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ТОМСКИЙ НАЦИОНАЛЬНЫЙ ИССЛЕДОВАТЕЛЬСКИЙ МЕДИЦИНСКИЙ ЦЕНТР РОССИЙСКОЙ АКАДЕМИИ НАУК

НИИ фармакологии и регенеративной медицины им. Е.Д. Гольдберга

ВСЕРОССИЙСКАЯ КОНФЕРЕНЦИЯ С МЕЖДУНАРОДНЫМ УЧАСТИЕМ «ПАТОФИЗИОЛОГИЯ: ПРОШЛОЕ, НАСТОЯЩЕЕ И БУДУЩЕЕ», ПОСВЯЩЕННАЯ 80-ЛЕТИЮ СО ДНЯ РОЖДЕНИЯ АКАДЕМИКА РАН ВЯЧЕСЛАВА ВИКТОРОВИЧА НОВИЦКОГО

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Глубокоуважаемые коллеги!

Сибирский государственный медицинский университет (СибГМУ) совместно с НИИ фармакологии и регенеративной медицины имени Е.Д. Гольдберга Томского НИМЦ (НИИФиРМ им. Е.Д. Гольдберга Томского НИМЦ) проводит Всероссийскую конференцию с международным участием «Патофизиология: прошлое, настоящее и будущее», посвященную 80-летию со дня рождения академика РАН Вячеслава Викторовича Новицкого – одного из ярчайших представителей отечественной медицинской науки, лидера томской научной школы специалистов по общей патологии и патофизиологии, почетного гражданина города Томска.

Конференция будет охватывать широкий спектр вопросов патологической физиологии, отражающих вклад учёного в развитие науки и медицины.

ОСНОВНЫЕ НАПРАВЛЕНИЯ:

1. Исторический экскурс в прошлое патофизиологии в Томске и роль академика РАН В.В. Новицкого в развитии томской научной школы патофизиологов;
2. Патофизиология в медицинской практике: тенденции и междисциплинарные подходы в изучении болезней;
3. Перспективы развития патофизиологии в условиях современных технологий: глобальные вызовы и пути их преодоления.

Данная конференция призвана объединить исследователей для обсуждения ключевых аспектов патофизиологии, её истории, текущего состояния и перспектив дальнейшего развития.

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СОДЕРЖАНИЕ

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Dear Authors and Readers,

On behalf of the editorial team, I am pleased to welcome you to the new issue of our scientific medical journal.

This year, we are committed to delivering the most relevant and valuable materials to enrich your scientific and practical knowledge, enhance your professional expertise, and broaden your professional horizons.

Each publication featured in this edition represents the culmination of rigorous research by leading specialists and a thorough analysis of current medical trends. We invite you to explore innovative diagnostic methods, effective approaches to disease prevention and treatment, and to engage in discussions on the most pressing issues facing the medical community today.

We continue to place special emphasis on promoting interdisciplinary research, which unites experts from various fields of medicine and related sciences. As experience shows, it is this integrative approach that fosters a holistic understanding of health and disease, enabling the development of comprehensive strategies for treatment and improving patients' quality of life.

In 2026, we will commemorate the 80th anniversary of the birth of Academician Vyacheslav Novitsky, an Honored Scientist of Russia, founder, and visionary leader of our journal. The memorial events will take place in September and will highlight his outstanding contribution to the development of pathophysiology – a fundamental discipline that unites diverse medical directions and approaches. The upcoming issues will feature recollections of Novitsky as a scholar, facilitator, and educator, as well as his significant achievements in science and education.

We extend our sincere gratitude to you – our authors – for your dedication and high professionalism, which ensure that our journal remains a vital source of cutting-edge scientific information.

We are confident that our collaborative efforts will make a meaningful contribution to the advancement of both national and global medicine.

We wish you inspiration, creative energy, and continued success in your noble profession!

