками про- и противовоспалительных факторов. В качестве отрицательного контроля использовали культуру макрофагов, культивируемых без антигенов микобактерий.

**Результаты.** Показано что, стимуляция макрофагов антигенами разных микобактерий приводит к формированию различных фенотипов клеток: про-тективного (*M. tuberculosis*) – с экспрессией генов IL6, IFN $\gamma$  и TNF; провоспалительного (*M. kansasii*) – с активной экспрессией генов IL6, IL1 $\beta$  и TNF и амбивалентного (*M. avium*) – с экспрессией генов IL6, IL12. Результаты исследования продукции цитокинов макрофагами человека в ответ на антигены различных микобактерий полностью подтвердили данные, полученные с помощью экспрессионного анализа.

**Заключение.** Выявленные в работе особенности реакции макрофагов на антигены различных видов микобактерий позволяют более детально охарактеризовать механизмы персистенции туберкулёзных и нетуберкулёзных микобактерий в макрофагах.

**Ключевые слова:** макрофаги, цитокины, хемокины, *M. tuberculosis*, *M. avium*, *M. kansasii*, исследование *in vitro*

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## INTRODUCTION

Although *Mycobacterium tuberculosis* is often regarded as a classical bacterial pathogen, a deeper understanding of its biology requires a more comprehensive investigation of its adaptation to its primary host – humans. Furthermore, it is important to consider that in the majority of individuals, *M. tuberculosis* infection may be asymptomatic, which may contribute to its transmission, including in regions with a high tuberculosis burden [1]. Upon reaching the alveoli, airborne bacteria are phagocytosed by macrophages, which become their primary niche. Mycobacteria can survive and replicate within these cells, thereby evading the immune system and migrating through the epithelium into the lung interstitium [2, 3]. The precise mechanisms underlying these processes remain hypothetical. However, observations in animal models and experiments using *ex vivo* systems suggest that early proliferation of *M. tuberculosis*-infected macrophages leads to the formation of cellular aggregates. These aggregates recruit monocytes and neutrophils, creating new cellular targets for *M. tuberculosis* dissemination [4].

Nontuberculous mycobacteria (NTM) are environmentally widespread pathogens that can cause severe disease, particularly in immunocompromised individuals, but also pose a threat to immunocompetent hosts [5]. The most common NTM species are the *Mycobacterium avium* complex (MAC) [6, 7]. The global incidence of NTM-associated diseases is rapidly increasing. Following inhalation or aspiration of NTM into the lower respiratory tract, the first cells to encounter them are airway epithelial cells, macrophages, and dendritic cells. All three cell types are capable of presenting bacterial antigens on major histocompatibility complex class II (MHC II) molecules and activating adaptive immune responses [8]. The immunopathogenesis of MAC involves a complex interplay between the bacteria and the human immune system. MAC survive and replicate within macrophages by preventing phagosome-lysosome fusion. Through their enzymatic machinery, mycobacteria are able to neutralize reactive oxygen and nitrogen species produced by macrophages. Furthermore, MAC modulate cytokine production, enabling them to suppress or regulate the host immune response [9, 10]. The second most common cause of NTM infections is *Mycobacterium kansasii*. Diseases caused by *M. kansasii* are frequently observed in immunocompromised individuals, such as HIV-infected patients [11]. Phylogenetic analysis places *M. kansasii* within the group of mycobacteria most closely related to *M. tuberculosis* [12]. However, *M. kansasii* is considered an opportunistic pathogen.

It is hypothesized that compared to NTM, *M. tuberculosis* exhibits higher virulence due to its enhanced ability to suppress the host immune response. However, the characterization of *M. tuberculosis* in the context of macrophage infection and the analysis of its effector

functions in comparison with various NTM species remain incomplete.

Currently, the complex interactions between mycobacteria and macrophages, characterized by the induction and production of various cytokines such as IFN- $\gamma$ , TNF, IL-1, IL-6, IL-12p40, and IL-12p70, remain insufficiently studied. It has been well established that cytokines exert a significant influence on the pathogenesis and protective immunity to tuberculosis and mycobacteriosis [13, 14].

## AIM

To investigate and compare the cytokine patterns produced by human macrophages in response to antigens of pathogenic and weakly pathogenic mycobacteria *in vitro*.

## MATERIALS AND METHODS

### Generation of Human Macrophage Cultures

Human macrophages were differentiated from peripheral blood mononuclear cells (PBMCs) obtained from healthy donors, as previously described [15]. Briefly, PBMCs were isolated from whole venous blood of healthy donors by density gradient centrifugation on Ficoll (density 1.077 g/mL, PanEco, Russia). Cells were then washed and incubated for 3 hours in DMEM medium at 37°C and 5 % CO<sub>2</sub> (PanEco, Russia). Adherent monocytes were differentiated into macrophages in the presence of 10 % “active” human serum. Serum was obtained either from the same donors or from donors with identical ABO and Rh blood groups. Macrophage enrichment was assessed by expression of the macrophage marker CD163.

Healthy donors included in the study provided written informed consent for participation in the scientific study prior to its initiation. All studies involving human biological material complied with the Declaration of Helsinki [16] and were approved by the local ethics committee of the Central Research Institute of Tuberculosis (Protocol No. 1/2 dated January 17, 2025). Biological material (whole venous blood) was obtained from healthy donors after January 2025.

### Mycobacterial Antigens

Culture filtrate (CF) proteins of *M. tuberculosis* H37Rv, *M. avium*, and *M. kansasii* from the collection of the Central Research Institute of Tuberculosis were used as antigens in this study, prepared as previously described [17].

CF proteins of *M. tuberculosis*, *M. avium*, and *M. kansasii* were used as mycobacterial antigens in *in vitro* experiments at a dose of 10  $\mu$ g/mL.

### Macrophage Culture

Activation of macrophages in response to mycobacterial antigens of *M. tuberculosis*, *M. avium*, and *M. kansasii* was compared using an *in vitro* human macrophage

model. Blood monocyte cultures were maintained for 5 days in the presence of 10 % active human serum. The resulting M0 macrophages were then cultured with mycobacterial antigens. After 24 hours of culture, changes in the gene expression profile of M0 macrophages and the production of pro- and anti-inflammatory factors by these cells were examined. Untreated macrophages cultured without mycobacterial antigens served as the negative control.

#### RNA Isolation and cDNA Synthesis

Total RNA was extracted from human macrophage cultures using TRIzol (Thermo Fisher Scientific (Invitrogen), USA) according to the manufacturer's instructions, without modifications. Complementary DNA (cDNA) was synthesized using the SuperScript™ III Reverse Transcriptase kit (Thermo Fisher Scientific, USA) according to the manufacturer's recommendations. One microgram of total RNA was used as a template for reverse transcription.

Gene expression levels were determined by semi-quantitative real-time PCR (TaqMan®) using TaqMan™ Gene Expression Assay kits (Thermo Fisher Scientific (ABI), USA). GAPDH was used as a housekeeping gene.

#### Protein Concentration Determination

Cytokine and chemokine concentrations in cell culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) using Human DuoSet ELISA kits (R&D Systems, USA) according to the manufacturer's instructions, without modifications.

#### Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8.4.3 (GraphPad Software, USA) with multiple

t-tests (Student's *t*-test) corrected for multiple comparisons using the Holm-Šidák method. Normality of distribution was assessed using the Shapiro – Wilk test.

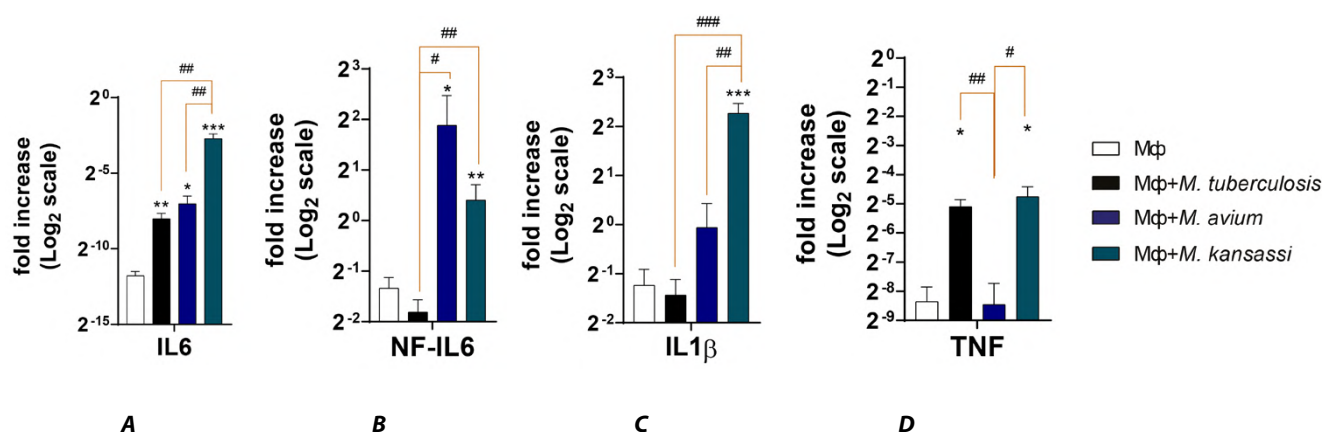
## RESULTS

It was demonstrated that stimulation of macrophages with *M. tuberculosis* antigens significantly up-regulated the expression of the cytokine genes *IL6*, *TNF*, and *IFNG* (Fig. 1A, D and Fig. 2A). *M. avium* antigens induced increased expression of *IL6*, *NF-IL6*, and *IL12* (Fig. 1A, B and (Fig. 2B). For *IL12*, expression was significantly higher than upon macrophage exposure to *M. tuberculosis* antigens (Fig. 2B). The effect of *M. kansasii* antigens on the macrophage expression profile was characterized by upregulation of *IL6*, *NF-IL6*, *IL1B*, *TNF*, and *IL12* (Fig. 1 and Fig. 25).

Notably, in the case of macrophage interaction with *M. kansasii* antigens, expression of *IL6* and *IL1B* was significantly higher than with *M. avium* and *M. tuberculosis* (Fig. 1A, C). Expression of *NF-IL6* was significantly higher with *M. avium* and *M. kansasii* compared to *M. tuberculosis*, with no difference between the two (Fig. 1B). The highest *TNF* expression was stimulated by *M. kansasii* and *M. tuberculosis* antigens (Fig. 1D).

Thus, stimulation of macrophages with antigens from different mycobacterial species leads to the formation of distinct cellular phenotypes:

- protective (*M. tuberculosis*), characterized by expression of *IL6*, *IFNG*, and *TNF*;
- pro-inflammatory (*M. kansasii*), with active expression of *IL6*, *IL1B*, and *TNF*;



**FIG. 1.**

Changes in expression of *IL6* (A), *NF-IL6* (B), *IL1B* (C), and *TNF* (D) in macrophages upon stimulation with *M. tuberculosis*, *M. avium*, and *M. kansasii* antigens

Data are presented as mean ± SEM (n = 3 biological replicates per group). \* – significant difference from the control group (macrophages cultured without mycobacterial antigens): \* – *p* < 0.05; \*\* – *p* < 0.01; \*\*\* – *p* < 0.001; # – significant differences between groups: # – *p* < 0.05; ## – *p* < 0.01; ### – *p* < 0.001.

• ambivalent (*M. avium*), expressing *IL6* and *IL12*.  
The findings from the study of cytokine production by human macrophages in response to antigens of various

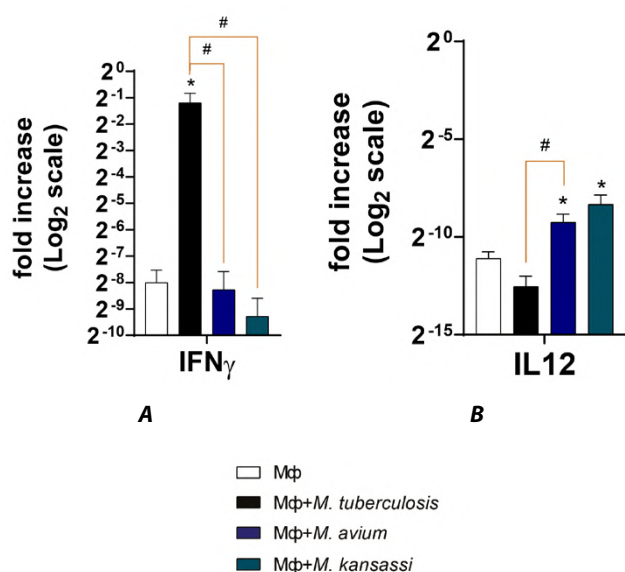
mycobacterial species were consistent with the expression profile data. It was demonstrated that macrophages cultured with *M. tuberculosis* antigens were characterized by production of *IL-6*, *IL-1β*, *TNF*, *IFN-γ*, and both forms of *IL-12* (*IL-12p70* and *IL-12p40*) (Fig. 3 and 4).

Macrophages cultured with *M. avium* antigens produced *IL-6*, *IL-1β*, *TNF*, *IFN-γ*, and *IL-12p40*, with *IFN-γ* production being significantly higher upon culture of macrophages with *M. tuberculosis* culture filtrate compared to the other groups (Fig. 3 and 4).

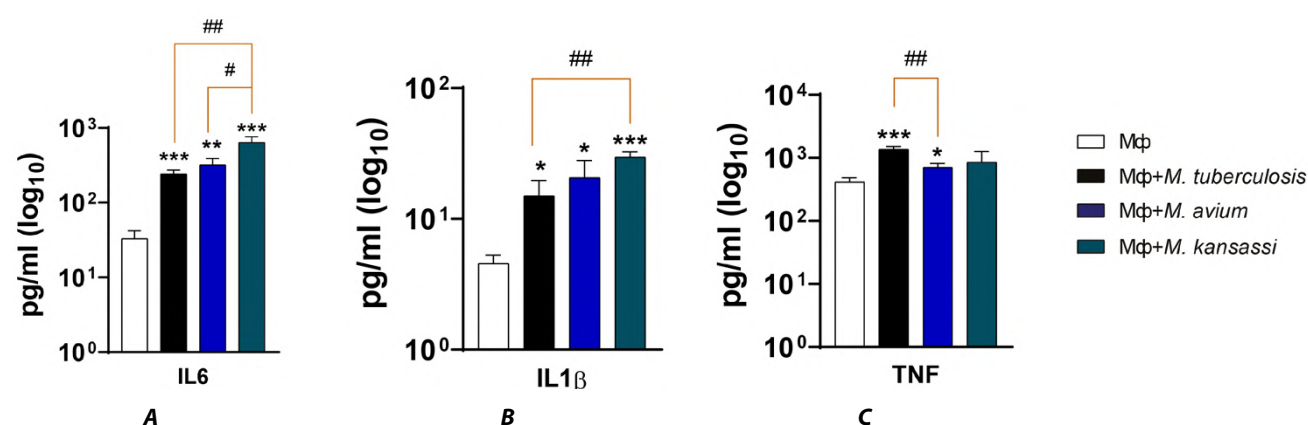
Cells cultured with *M. kansasii* antigens were characterized by production of *IL-6*, *IL-1β*, *IFN-γ*, and *IL-12p40* (Fig. 3 and 4). Notably, macrophages stimulated with *M. kansasii* culture filtrate secreted significantly higher levels of *IL-6*, *IL-1β*, and *IL-12p40* compared to the other experimental groups (Fig. 3, 4).

## DISCUSSION

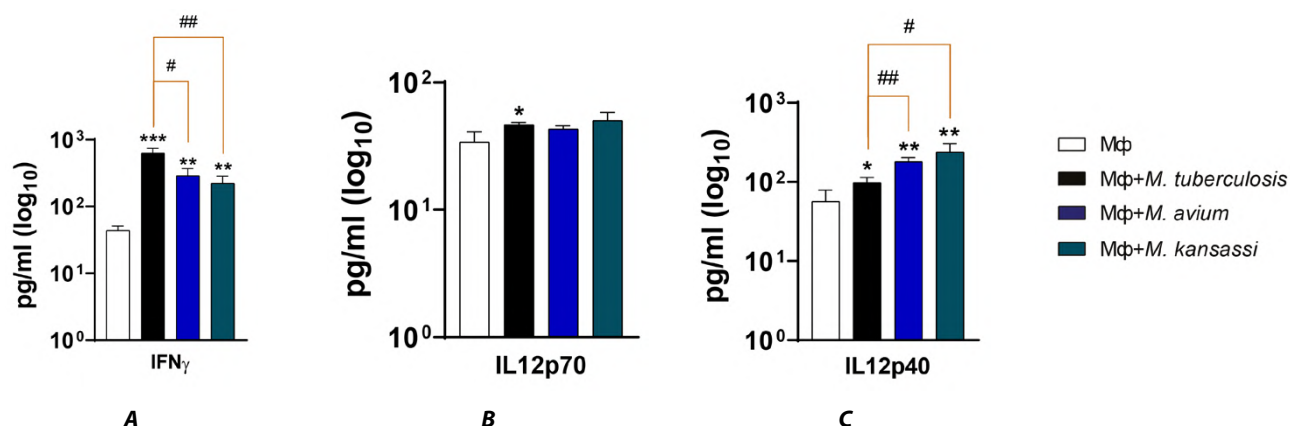
Understanding the distinct responses of macrophages to antigens of different mycobacterial species may aid in the identification of novel therapeutic targets. In our study, we compared the response of macrophages to complex antigens of *M. tuberculosis* H37Rv and two NTM species in terms of their ability to produce a panel of key cytokines *in vitro*. The results revealed an interesting pattern of differential cytokine expression and secretion by macrophages in response to stimulation with antigens of virulent and weakly virulent mycobacteria (Fig. 1–4). Stimulation with *M. tuberculosis* antigens resulted predominantly in *IFN-γ* and *TNF* production, whereas NTM antigens preferentially induced *IL-1*, *IL-6*, and *IL-12*. Importantly, this



**FIG. 2.** Changes in expression of *IFNγ* (A) and *IL12* (B) in macrophages upon stimulation with *M. tuberculosis*, *M. avium*, and *M. kansasii* antigens. Data are presented as mean ± SEM (n = 3 biological replicates per group). \* – significant difference from the control group (macrophages cultured without mycobacterial antigens); \* – p < 0.05. # – significant differences between groups: # – p < 0.05.



**FIG. 3.** Changes in production of *IL-6* (A), *IL-1β* (B), and *TNF* (C) in macrophages upon stimulation with *M. tuberculosis*, *M. avium*, and *M. kansasii* antigens. Data are presented as mean ± SEM (n = 3 biological replicates per group). \* – significant difference from the control group (macrophages cultured without mycobacterial antigens); \* – p < 0.05; \*\* – p < 0.01; \*\*\* – p < 0.001. # – significant differences between groups: # – p < 0.05; ## – p < 0.01.



**FIG. 4.**

Changes in production of IFN- $\gamma$  (A), IL-12p70 (B), and IL-12p40 (C) in macrophages upon stimulation with *M. tuberculosis*, *M. avium*, and *M. kansasii* antigens

Data are presented as mean  $\pm$  SEM ( $n = 3$  biological replicates per group). \* – significant difference from the control group (macrophages cultured without mycobacterial antigens): \* –  $p < 0.05$ ; \*\* –  $p < 0.01$ ; \*\*\* –  $p < 0.001$ . # – significant differences between groups: # –  $p < 0.05$ ; ## –  $p < 0.01$ .

pattern was consistent at both the transcriptional level and the protein level.

Other researchers have also compared various NTM species with *M. tuberculosis* in macrophage infection models [18–20]. Although, in contrast to our study, the majority of these investigations employed live mycobacterial infection models and quantitative assessment of mycobacterial burden, the considerable differences in observation duration and data analysis still allow for meaningful comparison with our findings.

Mononuclear cells, such as monocytes and macrophages, are professional phagocytes with high efficiency in defending the host against various pathogens, including tuberculous and NTM mycobacteria. Advances in the classification of mononuclear cell subsets, the study of their phenotypic and functional properties, and their modulation in various diseases have prompted research aimed at identifying monocyte-derived biomarkers that could be used for diagnostic and therapeutic purposes.

Host defense against NTM is regulated by macrophages through direct killing of mycobacteria and production of IL-12, which activates natural killer cells or T cells to secrete IFN- $\gamma$ . IL-12 and IFN- $\gamma$  mediate cross-talk between innate and adaptive immunity against NTM. Effective Th1 immunity, mediated by IL-12, IFN- $\gamma$ , and TNF, is essential for the elimination or containment of intracellular NTM infection.

Beltan E. et al. (2000) conducted a study in which human monocyte-derived macrophages were infected with various pathogenic mycobacteria (*M. tuberculosis*,

*M. avium*, *M. kansasii* or *M. xenopi*) and non-pathogenic mycobacteria (*M. smegmatis*, *M. phlei*), followed by measurement of cytokine induction levels. The study found that non-pathogenic mycobacteria induced higher levels of TNF and GM-CSF, and, to a lesser extent, more IL-6, compared to pathogenic mycobacteria [18].

In a study conducted by Song C.H. et al. (2003), IL-8 levels were measured in human monocytes. It was found that *M. smegmatis* induced significantly higher IL-8 levels compared to cells infected with *M. tuberculosis* H37Rv [19].

Using the THP-1 macrophage cell line, Feng Z. et al. (2020) demonstrated an interesting pattern of differential cytokine and chemokine production in macrophages infected with various mycobacterial species: rapid growers, slow growers, and *M. tuberculosis*. Overall, *M. smegmatis* stimulated higher production of pro-inflammatory cytokines (IL-1 $\beta$ , IL-6, IL-12, and TNF) as well as IL-10 compared to *M. abscessus*. However, these two rapid-growing species induced comparable amounts of chemokines (IL-8, MCP-1, and MIP-1 $\alpha$ ). When comparing *M. tuberculosis* and slow-growing *M. avium*, the latter stimulated higher production of IL-1 $\beta$ , IL-6, IL-12p70, TNF, and IL-10. At the same time, all three NTM species induced similar levels of IL-8, MCP-1, and MIP-1 $\alpha$  [20].

Zhao S. et al. (2024) investigated plasma cytokine levels in patients with NTM-associated lung disease as markers of pathogenesis and treatment response. Analysis of combinations of various plasma cytokines, particularly IL-1 $\beta$ , IL-6, and IL-10, revealed their potential utility in the differential diagnosis of patients

with different mycobacterial burdens. IL-6 and IL-10 emerged as promising biomarkers of early treatment response. The combination of IL-6 and IL-1 $\beta$  demonstrated high discriminatory capacity between patient groups with and without a positive response to anti-bacterial chemotherapy, as well as between patients with NTM infection and those with TB [13].

Using digital spatial profiling of lung tissue from patients with mycobacterial lung disease, Koh J. et al. (2024) demonstrated unique gene expression patterns in T cells and macrophages. Gene set enrichment analysis revealed that pathways related to antigen presentation and processing were predominantly activated in lung macrophages of these patients. Affected lung tissues showed increased numbers of macrophages with elevated expression of M1-associated genes (CD40 and CD80), whereas T cell activation markers remained unchanged [21].

Therefore, the results of our study, together with the findings reviewed here, indicate that macrophages may serve as therapeutic targets for focusing antibacterial immunity. Novel therapeutic strategies for TB, leprosy, and NTM infections that target specific cell types are currently being explored.

## CONCLUSION

The present study identified distinct patterns of macrophage responses to antigens of different mycobacterial species. This provided a more comprehensive characterization of the mechanisms through which tuberculous and NTM mycobacteria establish persistent macrophage infection. Further investigation of host-mycobacterium interactions in macrophages and other cell types may lead to the discovery of novel therapeutic targets for combating difficult-to-treat infections.

### Conflicts of interest

The authors declare no conflicts of interest.

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The authors declare that their authorship complies with the ICMJE international criteria.

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# ECONOMICS AND MANAGEMENT IN PUBLIC HEALTH SERVICE

## TWO-COMPONENT ORGANIZATIONAL MODEL FOR CLINICAL AND RADIOLOGICAL SCREENING FOR PULMONARY TUBERCULOSIS IN A SPARSELY POPULATED TERRITORY OF THE SAKHA REPUBLIC (YAKUTIA)

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### ABSTRACT

**Background.** The challenges of organizing tuberculosis detection in sparsely populated areas are primarily associated with poor transportation access and a shortage of medical personnel. Under these conditions, the implementation of modern information technologies in clinical and radiological screening for the disease becomes highly relevant.

**Aim.** To optimize methods for the detection and diagnosis of pulmonary tuberculosis using information technologies in the sparsely populated territory of the Republic of Sakha (Yakutia).

**Methods.** A pilot study was conducted in seven medical organizations of the Republic of Sakha (Yakutia) to test a two-component organizational model for clinical and radiological tuberculosis screening utilizing information technologies: a central medical image archive and telemedicine for remote diagnosis, as well as artificial intelligence for double reading of fluorograms. Effectiveness was assessed by monthly monitoring of population coverage with fluorographic examinations, measuring the time spent on double reading of fluorograms, and assessing the time to tuberculosis diagnosis.

**Results.** The pilot implementation of the two-component organizational model for clinical and radiological tuberculosis screening, employing information technologies and artificial intelligence in medical organizations, led to increased fluorographic examination coverage of the general population and individuals at risk for tuberculosis. This implementation also reduced the time required for double reading of fluorograms and shortened tuberculosis diagnostic timelines, both in the general medical network and at the specialized tuberculosis service level. Considering the specific characteristics of tuberculosis detection organization across different socio-territorial zones of the Far North, a model for clinical and radiological screening of pulmonary tuberculosis was developed. This model comprises two organizational variants tailored for use in the Arctic and industrial zones. The model consists of three stages (radiological, clinical, and specialized) implemented at the level of the central district hospital departments.

**Conclusion.** The proposed model for tuberculosis screening using information technologies is recommended for implementation in sparsely populated districts of the Far North.

**Keywords:** pulmonary tuberculosis, mass screening, diagnosis, information technology, telemedicine, artificial intelligence

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## ОРГАНИЗАЦИОННАЯ ДВУХКОМПОНЕНТНАЯ МОДЕЛЬ КЛИНИКО-РЕНТГЕНОЛОГИЧЕСКОГО СКРИНИНГА ТУБЕРКУЛЕЗА ЛЕГКИХ В УСЛОВИЯХ МАЛОНАСЕЛЕННОЙ ТЕРРИТОРИИ РЕСПУБЛИКИ САХА (ЯКУТИЯ)

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### РЕЗЮМЕ

**Обоснование.** Сложности организации выявления туберкулеза в малонаселенных территориях связаны в первую очередь с низким уровнем транспортной доступности и дефицитом медицинских кадров. В этих условиях является актуальным внедрение современных информационных технологий (ИТ) в процессы клиничко-рентгенологического скрининга заболевания.

**Цель исследования.** Оптимизация методов выявления и диагностики туберкулеза легких с использованием ИТ в условиях малонаселенной территории Республики Саха (Якутия).