Sincerely,
Olga Urazova
Editor-in-chief

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The Differentiation Status of Breast Cancer Cells, its Association with Metastasis and the Production of Cytokines by the Tumor in Various Molecular Subtypes

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ABSTRACT

Aim. To investigate the relationship between the differentiation status of breast cancer (BC) tumor cells (TC) of various molecular subtypes, the ability to metastasize, and the modulation of various cytokines in BC samples *in vitro*.

Materials and methods. Biopsies from invasive breast carcinoma of a non-specific type in 50 women, classified by the presence (Met+) and absence (Met–) of metastases in regional lymph nodes, were examined and categorized into 4 groups based on their molecular subtypes: luminal A (Lum A), luminal B (Lum B), HER2/neu positive (HER2+), and triple negative (TN). Using enzyme-linked immunosorbent assay, spontaneous and mitogen complex-stimulated (MC) production of 14 cytokines was determined in the supernatant of cultured BC samples (IL-2, IL-6, IL-8, IL-10, IL-17, IL-18, IL-1β, IL-1Ra, TNFα, IFNγ, G-CSF, GM-CSF, VEGF, and MCP-1), on the basis of which the index of the MC effect (IMCE) on cytokine production was calculated. The effect of stimulating the differentiation of TC was evaluated on the basis of cytomorphological criteria for reducing the relative content of low-grade cells (LGC) in BC samples.

Results. A statistically significant decrease in the relative content of LGC under the influence of MC was detected in the Met+ and Met– groups. MC had a differentiating effect in luminal and HER2-positive subtypes, while in TN breast cancer samples, there was only a downward trend in the proportion of LGC. To assess the role of cytokines in the mechanism of the differentiating effect of MC on LGC, a neural network analysis of IMCE in the relationship of various cytokines was carried out. According to the results obtained, in Lum A, the leading role in the manifestation of the differentiation effect was associated with IL-1Ra and IL-6; in Lum B – with MCP-1 and GM-CSF; in HER2+ – with IFN-γ and MCP-1, and in TN – with IL-1β and IL-6.

Conclusion. The data obtained indicate that the immune mechanisms mediating the differentiating MC effect on BC may vary in different molecular subtypes of BC.

Keywords: breast cancer, cytokines, molecular subtypes, cellular differentiation

Conflict of interest. The authors declare the absence of obvious and potential conflicts of interest related to the publication of this article.

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Дифференцировочный статус клеток рака молочной железы, его сопряженность с метастазированием и с продукцией опухолью цитокинов при различных молекулярных подтипах

Архипов С.А.[1,2], Михайлова Е.С.[1,2], Архипова В.В.[1], Дружинина Ю.Г.[3], Яковлева К.И.[3], Аутеншлюс А.И.[1,2]

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РЕЗЮМЕ

Цель. Исследовать взаимосвязь между дифференцировочным статусом опухолевых клеток (далее КО) рака молочной железы (РМЖ) различных молекулярных подтипов, способностью к метастазированию и модуляции в образцах РМЖ продукции различных цитокинов *in vitro.*

Материалы и методы. Исследовали биоптаты РМЖ (инвазивной карциномы молочной железы неспецифического типа) 50 женщин при наличии (Мет+) и отсутствии (Мет-) метастазов в регионарных лимфатических узлах, разделенных на четыре группы по молекулярным подтипам РМЖ: люминальный А (Люм А), люминальный В (Люм В), HER2/neu позитивный (HER2+) и тройной негативный (ТН). С помощью иммуноферментного анализа в супернатанте культивируемых образцов РМЖ определяли спонтанную и стимулированную комплексом митогенов (КМ) продукцию 14 цитокинов (интерлейкин (IL) 2, IL-6, IL-8, IL-10, IL-17, IL-18, IL-1β, IL-1Ra, фактор некроза опухоли альфа, интерферон-гамма (IFNγ), гранулоцитарный колониестимулирующий фактор, гранулоцитарно-макрофагальный колониестимулирующий фактор (GM-CSF), фактор роста эндотелия сосудов и моноцитарный хемоаттрактантный белок-1 (MCP-1)), на основе которой рассчитывали индекс влияния КМ на продукцию цитокинов (ИВКМ). Эффект стимуляции дифференцировки КО *in vitro* оценивали на основе цитоморфологических критериев по снижению относительного содержания низкодифференцированных клеток (НДКО) в образцах РМЖ.