**Методы.** В 7 пилотных медицинских организациях Республики Саха (Якутия) проведена апробация Организационной двухкомпонентной модели клиничко-рентгенологического скрининга туберкулеза с применением ИТ: центрального архива медицинских изображений и телемедицины для дистанционной постановки диагноза, а также искусственного интеллекта для двойного чтения флюорограмм. Оценка эффективности проводилась путем ежемесячного контроля охвата населения флюорографическим осмотром, учета временных затрат на двойное чтение флюорограмм и сроков установления диагноза туберкулеза.

**Результаты.** Апробация в пилотных медицинских организациях Организационной двухкомпонентной модели клиничко-рентгенологического скрининга туберкулеза с применением ИТ и искусственного интеллекта привело к повышению охвата населения и лиц из групп риска по туберкулезу флюорографическими осмотрами, а также к сокращению временных затрат на двойное чтение флюорограмм и сроков диагностики туберкулеза на уровне общей лечебной сети и противотуберкулезной службы. С учетом наличия особенностей в организации выявления туберкулеза в различных социально-территориальных зонах региона Крайнего Севера разработана модель клиничко-рентгенологического скрининга туберкулеза легких, состоящая из двух организационных вариантов для использования в условиях Арктической и Промышленной зон. Модель состоит из трех этапов: рентгенологического, клиничко-специализированного, осуществляемых на уровне подразделений центральной районной больницы.

**Заключение.** Предложенная модель скринингового обследования на туберкулез с использованием ИТ рекомендована для применения в малонаселенных районах Крайнего Севера.

**Ключевые слова:** туберкулез легких, скрининг, выявление, диагностика, информационные технологии, телемедицина, искусственный интеллект

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## BACKGROUND

The organization of early tuberculosis detection among the population is a crucial component of tuberculosis control efforts, as delayed or late diagnosis negatively affects the epidemiological situation [1, 2]. This is due to the fact that late detection of pulmonary tuberculosis results in prolonged massive bacterial shedding, creating a significant epidemiological risk for the surrounding population, while the morphological changes that develop are often irreversible or only partially reversible, leading to low treatment efficacy [3].

According to current regulatory documents, fluorography (FLG) is the screening method of choice for mass radiographic examination aimed at early detection of pulmonary tuberculosis among the adult population in the Russian Federation. When pulmonary tuberculosis is suspected following FLG screening at a primary healthcare facility (PHC), a diagnostic workup is performed, including medical history collection, physical examination, complete blood count, three-fold microscopic examination of biological material (sputum) for acid-fast bacilli (AFB), and chest X-ray. The patient is then referred to a tuberculosis facility, where additional diagnostic methods are employed: two-fold sputum microscopy for AFB, culture and molecular genetic testing for etiological diagnosis and drug susceptibility testing (DST), and immunodiagnosics.

The diagnosis of tuberculosis is confirmed by a medical commission of the tuberculosis medical organization (TB dispensary, hospital, center, or clinic), which makes decisions regarding hospitalization, treatment, and follow-up care. TB dispensaries (departments, offices) submit medical documentation and radiological findings to the commission for review. In complex diagnostic cases, an in-person consultation with the patient is arranged.

As is well known, the key challenges of healthcare delivery in the Arctic zone and the Far North, which are sparsely populated territories, include limited transport accessibility and a shortage of medical personnel [4–6]. In this study, we analyzed the standard algorithm for tuberculosis detection and diagnosis in the Republic of Sakha (Yakutia) for 2017–2019 and identified the following issues that negatively impact the effectiveness and timeliness of detection and diagnosis:

- Staffing shortages, including radiologists: 7 out of 13 (53.8 %) Arctic districts lack radiologists, and in 5 out of 34 (14.7 %) districts, their numbers fall below the required levels;
- The shortage of radiologists in the districts creates difficulties in organizing mandatory double reading of fluorograms during screening, resulting in missed pulmonary pathologies;
- Poorly developed transport infrastructure, especially in Arctic districts with seasonal accessibility to remote settlements, leads to prolonged delays in the delivery of diagnostic material for culture and molecular

genetic testing, as well as patient medical documentation, to the main TB facility of the republic, extending the time to diagnosis to one month or more.

Numerous studies have addressed the optimization of pulmonary tuberculosis detection and diagnosis, including through the use of modern information technologies (IT), in various regions of the Russian Federation. However, no studies addressing this issue in the context of sparsely populated territories of the Far North were found in the available literature. Most studies focus on the use of computer programs for recording and monitoring tuberculosis foci [7, 8] and intelligent systems for detecting pulmonary pathology based on medical images [9–11]. The combined use of IT, including telemedicine and artificial intelligence, to improve tuberculosis detection and diagnosis has not been investigated. Issues related to the identification of pathology during screening fluorographic examination, from both clinical and organizational perspectives, remain insufficiently studied.

The above confirms the relevance of developing measures for the implementation of modern information and digital technologies to improve methods for tuberculosis detection and diagnosis in the Far North region with low population density.

## AIM

To optimize methods for the detection and diagnosis of pulmonary tuberculosis using information technologies in the sparsely populated territory of the Republic of Sakha (Yakutia).

## MATERIALS AND METHODS

This study employed a zoning scheme of the Republic of Sakha (Yakutia) divided into five socio-territorial zones according to the method of M.A. Tyrylgina [12], modified in accordance with Presidential Decree No. 296 of May 2, 2014 “On the Land Territories of the Arctic Zone of the Russian Federation”:

I. Yakutsk City, the capital of the Republic of Sakha (Yakutia).

II. Arctic Zone, comprising 13 polar and subpolar districts with extensive territory accounting for 45 % of the republic’s area. The lowest population density in the region is 0.04 persons/km<sup>2</sup>, which is 8 times lower than the republic average (0.32 persons/km<sup>2</sup>). The proportion of indigenous small-numbered peoples of the North is 24 % of the population, significantly higher than in other zones. The main economic activities are reindeer herding, hunting, and fishing.

III. Industrial Zone, comprising 5 districts with developed mining industries (diamond, coal, oil, gas). In this zone, the urban population accounts for 92.6 %, and the non-indigenous population accounts for 92.4 %.

IV. Rural (Agricultural) Zone, consisting of 11 districts of Central Yakutia with stall-based animal husbandry and herd horse breeding, as well as risky agriculture. Here, the rural population accounts for 87.7 %, and the indigenous population accounts for 94.8 %.

V. Mixed Zone, comprising 5 scattered districts with stall-based animal husbandry, herd horse breeding, reindeer herding, risky agriculture, and a small share of mining industry. In the mixed zone, the urban population accounts for 49.4 %, rural – 50.6 %, and indigenous – 53.7 %.

All districts of the Republic of Sakha (Yakutia) are included in the list of territories with low population density, except for 5 districts of the Rural (Agricultural) Zone. Indigenous (native) peoples included the Yakuts (Sakha) and the small-numbered peoples of the North (Even, Evenki, Yukaghir, Chukchi, Dolgan). All other population groups were considered non-indigenous [13].

Starting from 2020, the Scientific and Practical Center “Phthisiology” began developing an algorithm for tuberculosis detection and diagnosis among the adult population using information technologies (IT). Monthly monitoring of population coverage with fluorographic (FLG) examinations was introduced. Telemedicine consultations (TMC) were organized between district primary healthcare physicians and TB specialists at TB dispensary departments of central district hospitals (CDHs) and Yakutsk through the “Cloud Polyclinic” application for diagnostic clarification and registration for follow-up. The telemedicine service “Cloud Polyclinic”, launched in the Republic of Sakha (Yakutia) in 2020, is designed for remote patient-specialist interaction and physician-to-physician consultations.

To evaluate the effectiveness of IT implementation in the TB service, two periods were compared: Period 1 (2014–2016) – traditional methods (pre-IT implementation); Period 2 (2020–2023) – widespread IT implementation (“Cloud Polyclinic”, ArchiMed system, TMC, and “Predict” software).

From 2022, work was conducted jointly with the Faculty of Computational Mathematics and Cybernetics of Lomonosov Moscow State University to process the FLG image database using a prototype interactive tool for radiographic image annotation to train diagnostic deep neural networks. The effectiveness of primary and secondary reading of digital fluorograms with a clinical decision support system in radiological screening for pulmonary tuberculosis was compared using expert evaluation, with accuracy, sensitivity, and specificity calculated [14]. As a result, a neural network diagnostic model for tuberculosis, the “Predict” software, was developed and included in the algorithm.

In 2022, the Ministry of Health of the Republic of Sakha (Yakutia) approved a pilot project for the implementation and testing of new information technologies to improve the effectiveness of tuberculosis detection and diagnosis among the republic’s population.

Seven pilot healthcare facilities were selected from each socio-territorial zone for algorithm implementation:

- Yakutsk City (Yakutsk City Hospital No. 2, Yakutsk City Hospital No. 3);
- Arctic Zone (Bulunskaya Central District Hospital);
- Industrial Zone (Aldanskaya Central District Hospital);
- Rural (Agricultural) Zone (Verkhnevilyuyanskaya Central District Hospital; Megino-Kangalasskaya Central District Hospital);
- Mixed Zone (Khangalasskaya Central District Hospital).

As shown in Figure 1, within the pilot healthcare facilities, at the first (radiological) stage of the algorithm for pulmonary tuberculosis detection and diagnosis during FLG screening of the adult population, the radiologist at the FLG unit uploads digital fluorograms to the Central Archive of Medical Images (ArchiMed system). For primary reading of fluorograms, the “Predict” software is used to prioritize images for radiologist review, highlighting radiological syndromes as a clinical decision support system. This is followed by secondary reading by a radiologist. The list of cases that have been identified as having pathological changes in their chest organs (referred to as “FLG-positive”) is transmitted by the authorized facility’s official to the primary care physicians, with the results of any further examinations being returned to the FLG unit. An electronic record of the subsequent follow-up for these cases is maintained within the FLG department.

At the second (clinical) stage, the district primary care physician at the healthcare facility performs a follow-up examination of “FLG-positive” individuals. When symptoms suggestive of tuberculosis are identified, the patient is referred to a phthiatrician (TB specialist) at the TB Dispensary Department of the Central District Hospital or the Yakutsk City TB Dispensary Department.

At the third (specialized) stage, following completion of the patient’s diagnostic workup, the phthiatrician conducts a telemedicine consultation (TMC) with the Scientific and Practical Center for Phthisiology via the Cloud Polyclinic platform to confirm the diagnosis of tuberculosis and to enroll the patient in dispensary follow-up. The results of the follow-up examination are transmitted back to the district primary care physician.

The effectiveness of algorithm implementation was assessed through monthly monitoring of the following parameters: population coverage with fluorographic (FLG) screening; proportion of “FLG-positive” individuals who completed follow-up examination; coverage of preventive screenings among tuberculosis risk groups; time intervals to tuberculosis diagnosis, measured from both the date of initial presentation to the primary healthcare facility and the date of referral to the phthiatrician.

For statistical analysis, descriptive statistics methods were employed, including calculation of means

Algorithm for detection and diagnosis of respiratory tuberculosis during FLG screening of the adult population

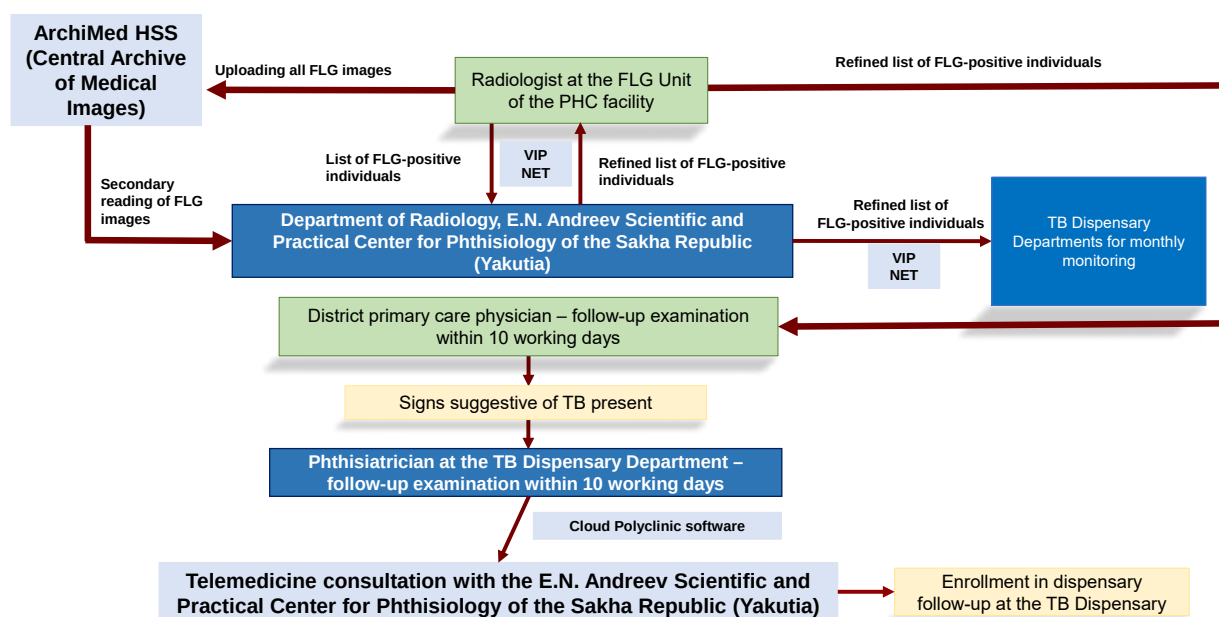


FIG. 1.

Algorithm for the detection and diagnosis of pulmonary tuberculosis during mass X-ray screening of the adult population in pilot health-care facilities

(M), standard errors (m), and proportions (%), allowing assessment of preventive screening coverage, diagnostic timeframes, and other indicators. To compare algorithm effectiveness before and after information technology implementation, Student's *t*-test was used, confirming the statistical significance of differences ( $p < 0.05$ ). Diagnostic accuracy of the methodology was evaluated through calculation of sensitivity, specificity, and overall accuracy, as well as by expert assessment during validation of primary and secondary fluorogram readings.

## RESULTS

To evaluate the effectiveness of the algorithm for tuberculosis detection and diagnosis among the adult population using information technologies, the Scientific and Practical Center for Phthisiology conducted monthly monitoring of the following parameters: fluorography (FLG) screening coverage of the catchment population; timeframes and proportion of FLG-positive individuals who completed follow-up examination; and monthly monitoring of preventive screening coverage for tuberculosis among risk groups in pilot healthcare facilities, compared with other healthcare facilities in the republic.

In healthcare facilities not included in the pilot project, the coverage of preventive screening for tuberculosis by FLG during the period 2017–2019 ranged

from 85.0 % to 87.9 %, averaging 2.0 % higher than in the pilot facilities. Following the launch of the project, the coverage rate in pilot facilities increased, reaching 82.6–88.7 % over the period 2020–2023, which exceeded the corresponding rate in the remaining healthcare facilities by an average of 3.2 %.

Analysis of preventive screening coverage among tuberculosis risk groups in pilot versus non-pilot healthcare facilities in 2022–2023 demonstrated that coverage of both medical and social risk groups was higher in pilot facilities. In 2022, preventive screening coverage of medical risk groups in pilot Central District Hospitals (CDHs) was 92.3 %, increasing to 95.2 % in 2023, thereby reaching the target indicator of 95 %. In the remaining CDHs, the corresponding rates were 88.0 % and 88.3 %, respectively. In pilot healthcare facilities of Yakutsk City, coverage decreased from 99.8 % in 2022 to 95.9 % in 2023, remaining above the target threshold. In non-pilot facilities, the indicator remained below the required level (90.8 % in 2022 and 84.0 % in 2023).

Coverage of preventive screening among social risk groups in pilot CDHs increased from 80.9 % in 2022 to 86.9 % in 2023, though it did not reach the target indicator. In non-pilot CDHs, the indicator was insufficient, although it increased from 79.9 % to 84.3 %. In pilot healthcare facilities of Yakutsk City, screening coverage for social risk groups during the study period ranged from 96.6 % to 100 %, compared with 90.6–97.6 % in non-pilot facilities.

Analysis of the proportion of FLG-positive individuals who did not complete follow-up examination in pilot versus non-pilot healthcare facilities in 2022 and 2023 revealed that, in facilities not currently included in the project, the proportion of individuals with FLG changes who were not fully examined decreased in both CDHs (1.4–1.6 % vs. 2.1–2.2 %) and Yakutsk City healthcare facilities (3.1–6.8 % vs. 12.8–14.6 %).

The implementation of information technologies in pilot healthcare facilities enabled minimization of missed pathology during FLG screening of the population for tuberculosis and reduced the timeframes for diagnosis and enrollment of patients in dispensary follow-up. As shown in Table 1, following IT implementation in pilot CDHs, the mean time interval from initial presentation at primary healthcare facilities to referral to a phthisiatrician was reduced by 22.7 days, and the mean time interval from presentation at the CDH TB Dispensary Department to confirmation of tuberculosis diagnosis was reduced by 32.9 days. Both differences were statistically significant ( $p = 0.001292$ ;  $p = 0.001925$ , respectively).

Thus, the pilot implementation of the developed algorithm for clinical and radiological tuberculosis screening using information technologies in pilot healthcare facilities has led to positive changes in pulmonary tuberculosis detection through the organization of secondary reading of FLG images in the Central Archive of Medical Images by radiologists from the core institution, monthly monitoring of population coverage with FLG screening, preventive screening coverage of medical and social risk groups, and follow-up examination of FLG-positive individuals, as well as a significant reduction in tuberculosis diagnostic timeframes through the transmission of patient medical documentation via the Cloud Polyclinic platform and the conduct of telemedicine consultations for diagnosis confirmation.

To test the “Predict” software in 2023–2024, double reading of digital fluorograms using artificial intelligence (AI) was organized and conducted at primary healthcare facilities. The AI performed

the primary reading of all FLG images, while the radiologist at the primary healthcare facility performed the secondary reading of the same images, taking into account the AI results. During the testing phase, radiologists from the Scientific and Practical Center for Phthisiology performed quality control of the primary and secondary reading reports for 16,427 FLG images included in the study. Based on the results of double reading (AI + radiologist), 468 cases of pathology were suspected, of which clinical and laboratory examination confirmed pulmonary tuberculosis in 135 patients. The diagnosis in all patients was verified through comprehensive examination, including medical history collection, analysis of clinical and laboratory data, chest radiography, and chest computed tomography.

In the current version of the “Predict” software, the system calculates the percentage probability of the presence of pulmonary abnormalities. The highest concordance rate between radiologist and AI readings was observed in the pathology probability range of 0 % to 25 % (93.3–99.7 %). In the 26–60 % range, the concordance rate was 61.9–82.4 %. When the AI-predicted pathology probability exceeded 61 %, radiologists most frequently identified pulmonary abnormalities, including fibrotic changes, inflammatory changes, postoperative changes, sequelae of trauma, as well as congenital pathology of the pulmonary, cardiovascular, and musculoskeletal systems.

ROC analysis of AI performance at the primary FLG screening stage revealed high performance metrics: sensitivity for pathology probability detection was 82 %, and specificity was 81 %.

Following the pilot testing of the algorithm for pulmonary tuberculosis detection and diagnosis in pilot healthcare facilities, in 2024 an Organizational Two-Component Model of Clinical and Radiological Screening for Pulmonary Tuberculosis was developed (hereinafter referred to as the “Model”). The Model is based on the comprehensive application of information and digital technologies to improve pulmonary tuberculosis detection and diagnosis in sparsely

**TABLE 1**

**TIMEFRAME FOR TUBERCULOSIS DIAGNOSIS IN PILOT CENTRAL DISTRICT HOSPITALS BEFORE AND AFTER IMPLEMENTATION OF IT-SOLUTIONS**

| Follow-up groups                                         | Time from presentation at HCF to referral to TBDD (CDH), days (M+m) | Time from presentation at TBDD (CDH) to diagnosis confirmation, days (M+m) |
|----------------------------------------------------------|---------------------------------------------------------------------|----------------------------------------------------------------------------|
| Pilot CDH before IT implementation, 2014-2016 ( $n=55$ ) | 31.3±6.1                                                            | 45.7±5.0                                                                   |
| Pilot CDH after IT implementation, 2020-2023 ( $n=31$ )  | 8.6±2.9                                                             | 12.8±3.9                                                                   |
| Student's <i>t</i> -test                                 | 3.33                                                                | 3.2                                                                        |
| <i>p</i>                                                 | 0.001292                                                            | 0.001925                                                                   |

populated territories. It comprises two Algorithms designed for use in the Arctic and Industrial zones of the Far North. The Organizational Two-Component Model was developed taking into account the existing differences in tuberculosis detection organization across the socio-territorial zones of the region, particularly with the predominantly mobile (outreach) mode of healthcare delivery, including FLG screening of the population in the districts of the Arctic zone.

The developed Algorithms of the Model include three stages: Radiological Stage at the Radiology Department of the Central District Hospital (hereinafter referred to as the First Stage); Clinical Stage at the Outpatient Department of the Central District Hospital (hereinafter referred to as the Second Stage); and Specialized Stage at the level of the phthisiatrician of the TB Dispensary Department of the Central District Hospital (hereinafter referred to as the Third Stage).

In the schematic representation of the Model Algorithms, asterisks with numbers indicate new organizational forms introduced in the work of Central District Hospitals for tuberculosis detection during population FLG screening.

The algorithm for the Arctic Zone (Fig. 2) has several distinctions due to infrastructure characteristics. At the first stage, screening FLG examination of the entire population is conducted by a mobile fluorography team, which provides the FLG Unit of the Central District Hospital with the completed FLG images on a digital storage medium. The radiologist uploads the FLG image database to the ArchiMed Central Archive of Medical

Images and simultaneously to the Predict software for primary reading. Following the receipt of fluorogram reading results from the Predict software, the radiologist performs secondary reading, prioritizing those images identified with a pathology probability of 1 % or higher. After reviewing all fluorographic images, the radiologist prepares a list of FLG-positive individuals and transmits it to the second stage of the Model – the Head of the Polyclinic Department of the Central District Hospital.

At the second stage of the Algorithm, the Head of the Polyclinic Department of the CDH transmits the lists of FLG-positive individuals to a mobile medical team, which specifically travels to populated areas to examine patients with pathology detected during FLG screening. The mobile medical team completes Block 1 of the insert in the outpatient medical record – “Clinical and Radiological Examination of FLG-positive Patient” (hereinafter referred to as the “insert”) and issues a referral for further examination. The insert with completed Block 1 is handed over by the mobile medical team physician to the Head of the Polyclinic Department, who then forwards it to the district primary care physician. The district primary care physician recalls and performs a follow-up examination of the FLG-positive patient within 10 working days and completes Block 2 of the insert. When signs suggestive of tuberculosis are identified, the patient is referred to the third stage – the TB Dispensary Department of the CDH.

At this stage, a new documentation form is introduced – the insert in the outpatient medical record

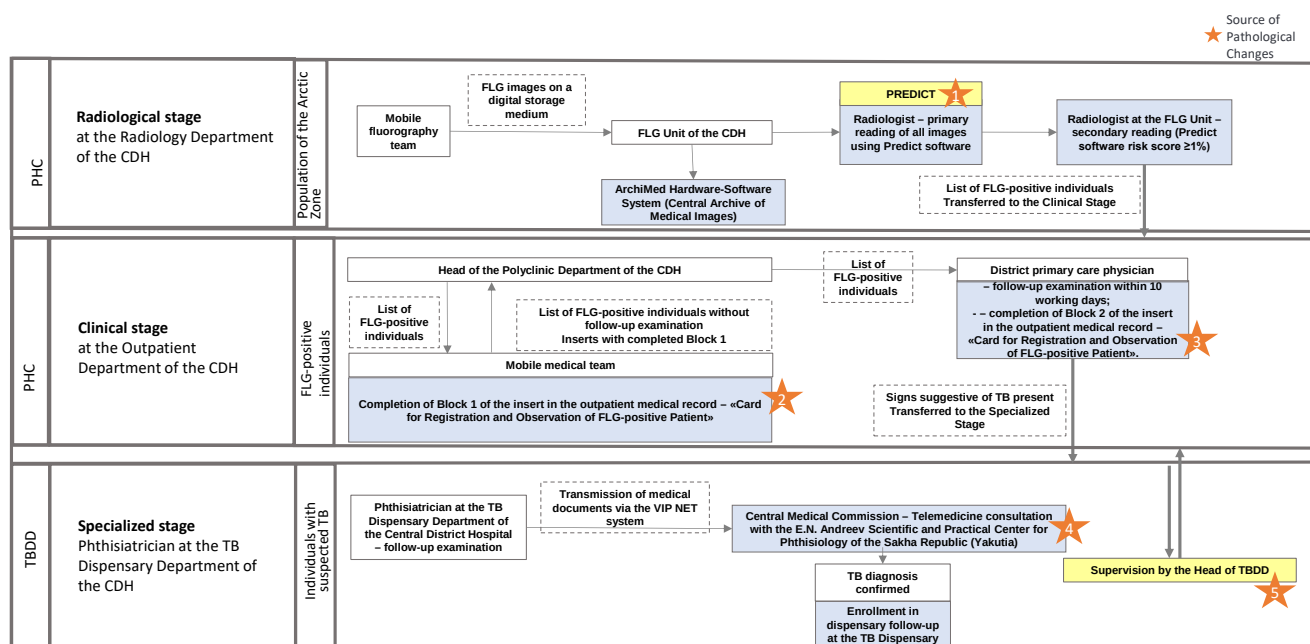


FIG. 2.

Algorithm for clinical and radiological screening of pulmonary tuberculosis based on the integrated use of information technology to improve detection and diagnosis in the Arctic zone of the Far North

“Clinical and Radiological Examination of FLG-positive Patient”, divided into two blocks: for the mobile medical team physician and for the district primary care physician. The insert for the mobile medical team includes patient identification information: demographic data, brief medical history, and the date of the most recent examination as reported by the patient. The insert for the primary care physician includes follow-up examination results and the final diagnosis.

At the third stage, individuals with suspected tuberculosis are referred for follow-up examination to the TB Dispensary Department of the CDH. The phthisiatrician at the TBDD transmits all patient examination results to the Scientific and Practical Center for Phthisiology via the secure ViPNet network for review by the Central Medical Commission using telemedicine technologies. When the diagnosis of tuberculosis is confirmed, the Central Medical Commission enrolls the patient in dispensary follow-up. Oversight of patient routing and compliance with the third stage procedures is the responsibility of the Head of the TB Dispensary Department of the CDH.

The Industrial Zone is characterized by more stable internet connectivity and better transport accessibility. Therefore, the Algorithm for this zone does not include the deployment of mobile teams (Fig. 3).