Результаты. Статистически значимое снижение НДКО под воздействием КМ выявлено в группах Мет+ и Мет-. Комплекс митогенов оказывал дифференцировочный эффект при люминальных и HER2-позитивном подтипах, в образцах ТН наблюдалась тенденция к снижению НДКО. Для оценки роли цитокинов в механизме дифференцирующего действия КМ проведен нейросетевой анализ ИВКМ в отношении различных цитокинов. Согласно полученным результатам, при Люм А ведущая роль в проявлении дифференцировочного эффекта была связана с IL-1Ra и IL-6, при Люм В – с MCP-1 и GM-CSF, при HER2+ – с IFNγ и MCP-1, а при ТН – с IL-1β и IL-6.

Заключение. Полученные данные свидетельствуют о том, что иммунные механизмы, опосредующие дифференцировочное воздействие КМ на КО, РМЖ могут отличаться при различных молекулярных подтипах РМЖ.

Ключевые слова: рак молочной железы, цитокины, молекулярные подтипы, клеточная дифференцировка

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источники финансирования. Финансирование тем государственных заданий: № госрегистрации 115060410035, 125031203556-7.

Соответствие принципам этики. Все пациенты подписали информированное согласие на участие в исследовании. Исследование одобрено этическим комитетом НИИМББ ФИЦ ФТМ (протокол № 28 от 27.09.2023).

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INTRODUCTION

Despite significant advances in developing new treatments for breast cancer (BC), including targeted therapy and immunotherapy, the problem of resistance of breast cancer tumor cells (TC) to anticancer therapy continues to be a pressing concern [1]. Traditional approaches to BC treatment, such as chemotherapy and radiation therapy, target rapidly dividing cells, but they are not always effective against TC that are dormant at the time of treatment but retain the potential for tumor progression. In this regard, alternative strategies aimed at changing the biological properties of TC rather than just eliminating them are gaining increased interest. One such strategy is differentiation therapy [2, 3].

Differentiation therapy is an approach aimed at stimulating the differentiation of TCs, leading to a reduction in their proliferative activity and malignant potential. This approach is based on the idea that TCs retain the capacity for differentiation, and that targeting specific signaling pathways can restore a normal cell phenotype [3]. Differentiation therapy is already used in the treatment of certain types of cancer, such as acute promyelocytic leukemia, where the use of retinoic acid leads to the differentiation of blast cells and disease remission [4]. However, the application of differentiation therapy to solid tumors, such as BC, presents a more complex challenge. BC is a heterogeneous disease that is divided into different molecular subtypes, characterized by different prognoses and sensitivity to therapy [5, 6]. These subtypes differ in the expression of estrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor receptor 2 (HER2), as well as in the levels of expression of the proliferation marker Ki-67. Each BC subtype has a unique gene expression profile, and, therefore, its response to differentiation therapy may vary [7]. One of the factors regulating the differentiation of BC cells is the tumor cell microenvironment producing cytokines [8]. Cytokines can affect directly the differentiation of TC, both stimulating and inhibiting this process. For example, interleukin-6 (IL-6) can promote TC proliferation and BC metastasis, while interferon gamma (IFN-γ) can have an anti-tumor effect, inducing differentiation or apoptosis of BC TC [9, 10].