At the first stage, screening FLG examination of the entire population is conducted at the FLG Units of the Central District Hospitals. The radiologist uploads the FLG image database to the ArchiMed Central Archive of Medical Images and simultaneously

to the Predict software for primary reading. Following the receipt of fluorogram reading results from the Predict software, the radiologist performs secondary reading, prioritizing those images identified with a pathology probability of 1 % or higher. After completing secondary reading of all FLG images, the radiologist generates a list of FLG-positive individuals and transmits it to the second stage of the Model – the Deputy Chief Physician for Outpatient Care / Head of the Polyclinic Department of the CDH.

At the second stage of the Algorithm for the Industrial Zone, the Head of the Polyclinic Department of the CDH transmits the lists of FLG-positive individuals to the district primary care physicians, who perform a follow-up examination of the patient within 10 working days and complete the insert in the outpatient medical record – “Clinical and Radiological Examination of FLG-positive Patient”. When signs suggestive of tuberculosis are identified, the patient is referred to the third stage – the TB Dispensary Department of the CDH for follow-up examination.

At the third stage, the phthisiatrician transmits all patient examination results to the Scientific and Practical Center for Phthisiology via the secure ViPNet network for review by the Central Medical Commission (CMC) using telemedicine technologies. When the diagnosis of tuberculosis is confirmed, the CMC enrolls the patient in dispensary follow-up. Oversight of patient routing and compliance with the third stage procedures is the responsibility of the Head of the TB Dispensary Department of the CDH.

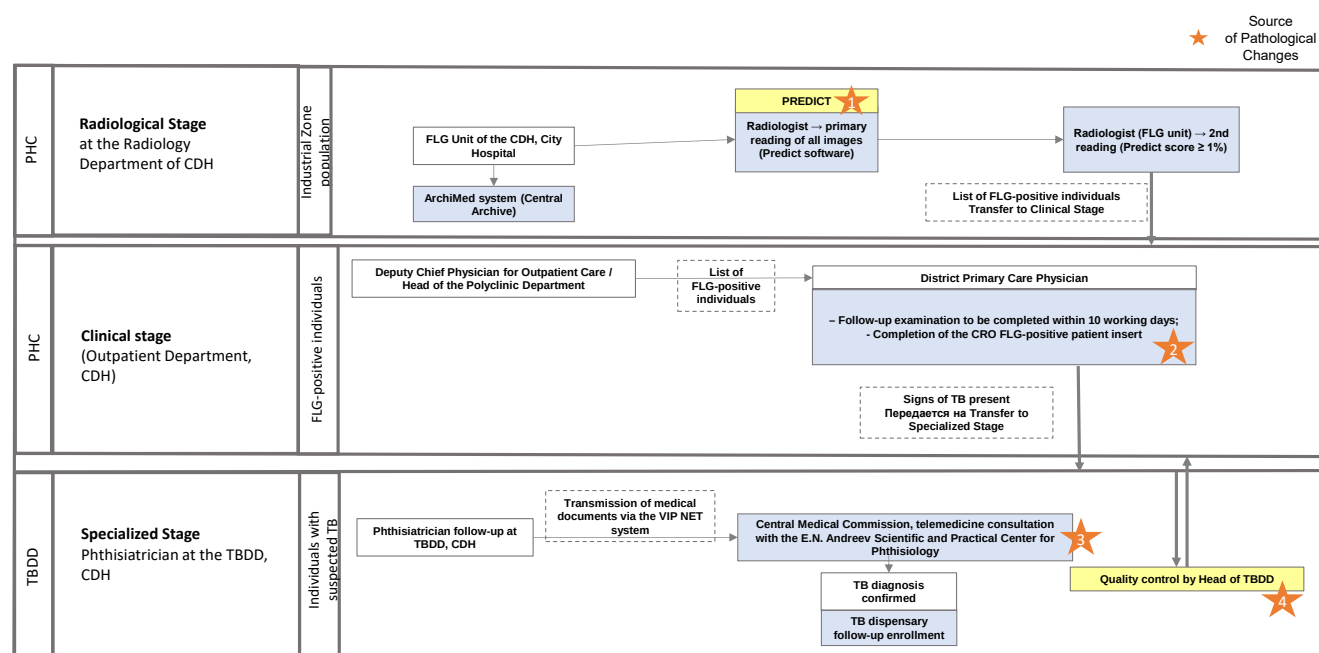


FIG. 3.

Algorithm for clinical and radiological screening of pulmonary tuberculosis based on the integrated use of information technology to improve detection and diagnosis in the Industrial zone of the Far North

At present, the Predict software continues to undergo pilot testing and refinement of artificial intelligence parameters to improve sensitivity and specificity.

Radiological images obtained during mass screening examinations are routinely reviewed by two radiologists to reduce the risk of missed pathology. Primary reading of the radiological image is performed immediately after the examination, taking 5–7 minutes per image. Secondary reading is performed either by a different radiologist or by the same radiologist after 24 hours. This system improves the detection rate of pathological changes to up to 99 %, but is time-consuming.

The use of AI eliminates the need for primary reading by a radiologist, reducing reading time to 1–2 seconds per image and increasing detection efficiency to 99.6 %. The radiologist performs only secondary reading of radiological images, and with AI assistance, reading efficiency increases to up to 100 %.

As a result of implementing the Organizational Two-Component Model with AI integration, the following objectives of the healthcare digitalization program are achieved: improved quality of pulmonary tuberculosis diagnosis; reduced examination time per patient; reduced workload on radiologists; and increased accessibility of medical care to the population.

## DISCUSSION

The key achievement of this work is the development of a comprehensive Organizational Two-Component Model, in which digital tools perform strictly defined functions within the overall tuberculosis care system. The main process improvement has been achieved through clear regulation of stages and data flows. Automated primary triage through the upload of fluorograms to the Predict software replaces the labor-intensive primary reading by a physician, instantly identifying images with low pathology probability and prioritizing those requiring specialist attention. This represents not diagnosis, but an efficient triage system that optimizes specialist workload.

An important element of the Model Algorithms is centralization of expert review, which provides for mandatory secondary reading of images from risk groups by a radiologist at the core institution via the Central Archive of Medical Images. This approach successfully addresses the shortage of local personnel and standardizes evaluation criteria. End-to-end electronic routing ensures the generation of a digital list of FLG-positive individuals and its automatic transmission via secure network from the radiologist to the Head of the Polyclinic Department and then to the district primary care physician, eliminating data loss and delays inherent in paper-based document management.

Of particular note is the development of differentiated processes for different settings. The creation of two separate algorithms for the Arctic and Industrial Zones

demonstrates a systematic approach to problem-solving. For the Arctic Zone, the algorithm is supplemented by the deployment of mobile teams and a new documentation form, compensating for the lack of direct access to healthcare facilities. For the Industrial Zone with developed infrastructure, the algorithm operates in a more direct and rapid mode. Thus, the Predict software functions not as an independent diagnostic tool, but as a critical filter-accelerator at the entry point of the established clinical process. The reduction in diagnostic timeframes should be considered a consequence of comprehensive process optimization rather than merely the use of technological solutions.

### Study Limitations

The presented algorithms have several limitations related to implementation stages and generalizability. Reliable operation critically depends on the uninterrupted functioning of all technological components, including the Central Archive of Medical Images, secure networks, and stable Internet connectivity. In the Arctic Zone, failure at any of these stages may paralyze the entire process. There is also a risk of operational errors, as the workload increases on the central hub – the radiologists at the core institution and healthcare organizers responsible for coordinating data flows.

The study documents improvement in performance indicators; however, to assess the ultimate effectiveness of the Organizational Two-Component Model, long-term data on epidemiological outcomes are required, which may only become apparent after several years of observation.

## CONCLUSION

The conducted research confirms that early tuberculosis detection in sparsely populated territories remains a challenging task due to low population density and limited accessibility of traditional screening methods. The implementation of telemedicine technologies and digital tools may improve diagnostic coverage; however, their application in the Arctic and Industrial Zones requires adaptation to the specific characteristics of the territories and approaches to healthcare service organization.

Key unresolved aspects include optimization of patient routing, reduction of time to diagnosis, and improvement of radiological data interpretation accuracy, particularly under conditions of specialist shortages.

In this study, an Organizational Two-Component Model for tuberculosis detection using information technologies, including AI-based Predict software, was developed and piloted. The implementation of the Model resulted in a 3.2 % increase in fluorographic screening coverage, a reduction in diagnostic timeframes by 22.7–32.9 days, and a 2–4 times reduction in the number of patients without follow-up completed.

The use of artificial intelligence in fluorogram analysis provides high accuracy (up to 99.6 %),

while minimizing missed pathology and reducing the workload on radiologists. The developed Algorithms for the Arctic and Industrial Zones have demonstrated their effectiveness through the combination of telemedicine consultations, automated image analysis, and optimized patient routing.

The obtained results have significant clinical implications, as they improve the accessibility and quality of tuberculosis diagnosis in hard-to-reach regions and reduce the time from detection to treatment. The scientific value of this work lies in substantiating an approach that combines digital technologies with adapted organizational solutions for different socio-territorial zones. For further research, it would be beneficial to extend the pilot implementation of the Model to other regions with similar conditions, refine AI algorithms to improve specificity in differential diagnosis, and study the long-term impact of the implemented methods on tuberculosis epidemiological indicators.

### Conflict of interest

The authors declare no conflict of interest.

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## EPIDEMIOLOGY

### COVID-19 PANDEMIC IMPACT ON PEDIATRIC HOSPITALIZATION DYNAMICS FOR ACUTE INTESTINAL INFECTIONS (INCLUDING ROTAVIRUS) IN CHITA

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#### ABSTRACT

**Background.** The COVID-19 pandemic and the associated non-pharmaceutical interventions (NPIs) significantly altered the global epidemiology of various infectious diseases. Studying the dynamics of hospitalizations for acute intestinal infections in a specific region during different periods of the pandemic allows for assessment of the effect of these measures and identification of patterns in the recovery of the epidemic process.

**Aim.** To evaluate the impact of the COVID-19 pandemic on the dynamics and structure of hospitalizations of children with acute intestinal infections (All), including rotavirus infection (RVI), in Chita over the period 2014–2023.

**Materials and Methods.** A retrospective analysis of statistical reporting data from the Regional Clinical Infectious Diseases Hospital in Chita was conducted. A total of 11,520 hospitalization cases of children aged 0–17 years with All over a 10-year period were analyzed. Descriptive and non-parametric statistical methods were used, including time series analysis, the  $\chi^2$  test, the  $\phi$ -coefficient, and Spearman's correlation analysis. Data processing was performed using IBM SPSS Statistics 26.

**Results.** During the peak pandemic period (2020–2021), the number of hospitalizations for All decreased 1.6-fold ( $\chi^2 = 9.680$ ;  $p = 0.002$ ), and for RVI – 1.4-fold ( $\chi^2 = 23.955$ ;  $p < 0.001$ ) compared to the pre-pandemic period. The etiological structure of All did not change significantly: the proportion of RVI among all cases was 24.48 % (pre-pandemic), 23.68 % (peak pandemic), and 22.87 % (post-pandemic) ( $p > 0.05$ ). A strong positive correlation was found between the total number of All hospitalizations and RVI cases ( $r = 0.789$ ;  $p = 0.008$ ). In the post-restriction period (2022–2023), an increase in hospitalizations was observed.

**Conclusions.** The anti-epidemic measures during the COVID-19 pandemic led to a significant reduction in hospitalizations for All and RVI without affecting their etiological structure. The subsequent increase in incidence following the lifting of restrictions confirms the restoration of the epidemic process, which underscores the need for ongoing epidemiological surveillance and the expansion of vaccination programs.

**Keywords:** acute intestinal infections, rotavirus infection, COVID-19 pandemic, children, hospitalization, epidemiology, Zabaykalsky Krai, retrospective study, SARS-CoV-2

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## ВЛИЯНИЕ ПАНДЕМИИ COVID-19 НА ДИНАМИКУ ГОСПИТАЛИЗАЦИЙ ДЕТЕЙ В ЧИТЕ С ОСТРЫМИ КИШЕЧНЫМИ ИНФЕКЦИЯМИ, В ТОМ ЧИСЛЕ С РОТАВИРУСНОЙ ИНФЕКЦИЕЙ

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### РЕЗЮМЕ

**Обоснование.** Пандемия COVID-19 и сопутствующие противоэпидемические меры оказали глобальное влияние на циркуляцию респираторных и кишечных вирусов. Изучение динамики госпитализаций с острыми кишечными инфекциями (ОКИ) в конкретном регионе в разные периоды пандемии позволяет оценить эффект этих мер и выявить особенности восстановления эпидпроцесса.

**Цель.** Оценить влияние пандемии COVID-19 на динамику и структуру госпитализаций детей с ОКИ, в том числе с ротавирусной инфекцией (РВИ), в Чите за 2014–2023 гг.

**Методы.** Проведён ретроспективный анализ данных статистической отчётности Краевой клинической инфекционной больницы г. Читы. Проанализированы 11520 случаев госпитализации детей 0–17 лет с ОКИ за 10-летний период. Использовались методы описательной и непараметрической статистики, анализ динамических рядов, критерий  $\chi^2$ , коэффициент  $\phi$ , корреляционный анализ Спирмена. Обработка данных выполнена в IBM SPSS Statistics 26.

**Результаты.** В период разгара пандемии (2020–2021 гг.) число госпитализаций с ОКИ снизилось в 1,6 раза ( $\chi^2 = 9,680$ ;  $p = 0,002$ ), с РВИ – в 1,4 раза ( $\chi^2 = 23,955$ ;  $p < 0,001$ ) относительно допандемийного периода. Структура ОКИ значительно не изменилась: доля РВИ от всех случаев составила 24,48 % (до), 23,68 % (разгар) и 22,87 % (после) ( $p > 0,05$ ). Выявлена прямая тесная связь между общим числом госпитализаций с ОКИ и РВИ ( $r = 0,789$ ;  $p = 0,008$ ). После отмены ограничений в 2022–2023 гг. наблюдался рост госпитализаций.

**Заключение.** Противоэпидемические меры в период пандемии COVID-19 привели к значимому снижению числа госпитализаций с ОКИ и РВИ, не повлияв на их этиологическую структуру. Последующий рост заболеваемости после отмены ограничений подтверждает восстановление эпидемиологического процесса, что подчеркивает необходимость постоянного эпидемиологического мониторинга и расширения программ вакцинопрофилактики.

**Ключевые слова:** острые кишечные инфекции, ротавирусная инфекция, пандемия COVID-19, дети, госпитализация, эпидемиология, Забайкальский край, ретроспективное исследование, SARS-CoV-2

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## BACKGROUND

Acute intestinal infections (All), particularly rotavirus infection (RVI), despite significant advances in vaccine prophylaxis and the improvement of epidemiological surveillance methods, remain one of the leading causes of morbidity and hospitalization in children worldwide [1, 2]. According to the World Health Organization (WHO, 2023), rotaviruses occupy a leading position among the etiological agents of severe gastroenteritis in young children, accounting for a substantial proportion of hospitalizations and deaths, especially in low-income and middle-income countries. Global estimates indicate that vaccination programs are being actively implemented worldwide, with coverage of the target cohort who completed vaccination reaching 59 % in 2024 [3], leading to a reduction in mortality and hospitalizations. However, the incidence of RVI remains high, and the economic burden associated with rotavirus diarrhea in Russia in 2024 ranks fifth among infectious diseases according to the State Report (2025).

In recent years, the COVID-19 pandemic has had a significant impact on the epidemiology of rotavirus infection worldwide [4, 5]. The introduction of restrictive measures, closure of educational institutions, and enhanced sanitary requirements led to a sharp decline in hospitalizations for All, including rotavirus-induced gastroenteritis [6, 7]. However, in the post-COVID period, following the lifting of strict restrictive measures, a recovery and even an increase in rotavirus infection incidence to pre-pandemic levels has been observed, as confirmed by both international and Russian data [8, 9].

In the Russian Federation, RVI occupies a leading position in the structure of acute intestinal infections of established etiology in children, accounting for 33.3–78.8 % [10, 11]. However, the actual number of cases may be significantly higher due to insufficient laboratory confirmation, registration peculiarities, and laboratory diagnostic capabilities. The introduction of routine rotavirus vaccination with high coverage of the target cohort in several regions of Russia (State Report, 2025), such as Sakhalin Oblast (coverage 88.3 %), Yamalo-Nenets Autonomous Okrug (82.0 %), and the Republic of Buryatia (69.1 %), has made it possible to reduce the incidence and severity of infection. However, vaccination coverage across the Russian Federation remains low, amounting to 15.09 % in 2024.

Of particular importance are the epidemiological features of rotavirus infection in the Far East, where the structure and dynamics of incidence differ from national averages due to geographical, climatic, and demographic factors [12]. According to regional studies, rotavirus infection in Chita, the capital of the Trans-Baikal Territory, occupies a leading position among viral All in children, with RVI accounting for 60–70 % of viral diarrheas [13]. In Chita, rotavirus vaccination is administered only for epidemic indications or on a commercial basis, with extremely low coverage, which does not

allow for the formation of herd immunity and contributes to the persistence of high incidence rates.

Over the past decade, significant variability in the structure of morbidity has been observed, which is associated both with changes in virus circulation, the organization of laboratory diagnostics, and the coronavirus pandemic. The dynamics of COVID-19 spread in Russia included four distinct epidemic waves. Following the identification of the first cases on January 31, 2020, the country experienced an initial surge by May of the same year, peaking at 11,656 infections per day. However, the autumn wave of the same year demonstrated a more intensive increase, exceeding the spring peaks. A third wave followed, peaking in July 2021, and by the autumn of that year, a fourth wave began. The final step in the easing of anti-COVID policies was the lifting of some restrictive measures, officially announced on July 2, 2022 [14]. Analysis of the structure and dynamics of All hospitalizations, as well as the patterns of rotavirus-induced gastroenteritis spread during different epidemiological periods, including the pre-pandemic, pandemic, and post-pandemic stages, allows not only the identification of regional epidemiological features but also the assessment of the effectiveness of anti-epidemic measures, as well as highlighting the relevance of introducing and expanding vaccination programs.

## AIM

To evaluate the impact of the COVID-19 pandemic and associated anti-epidemic measures on the dynamics and structure of hospitalizations of children with acute intestinal infections (All), including rotavirus infection (RVI), in a large infectious disease hospital in Chita over a 10-year period (2014–2023).

## MATERIALS AND METHODS

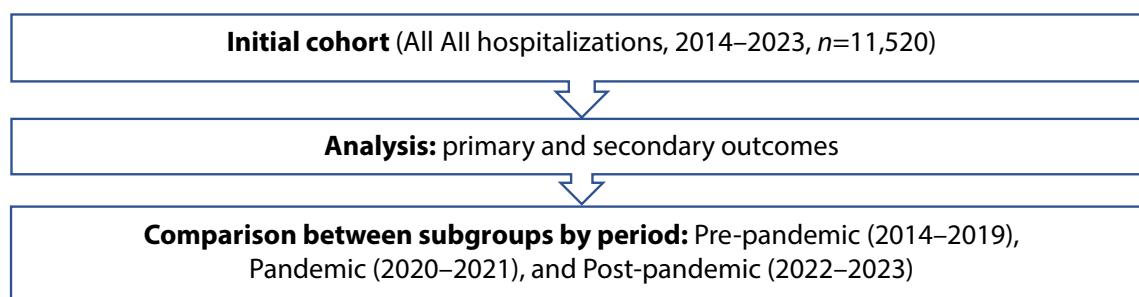
A retrospective cohort study was conducted based on the analysis of inpatient records (Fig. 1).

**Inclusion criteria.** All hospitalization cases of patients aged 0 to 17 years with a confirmed diagnosis of acute intestinal infection (regardless of etiology) at the Regional Clinical Infectious Diseases Hospital (RCH) in Chita between January 1, 2014, and December 31, 2023.

**Non-inclusion criteria.** Absence of data required for analysis (age, diagnosis, date of hospitalization) in the statistical records.

**Exclusion criteria.** Duplicate records of hospitalization for the same patient within a single disease episode.

**Study setting.** The study was conducted at the Regional Clinical Infectious Diseases Hospital (RCH) in Chita, which is a specialized inpatient facility for the hospitalization of children with All within the city district. The low rotavirus vaccination coverage in the region



**FIG. 1.**  
Study design

may limit the generalizability of the findings to populations with high vaccine coverage.

**Study duration.** The enrollment period for the study was 10 years (from January 1, 2014, to December 31, 2023). The observation period covered the entire duration of each patient's hospitalization. The analysis was performed across three time periods: pre-pandemic (2014–2019), pandemic peak (2020–2021), and post-pandemic (2022–2023). No shifts in the planned time intervals occurred.

**Description of the medical intervention.** No medical intervention was planned or applied within the framework of this observational study. The "exposure" under investigation was the introduction and subsequent lifting of state-imposed anti-epidemic measures during the COVID-19 pandemic (isolation measures, closure of educational institutions, and mandatory mask wearing).

**Study outcomes.** Primary outcome: dynamics of the absolute number of hospitalizations of children with All and RVI during different periods of the COVID-19 pandemic. *Secondary outcomes:* 1) etiological structure of All (viral, bacterial, rotaviral, unspecified); 2) statistical strength of association between the pandemic period and hospitalization indicators; 3) correlation between the number of hospitalizations for RVI and the total number of All hospitalizations.

**Subgroup analysis.** Additional analysis was performed in subgroups defined by COVID-19 pandemic periods: pre-pandemic, pandemic peak, and post-pandemic.

**Outcome registration methods.** Outcomes were recorded through a retrospective analysis of aggregated data from official inpatient records (Form No. 14). Laboratory diagnosis of All etiology was performed using standard methods (ELISA and PCR) as specified for the facility.

**Ethical approval.** Due to the retrospective nature of the study and the use of anonymized data, informed consent from patients was not required.

**Statistical analysis.** The sample size was not calculated a priori. The entire population of hospitalization cases for the specified period was analyzed ( $n = 11,520$ ). Analysis was performed using IBM SPSS Statistics 26.

Quantitative data are presented as absolute numbers and percentages. The  $\chi^2$  test was used to compare proportions between subgroups. The strength of association was assessed using the  $\phi$  coefficient. For time series analysis, chain and baseline absolute increases, growth rates, and growth increments were calculated. Spearman's rank correlation coefficient ( $r$ ) was used to assess correlations. Statistical significance was set at  $p < 0.05$ .

## RESULTS

The retrospective analysis included data from 11,520 hospitalization cases of children aged 0 to 17 years with a diagnosis of acute intestinal infection at the Regional Clinical Infectious Diseases Hospital (RCH) in Chita from 2014 to 2023. All cases meeting the inclusion criteria were analyzed. No subsampling was performed. The baseline characteristic of the study objects was aggregated inpatient record data.

Figure 2 shows the dynamics of hospitalizations relative to the multi-year average (MYA) (1,152, 100 %). In 2023, compared to 2022, hospitalizations increased by 45 cases (4.5 %). The maximum increase was observed in 2017 (234 patients, 18.22 %), while the minimum increase was recorded in 2020 (-439 children, -34.98 %). The overall trend was increasing, indicating a rise in hospitalizations by 2023.

On average, the growth rate decreased by 0.7 % annually. The mean annual number of hospitalizations declined by 7.78 cases, reaching nearly 2014 levels by 2023.

Over the 10-year observation period (Fig. 3), among confirmed acute intestinal infections, viral infections ranked first, accounting for an average of 35 %; rotavirus infection accounted for 24 % of all All cases, while a substantial proportion (59 %) was attributed to acute intestinal infections of unspecified etiology (AIUE).

Previously, **in the pre-pandemic period**, we conducted a study of the long-term dynamics of hospitalizations of children aged 0–17 years at the RCH for All from 2014 to 2019 [13]. Analysis showed that the MYA of hospitalizations of children aged 0–17 years

with acute diarrhea during this period was 1,306 cases. On average, the number of hospitalizations of children with AI in the pre-pandemic period increased by 2.3 % annually (Fig. 4). A one-year forecast was made using the absolute increase indicator, yielding a predicted number of 1,282.4 hospitalizations in 2020. However, the COVID-19 pandemic resulted in a deviation from the forecasted values.

The growth rate of RVI in the pre-pandemic period shows that the number of hospitalizations increased annually by an average of 11.7 % (Fig. 4). A one-year forecast using the absolute increase indicator predicted 365.6 cases. Hospitalizations for AI and rotavirus infection demonstrated steady growth, with viral diarrheas accounting for the majority, of which RVI constituted the main part.

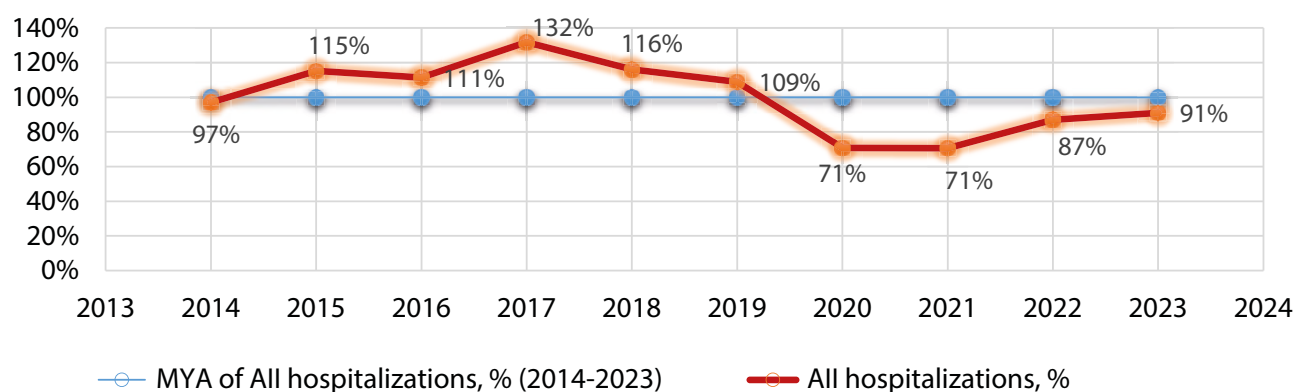
In the period from 2014 to 2019, the structure of acute diarrheal diseases was dominated by acute intestinal infections of unspecified etiology (AIUE) –  $59.07 \pm 7.44$  %;

viral diarrheas ranked second –  $33.92 \pm 9.78$  %; and bacterial AI were recorded less frequently –  $6.9 \pm 3.01$  %. RVI accounted for  $24.47 \pm 5.33$  % of all AI cases.

During the pandemic peak period from 2020 to 2021 (Table 1), the lowest hospitalization rates for children with AI were recorded in 2020 and 2021. These were 29 % below the multi-year average (Fig. 2).