We have previously obtained data indicating that a mitogen complex (MC) consisting of lectins (phytohemagglutinin M, phytohemagglutinin P, concanavalin A) and lipopolysaccharide (LPS) has the ability to reduce the content of low-grade tumor cells (LGC) and, accordingly, increase the content of more differentiated forms of TCs in samples of invasive breast carcinoma *in vitro* [11]. In the present study, we investigated the effect of MC on the differentiation status of TC in BC samples of various molecular subtypes, as well as on the production of various cytokines by BC biopsy specimens. It is known that counting BC TC with varying grades of differentiation is critical for both diagnosis and prognosis of malignant neoplasms [12]. LGC contribute to a higher level of malignancy and aggressiveness of BC compared to more differentiated cells [13]. Clinical studies show that the presence of LGC in the tumor correlates with a poor prognosis. Their level is associated with a lower survival rate of patients and an increased risk of recurrence [14]. Therefore, when analyzing the results obtained, we primarily focused on assessing changes in the relative content of LGC, the counting accuracy of which due to their morphological characteristics is higher compared to differentiated TC.

The aim of the study was to investigate the relationship between the differentiation status of TC of different molecular subtypes of BC, the BC ability to metastasize, and the modulation of the production of various cytokines in BC samples *in vitro*.

MATERIALS AND METHODS

The study material comprised biopsy samples of BC (grade 2-3 invasive breast carcinoma of non-specific type) collected from 50 women aged 35–76 years with (Met+) or without (Met–) metastases to regional lymph nodes, as well as supernatants obtained during *in vitro* culturing of biopsy samples in a medium without MC

and in a medium containing MC, who were treated at Novosibirsk Regional Oncology Dispensary. The Ethics Committee of the Research Institute of Molecular Biology and Biophysics of the Federal Research Center for Fundamental and Translational Medicine approved this study (Minutes No. 28 dated September 27, 2023). The inclusion criterion for the study was newly diagnosed BC. Exclusion criteria were the presence of distant metastases, exacerbation of chronic diseases, and neoadjuvant therapy. Patients were divided into 4 groups depending on a particular molecular subtype of the tumor: Luminal A (Lum A), Luminal B (Lum B), HER2/neu positive (HER2+), and triple negative (TN).

To stimulate the differentiation of BC TC and modulate cytokine production *in vitro*, we used a composition of four MCs, specifically: phytohemagglutinin M at a concentration of 2.0 μg/ml, phytohemagglutinin P at a concentration of 2.0 μg/ml, concanavalin A at a concentration of 4.0 μg/ml, and lipopolysaccharide (LPS) at a concentration of 2.0 μg/ml [11]. Tumor biopsy samples obtained by core needle biopsy with an 8 mm^3 volume were placed into two glass vials. One vial contained only DMEM-F12 nutrient medium, and the other contained MC in the same volume of the medium. All samples were then cultured for 72 hours at 37 ^{0}C. Next, the biopsy samples were removed from the culture medium and fixed in a neutral formalin solution for further morphological examination, and cytokine concentrations in the obtained supernatants were determined using solid-phase enzyme-linked immunosorbent assay: IL-2, IL-6, IL-8, IL-10, IL-17, IL-18, IL-1b, IL-1Ra, TNFα, IFNγ, G-CSF, GM-CSF, VEGF, and MCP-1. The index of the MC effect on cytokine production by tumor samples was calculated using the formula: IMCE = A / B, where A is the concentration of the cytokine in the supernatant of the BC biopsy sample after stimulation of the tumor with CM (CM+), B is the concentration of the cytokine in the supernatant of the BC biopsy sample without MC stimulation (MC–). For morphological examination, BC biopsy samples fixed in formalin were dehydrated and embedded in paraffin, followed by preparation of sections with thickness of 4–5 μm. Deparaffinization and rehydration of BC samples were carried out according to the standard method using a xylene – ethanol sequence. The obtained sections were stained with hematoxylin and eosin according to the standard method. The study was conducted using light microscopy at ×400 magnification. The grade of differentiation of TC in BC samples was evaluated according to the cytomorphological criteria described in our study [11], taking into account the Nottingham histologic grade [15]. The relative content (%) of low-grade TC (LGC), as well as intermediate-grade (IGC) and high-gradeC (HGC), was counted. The presence of the stimulating effect of BC TC differentiation when exposed to MC was determined by a decrease in the relative content of LGC in BC samples and expressed as an index of the MC effect on the differentiation of (IEMC LGC) equal to the ratio of the amount of LGC in BC samples without MC exposure to the amount of LGC after MC exposure [11].