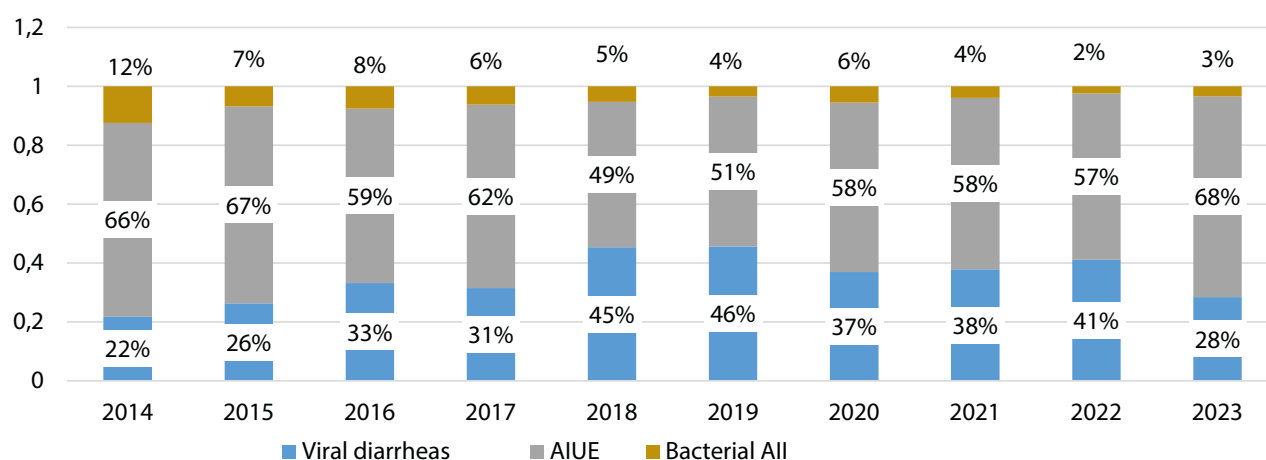
In 2020 and 2021, compared to 2014, hospitalizations decreased by 27 %. The mean growth rate during the pandemic peak period was low (17.61 %).

On average, during the pandemic peak years, the percentage of hospitalizations for AI decreased 1.6-fold relative to the pre-pandemic period ( $\chi^2 = 9.680$ ;  $p = 0.002$ ). To assess the strength of association between the pandemic factor and the mean AI hospitalization rate, the  $\phi$  coefficient and Pearson's contingency coefficient (C) were used. The calculated values indicated a moderate strength of association ( $\phi = 0.220$ ;  $C = 0.215$ ). The results are presented in Table 2.



**FIG. 2.**

Dynamics of hospitalizations for acute intestinal infections among children aged 0-17 years, 2014-2023



**FIG. 3.**

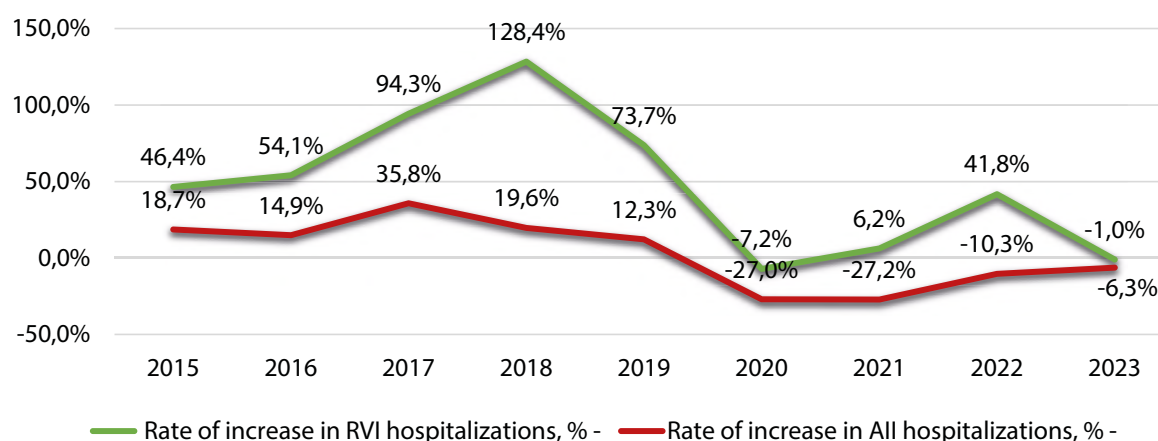
Etiological structure of acute intestinal infections (AI) in hospitalized children aged 0-17 years, 2014-2023

In 2020 compared to 2014, hospitalizations decreased by 7 %. Notably, 2014 was the lowest pre-pandemic value. In 2021 compared to 2020, an increase of 14.4 % was observed. The maximum increase during this period was in 2021 (26 cases), while the minimum (-157 cases) was recorded in 2020. On average, the rate of increase declined by 16.07 % annually during 2020–2021.

On average (Fig. 5), during the pandemic peak years, the percentage of hospitalizations for RVI

decreased 1.4-fold relative to the pre-pandemic period ( $\chi^2 = 23.955$ ;  $p < 0.001$ ). To assess the strength of association between the pandemic factor and the mean RVI hospitalization rate, the  $\phi$  coefficient and Pearson's contingency coefficient ( $C$ ) were used. According to Rea and Parker's recommendations, the strength of association was moderate ( $\phi = 0.346$ ;  $C = 0.327$ ).

The introduction of strict anti-epidemic measures (isolation, closure of schools and kindergartens, and mandatory mask wearing) led to a sharp decline



**FIG. 4.**

Rate of change in hospitalizations for acute intestinal infections and rotavirus infection among children aged 0–17 years, 2014–2023

**TABLE 1**

**HOSPITALIZATION DYNAMICS INDICATORS FOR CHILDREN WITH ACUTE INTESTINAL INFECTIONS, 2019-2021**

| Year | Number of All hospitalizations, abs. | Absolute increase |          | Growth rate |          | Rate of increase |          | Absolute value of 1% increase |
|------|--------------------------------------|-------------------|----------|-------------|----------|------------------|----------|-------------------------------|
|      |                                      | chain             | baseline | chain       | baseline | chain            | baseline |                               |
| 2019 | 1255                                 | -82               | 137      | 93.87       | 112.25   | -6.13            | 12.25    | 13.37                         |
| 2020 | 816                                  | -439              | -302     | 65.02       | 72.99    | -34.98           | -27.01   | 12.55                         |
| 2021 | 814                                  | -2                | -304     | 99.75       | 72.81    | -0.25            | -27.19   | 8.16                          |

**TABLE 2**

**HOSPITALIZATION DYNAMICS INDICATORS FOR CHILDREN WITH ROTAVIRUS INFECTION, 2019-2021**

| Year | Number of RVI hospitalizations, abs. | Absolute increase |          | Growth rate |          | Rate of increase |          | Absolute value of 1% increase |
|------|--------------------------------------|-------------------|----------|-------------|----------|------------------|----------|-------------------------------|
|      |                                      | chain             | baseline | chain       | baseline | chain            | baseline |                               |
| 2019 | 337                                  | -106              | 143      | 76.07       | 173.71   | -23.93           | 73.71    | 4.43                          |
| 2020 | 180                                  | -157              | -14      | 53.41       | 92.78    | -46.59           | -7.22    | 3.37                          |
| 2021 | 206                                  | 26                | 12       | 114.44      | 106.19   | 14.44            | 6.19     | 1.8                           |

in hospitalizations below the multi-year average for All by 29 % (Fig. 2) and for RVI by 35 % (Fig. 5).

During this period, the etiological structure of All showed no significant differences compared to the previous period: AIUE accounted for  $57.91 \% \pm 0.45$  ( $\chi^2 = 0.021$ ;  $p > 0.05$ ), viral diarrheas –  $37.36 \% \pm 0.67$  ( $\chi^2 = 0.197$ ;  $p > 0.05$ ), bacterial –  $4.72 \% \pm 1.12$  ( $\chi^2 = 0.355$ ;  $p > 0.05$ ), and rotavirus diarrhea among all All –  $23.68 \%$  ( $\chi^2 = 0.027$ ;  $p > 0.05$ ).

Subgroup analysis stratified by pandemic periods did not reveal statistically significant differences in the proportion of bacterial All or in the ratios of other etiological forms. During the pandemic peak period, a tendency toward a decrease in the proportion of bacterial All to 4.7 % was observed compared to 6.6 % in the pre-pandemic period. However, the differences did not reach statistical significance ( $\chi^2 = 0.355$ ;  $p = 0.552$ ), with a negligible strength of association ( $\phi = 0.042$ ). The prevalence of RVI in the structure of All also showed no statistically significant differences ( $\chi^2 = 0.559$ ;  $p = 0.212$ ), with a negligible strength of association ( $\phi = 0.027$ ).

**In the post-pandemic period**, the following changes were observed (Table 3).

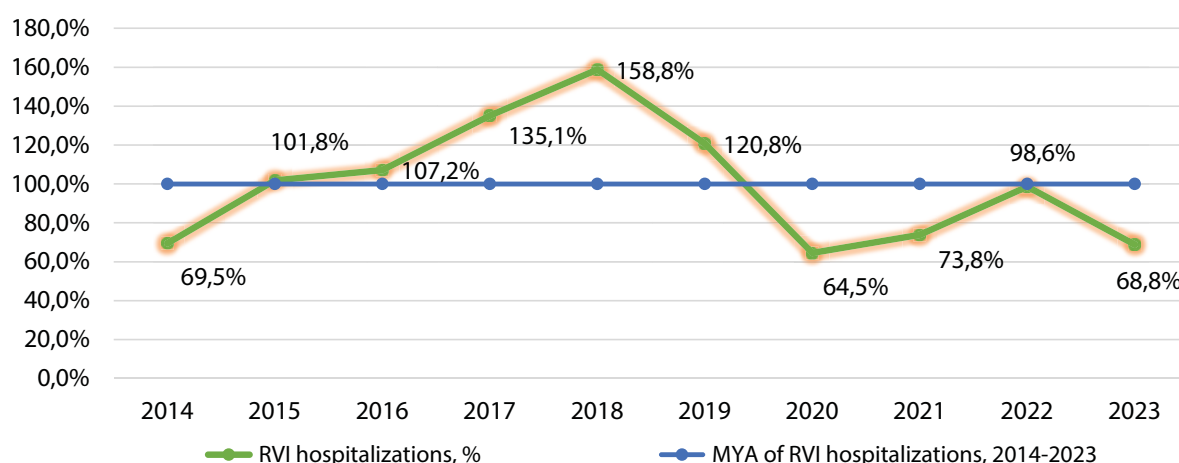
In 2022–2023, following the lifting of strict anti-epidemic restrictions, the number of reported All cases increased significantly worldwide.

Compared to 2021, the number of hospitalizations of children with All increased in 2022–2023, with the largest increase recorded in 2022. The mean annual number of hospitalizations increased by 117 cases, although the growth rate in 2023 compared to 2014 was low at 6.26 %. Similar changes were observed for rotavirus infection (Table 4).

The number of hospitalizations increased by 41.75 % in 2022 compared to 2014, and by 52.3 % compared to 2020.

On average, in the post-pandemic period, the period of lifting restrictive measures, the percentage of hospitalizations for RVI increased 1.3-fold relative to the pandemic period ( $\chi^2 = 10.125$ ;  $p = 0.002$ ). To assess the strength of association between the pandemic factor and the mean RVI hospitalization rate, the  $\phi$  coefficient was used. According to Rea and Parker's recommendations, the strength of association was moderate ( $\phi = 0.225$ ;  $C = 0.220$ ).

Correlation analysis revealed a strong direct correlation between the total number of All hospitalizations



**FIG. 5.**

*Dynamics of hospitalizations for rotavirus infection among children aged 0–17 years, 2014–2023*

**TABLE 3**

**HOSPITALIZATION DYNAMICS INDICATORS FOR CHILDREN AGED 0-17 WITH ACUTE INTESTINAL INFECTIONS, 2021-2023**

| Year | Number of All hospitalizations, abs. | Absolute increase |          | Growth rate |          | Rate of increase |          | Absolute value of 1% increase |
|------|--------------------------------------|-------------------|----------|-------------|----------|------------------|----------|-------------------------------|
|      |                                      | chain             | baseline | chain       | baseline | chain            | baseline |                               |
| 2021 | 814                                  | -2                | -304     | 99.75       | 72.81    | -0.25            | -27.19   | 8.16                          |
| 2022 | 1003                                 | 189               | -115     | 123.22      | 89.71    | 23.22            | -10.29   | 8.14                          |
| 2023 | 1048                                 | 45                | -70      | 104.49      | 93.74    | 4.49             | -6.26    | 10.03                         |

TABLE 4

HOSPITALIZATION DYNAMICS INDICATORS FOR CHILDREN AGED 0-17 WITH ROTAVIRUS INFECTION, 2021-2023

| Year | Number of RVI hospitalizations, abs. | Absolute increase |          | Growth rate |          | Rate of increase |          | Absolute value of 1% increase |
|------|--------------------------------------|-------------------|----------|-------------|----------|------------------|----------|-------------------------------|
|      |                                      | chain             | baseline | chain       | baseline | chain            | baseline |                               |
| 2021 | 206                                  | 26                | 12       | 114.44      | 106.19   | 14.44            | 6.19     | 1.8                           |
| 2022 | 275                                  | 69                | 81       | 133.5       | 141.75   | 33.5             | 41.75    | 2.06                          |
| 2023 | 192                                  | -83               | -2       | 69.82       | 98.97    | -30.18           | -1.03    | 2.75                          |

and the number of RVI hospitalizations ( $r = 0.789$ ;  $p = 0.008$ ).