The level of statistical significance of the differences between the groups was determined using the non-parametric paired Wilcoxon test. Indicators were expressed as the median (*Me*), lower and upper quartiles (Q_1; Q_3). Calculations of medians and lower and upper quartiles were performed using the Statistica v. 7.0 software package. Neural network analysis was performed using the IBM SPSS Statistics v. 22.0 software package. The study used a neural network model (NNM) generated based on the Multilayer Perceptron module. The input layer included 15 neurons, one hidden – three neurons, and the output – one neuron. The activation function of the hidden layer is "hyperbolic tangent", the activation function of the output layer is "sigmoid". The use of a "hyperbolic tangent" in the hidden layer allowed the NNM to learn on non-linear dependencies between input and output data. The activation function of the output layer "sigmoid" allowed to interpret the output value as the probability of belonging to the class "differentiation stimulation" (value 0 or 1). The choice of the Multilayer Perceptron"architecture (one hidden layer with three neurons) was due to the relatively small amount of data, in order to reduce the probability of overfitting the NNM. The input parameters for the perceptron were the values of the IMCE. The target variable in the NNM was the presence or absence of the effect of TC differentiation stimulation in the corresponding BC samples (values 0 and 1). Since the relationship between cytokines and the effect of TC differentiation could vary for different subtypes of BC, a separate NNM was formed for each subtype. The data of each group of patients were divided into training and test samples in an approximate ratio of 80/20 (%) or 70/30 (%). The division was performed randomly, but with the preservation of the proportions of classes (0 and 1) in both samples (stratified division). The neural network was trained on the training sample, and the accuracy of the NNM was evaluated on the test sample. For Lum A, the data were randomly divided into training and test samples in a ratio of 14/4. For

Lum B, HER2+, and TN subtypes, the data were randomly divided into training and test samples in the ratios 9/3, 5/2, and 9/3, respectively. The batch training method and the gradient descent algorithm were used. NNM training was performed under similar training parameters for different BC subtypes: standardization of input predictors using normalization: (x – min) / (max – min)); initial learning rate was 0.4; moment – 0.9; center of the interval – 0; interval bias – ±0.5. The accuracy of the neural network model (ANNM) for determining class 1 – "presence of differentiation effect" (Positive Predictive Value) was calculated using the formula: ANNM = (TP / (TP + FP)), where TP (True Positive) is the number of samples with a "differentiation effect" that are correctly classified by the model as having a "differentiation effect", FP (False Positive) – the number of samples without a "differentiation effect" that are erroneously classified by the model as having a "differentiation effect". The ANNM, obtained on the training and test sets was the following: for NNM LumA – 92.31% and 100%, for NNM LumB – 87.5% and 100%, for HER2+ – 80% and 100%, for NNM TN – 75% and 66.7%, respectively. After training the NNM, the "normalized importance" (NI) of each input predictor was calculated, which is an estimate of the relative strength of the influence of the input variable on predicting the target variable – the presence or absence of the effect of stimulation of TC differentiation in BC samples after MC exposure.

RESULTS

Table 1 presents data demonstrating the effect of MC on the relative content of LGC, IGC, and HGC in BC samples from patients with (Met+) and without (Met–) metastases to regional lymph nodes, as well as in patients not separated by the presence or absence of lymphogenous metastasis.