This study demonstrates that the introduction of anti-epidemic measures during the COVID-19 pandemic led to a statistically significant reduction in hospitalizations of children with All and RVI in Chita. At the same time, the etiological structure of All remained stable across all three analyzed periods. In the post-pandemic period, a marked increase in hospitalizations was recorded, indicating restoration of the epidemic process. AIUE accounted for  $62.33 \% \pm 8.2$ , viral diarrheas –  $34.76 \% \pm 8.93$ , bacterial –  $2.91 \% \pm 0.74$ , and RVI among all All –  $22.87 \% \pm 6.43$ . No statistically significant differences in pathogen detection were found across the different periods ( $p < 0.05$ ).

Comparing the rates of confirmed All across different pandemic periods, it was noted that the level of laboratory diagnostics of diseases did not change over the 10-year period. According to the Clinical Infectious Diseases Hospital, no decline in the level of laboratory diagnostics was recorded, which is likely due to the fact that the priorities of laboratory diagnostics at this facility did not change during the pandemic. This fact excludes the influence of diagnostic shifts on the structure of detected infections. This consistent approach of the RCH allows the use of data from this facility for objective long-term monitoring of All pathogens. Intestinal infections of unspecified etiology were recorded at their lowest levels in 2018–2019, parallel to the maximum increase in viral infections. As viral diarrhea incidence decreased, the number of unspecified diarrheas increased, reaching 2015 levels by 2023, with viral diarrhea rates similarly declining to 2015 levels.

Among All cases of established etiology, as in many other regions of Russia, viruses predominate (rota-, noro-, and astroviruses), with rotaviruses being the leading cause [15].

## DISCUSSION

The data obtained on the sharp decline in hospitalizations for All and RVI in 2020–2021 are fully consistent with the results of studies conducted in other regions of Russia and worldwide [6, 16]. This supports

the hypothesis that non-pharmaceutical interventions (social distancing, mask wearing, and closure of schools and kindergartens) effectively limit the transmission not only of SARS-CoV-2, but also of other respiratory and enteric pathogens transmitted via the contact-household and fecal-oral routes.

A key finding of our study is the stability of the etiological structure of All. Despite a significant reduction in absolute numbers, the proportion of RVI among all All hospitalizations did not undergo statistically significant changes and retains its leading role. This suggests that the anti-epidemic measures had a universal, non-specific effect on the entire group of All, proportionally reducing the circulation of all pathogens rather than selectively suppressing rotavirus transmission. The strong positive correlation ( $r = 0.789$ ;  $p = 0.008$ ) between the dynamics of All and RVI hospitalizations further supports this conclusion, indicating their shared dependence on environmental factors and population behavior.

The subsequent increase in hospitalizations in the post-pandemic period, particularly pronounced in 2022, is consistent with the concept of epidemic rebound. The accumulation of a cohort of susceptible children who had no contact with the virus during the period of restrictions naturally led to a surge in incidence following the resumption of social activity, which has also been observed in other studies [8, 17].

**Study limitations.** This study has several limitations. First, its retrospective design and the use of aggregated data from official records do not allow for in-depth analysis of individual cases, such as disease severity, presence of comorbidities, or vaccination status. Second, the substantial proportion of cases classified as AIUE may have influenced the accuracy of the All structure assessment, although the stable level of laboratory diagnostics at the facility minimizes this risk. Third, the study was conducted at a single hospital in a specific region with unique climatic, geographical, and socio-demographic conditions, as well as extremely low rotavirus vaccination coverage, which limits the generalizability of the findings to populations with different characteristics. Finally, other unaccounted factors, such as changes in healthcare-seeking behavior, may have also influenced hospitalization dynamics.

The conducted study demonstrates that the introduction of anti-epidemic measures during the COVID-19 pandemic led to a statistically significant reduction in hospitalizations of children with All and RVI in Chita. At the same time, the etiological structure of All remained stable across all three analyzed periods. Following the lifting of restrictions, a marked increase in hospitalizations was recorded, indicating restoration of the epidemic process.

## CONCLUSION

Based on a decade-long monitoring study conducted at a large infectious disease hospital in the Trans-Baikal Territory, this research deepens our understanding of the regional consequences of the COVID-19 pandemic. It documents not only the marked but transient nature of the reduction in hospital burden associated with All and rotavirus infection during the period of restrictions, but also reveals a key feature: the persistent preservation of the established etiological structure, in which rotavirus gastroenteritis maintains its dominant position despite fluctuations in absolute indicators. In the context of persistently low rotavirus vaccination coverage, the data obtained convincingly demonstrate that long-term control of RVI in the region requires a combination of continuous epidemiological surveillance and the implementation of specific prophylaxis.

## Conflicts of interest

The authors declare no conflicts of interest.

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## HISTORY OF MEDICINE AND ANNIVERSARIES

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### ON THE 60<sup>TH</sup> ANNIVERSARY OF OLEG L. LAKHMAN'S BIRTH



On May 31, Oleg L. Lakhman, Professor of the Russian Academy of Sciences, Doctor of Medical Sciences, Professor, and Director of the East Siberian Institute of Medical and Environmental Research, celebrates his 60th birthday.

The life and professional journey of Oleg Leonidovich is an example of rare devotion to his work and to his home institute, where he came in 1991 after graduating from the Faculty of General Medicine of the Irkutsk State Medical Institute and completing his clinical residency in the specialty "Nervous Diseases". Oleg Leonidovich progressed through all stages of professional growth, from Junior Researcher to the position of Chief Physician of the clinic, where he worked for 22 years. In 2017, he was elected by the institute's staff to the position of Director.

In 1997, Oleg Leonidovich defended his Candidate of Sciences dissertation on "Clinical, electrophysiological, hormonal and immunological characteristics of vibration disease and its differentiated therapy", and in 2004 – his Doctoral dissertation on "Long-term consequences of occupational neurointoxication (mechanisms of development, clinical presentation, diagnosis, treatment)". In 2008, he was awarded the academic title of Professor, and in 2016 – the title of Professor of the Russian Academy of Sciences. He holds the highest qualification category in the specialty "Neurology" and is accredited in the specialties of neurology and occupational pathology.

Oleg L. Lakhman is a recognized scientist in the field of preventive medicine and occupational medicine in Russia. His scientific work is aimed at developing the theory of formation of the leading forms of occupational neurological pathology and addresses issues of their diagnosis, clinical presentation, and treatment. He has established an original scientific school of occupational pathologists in Siberia, focused on studying and substantiating the development of occupational diseases in workers exposed to chemical and physical occupational factors.

Oleg Leonidovich has developed a methodology for the etiological and differential diagnosis of long-term manifestations of occupational neurointoxications, substantiated the concept of the pathogenesis of toxic encephalopathy, and developed and implemented new medical technologies for the diagnosis of toxic encephalopathy in occupational neurotoxicoses. Lakhman O.L. was the first to substantiate the classification criteria for the severity of cognitive impairment in chronic mercury poisoning and developed neuropsychological criteria for the diagnosis of cognitive impairment in various occupational intoxications. With the participation of Oleg Leonidovich, the pathogenesis of a new form of occupational disease under chronic mercury exposure, ophthalmomercurialism, was studied and scientifically substantiated.

Under the leadership of Oleg Leonidovich, neurointoxications in firefighters have been studied for many years. As a result of the research, the clinical manifestations in the acute and long-term periods of intoxication with a complex of neurotropic toxic substances were systematized, and diagnostic criteria for occupational neurointoxication

in firefighters were established. This made it possible to establish occupational diseases in 102 firefighters who suffered during the suppression of a highest-category-complexity fire.

One of the scientific directions of O.L. Lakhman's activities is the identification of mechanisms underlying the development of occupational diseases of the peripheral nervous system under the influence of physical occupational factors. The peculiarities of changes in the sensory systems of the body under the influence of local and general vibration have been established. Under the guidance of Lakhman O.L., the modern clinical manifestations of vibration disease have been defined and its classification has been improved.

With his direct participation, original technologies for the diagnosis and treatment of polyneuropathy, vibration disease, fluorosis, and sensorineural hearing loss have been developed and implemented into practice, protected by patents. These developments make it possible to reduce the risk of occupational diseases and, when their initial manifestations are detected, to avoid the development of pathological processes and their progression with continued worker exposure to harmful occupational factors.

In recent years, the scope of O.L. Lakhman's scientific interests has included the study of the mechanisms of development of occupational stress caused by high neuropsychiatric loads and physical factors, and the substantiation of the possibility of establishing an occupational disease in the form of burnout syndrome in healthcare workers. With his direct participation, research is being conducted to identify post-traumatic stress disorder in participants of military conflicts and to implement therapeutic and rehabilitation measures.

Oleg L. Lakhman makes a significant contribution to the organization and improvement of occupational health services for the working population of the Irkutsk Region. He is the initiator, organizer, and executor of a number of regional targeted programs for environmental protection and public health preservation. The most important of these are "Medical and social measures to eliminate the consequences of the fire at Irkutskkabel OJSC" (2000–2002), "Improvement of working conditions for workers in the Irkutsk Region for 2003–2005, 2006–2010". Under his leadership, work was organized to provide medical support for the cleanup of accumulated environmental damage at the industrial site of Usolyekhimprom LLC in accordance with the Action Plan for the elimination of environmental pollution on the territory of Usolyekhimprom LLC (Order of the Government of the Russian Federation No. 3136-r dated November 27, 2020).

During O.L. Lakhman's tenure as Chief Physician of the clinic and Director of the institute, activities were organized within the compulsory health insurance system; new departments of radiology, physical and rehabilitation medicine, and an occupational pathology department were opened; the institute's material and technical base was significantly strengthened; and the modernization of medical and scientific activities of the institute's clinic was implemented.

Despite his enormous administrative workload, Oleg Leonidovich remains a practicing physician and mentor. Under his supervision, 14 Candidate of Sciences dissertations and 1 Doctoral dissertation have been defended. Currently, 2 Doctoral and 3 Candidate dissertations are being completed, and residents and postgraduate students are undergoing training. Oleg Leonidovich is a member of the Dissertation Councils D 001.058.01 at the East Siberian Institute of Medical and Environmental Research in the specialties "Occupational Medicine" (medical sciences) and "Hygiene" (medical sciences), and since January 2026, he has been its Chairman, as well as a member of D 999.184.02 at the Ural State Medical University and the Yekaterinburg Medical Research Center for Prophylaxis and Health Protection of Industrial Workers.

Since 2011, Oleg Leonidovich has been the Head of the Department of Occupational Pathology and Hygiene at the Irkutsk State Medical Academy of Postgraduate Education, a branch of the Russian Medical Academy of Continuous Professional Education, as well as the Head of the Occupational Pathology Course at the Educational Center of the East Siberian Institute of Medical and Environmental Research.

Oleg Leonidovich annually organizes and actively participates in seminars for physicians of the Irkutsk Region on current issues of occupational pathology.

Lakhman O.L. has 459 publications in leading journals indexed in Russian and international databases, including 12 monographs. With the participation of Oleg Leonidovich Lakhman, 33 regulatory and methodological documents have been developed, prepared, and approved at the federal and regional levels, including 8 new medical technologies, 7 teaching aids approved by the Educational and Methodological Association, and 41 patents for inventions have been obtained.

Oleg L. Lakhman is a member of the Editorial Boards of the journals "Medicine in Kuzbass", "Transbaikalian Medical Bulletin", and "Baikal Medical Journal".

Oleg L. Lakhman is actively involved in public service. He is an expert of the Russian Academy of Sciences, the Chief External Specialist in Occupational Pathology of the Ministry of Health of the Irkutsk Region, and a member of the Profile Commission on Occupational Pathology of the Ministry of Health of the Russian Federation. He is a member of the Central Attestation Commission for the Siberian Federal District and the Attestation Commission of the Ministry of Health of the Irkutsk Region, a member of the Interdepartmental Regional Commission on Occupational Safety, a member of the Presidium of the Russian Association of Physicians and Specialists in Occupational Medicine, the Medical Council under the Governor of the Irkutsk Region, the Coordination Council under the Government of the Irkutsk Region on the prevention of non-communicable diseases and the promotion of a healthy lifestyle among citizens in the Irkutsk Region, and the Environmental Council of the Irkutsk Region. During O.L. Lakhman's tenure as the Chief External Specialist in Occupational Pathology of the Ministry of Health of the Irkutsk Region, a clearly functioning occupational

pathology service was established in the region and its structure was proposed. Currently, all tasks facing specialists are being fulfilled, and extensive work is being carried out on the early detection of occupational diseases, their prevention, and treatment.

Oleg L. Lakhman has been awarded the badge of "Excellence in Healthcare", the Academician M.A. Lavrentiev Commemorative Medal for his achievements in science and effective leadership of the institute; he has been awarded the honorary titles of "Honorary Worker of Science and High Technologies of the Russian Federation" and "Honored Scientist of the Siberian Branch of the Russian Academy of Sciences" with the award of the "Golden Sigma" badge, and "Honored Veteran of the Siberian Branch of the Russian Academy of Sciences". For the development and implementation of various diagnostic and therapeutic-rehabilitation methods for occupational diseases in Eastern Siberia, Oleg Leonidovich has three times become a laureate of the regional competition in the field of science and technology. He has been awarded the Honorary Badge of the Russian Academy of Natural Sciences "For Services to the Development of Science and Economy of Russia" and the Prize "For Achievements in the Preservation and Promotion of Health of the Working Population of Russia" of the All-Russian Congress "Profession and Health", as well as awards from the Irkutsk Region and the city of Angarsk.

***Dear Oleg Leonidovich!***

***The staff of the East Siberian Institute of Medical and Environmental Research congratulates you on your 60th anniversary! Your jubilee marks a period of flourishing, when immense experience combines with inexhaustible energy for new accomplishments. We wish you robust health, scientific inspiration, and well-being. May every new project bring satisfaction, and may your scientific school continue to enhance the prestige of Russian science!***

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