In all subgroups of patients (with or without metastases), a statistically significant decrease in the

Table 1

Effect of MC on the Relative Content of LGC, IGC, and HGC in BC Samples, *Me* (Q_1; Q_3), %

BC Patients	Samples	Relative content of LGC, %	Relative content of HGC+IGC, %
Without separation for metastasis	MC – (n = 50)	22.32 (16.67; 27.06)	77.68 (72.94; 83.33)
	MC+ (n = 50)	16.36*** (12.07; 21.55)	83.64*** (78.45; 87.93)
Without metastasis (Met–)	MC – (n = 29)	18.57 (15.00; 24.59)	81.43 (75.41; 85.00)
	MC + (n = 29)	14.89*** (12.07; 18.22)	85.11*** (81.78; 87.93)
With metastasis (Met+)	MC – (n = 21)	23.45 (21.11; 30.38)	76.55 (69.62; 78.89)
	CM+ (n = 21)	17.64*** (15.12; 23.61)	82.36*** (76.39; 84.88)

Differences are statistically significant when comparing MC+ samples with MC– samples. **$p < 0.0001$ (paired Wilcoxon test)

relative content of LGC was observed in BC samples in response to MC exposure (MC+), which indicates that MC can affect the differentiation of BC TC, shifting them towards more mature forms. Analysis of the data obtained, depending on the metastatic status (Met– and Met+), showed that the effect of MC on TC differentiation was maintained regardless of the presence or absence of metastases in the lymph nodes. The differences between the MC+ and MC– sample groups were statistically significant for both patients with metastases (Met+) and patients without metastases (Met–). Comparing the medians (Me) for each sample group, one can estimate the degree of a decrease in LGC content under the influence of MC. In the overall patient cohort, without segregating bu the presence or absence of metastases, the median decreased from 22.32% (MC–) to 16.36% (MC+). In the group of patients without metastases (Met–), the median relative content of LGC decreased from 18.57% (MC–) to 14.89% (MC+), and in the group of patients with metastases (Met+) – from 23.45% (MC–) to 17.64% (MC+).

Table 2 presents the results of studying the MC effect on the relative content of cells with varying grades of differentiation in BC samples of different molecular subtypes: Lum A, Lum B, HER2+, and TN. It was established that in Lum A samples, a statistically significant decrease in the relative content of LGC was observed in MC+ samples compared to MC– samples (p = 0.00159). This suggests that MC may promote TC differentiation in this BC subtype. Similarly, in the Lum B subtype, a statistically significant decrease in LGC content was also observed in MC+ samples (p = 0.00963). The median decreased from 22.18% to 17.26%, which also indicates the ability of MC to induce differentiation in Lum B cells. In the HER2+ subtype, a statistically significant decrease in the

Table 2

Effect of MC on the Relative Content of LGC, HGC and MGC in BC Samples of Different Molecular Subtypes

Molecular BC Subtype	Samples	Relative content of LGC, %	Relative content of HGC+IGC, %
Lum A	MC– (n = 18)	18.52 (15.61; 25.32)	81.48 (74.62; 84.39)
	MC+ (n = 18)	15.77** (11.01; 20.08)	84.23** (79.92; 88.99)
Lum B	MC– (n = 12)	22.18 (18.37; 27.06)	77.82 (72.94; 81.63)
	MC+ (n = 12)	17.26** (13.19; 22.5)	82.74** (77.5; 86.81)
HER2+	CM– (n = 7)	17.38 (12.71; 24.57)	82.62 (75.43; 87.30)
	CM+ (n = 7)	15.67* (9.68; 24.55)	84.33* (75.45; 90.32)
TN	CM– (n = 12)	23.26 (21.87; 29.98)	76.74 (70.03; 77.69)
	CM+ (n = 12)	15.79 (14.23; 18.76)	84.21 (77.84; 85.48)

Differences are statistically significant when comparing MC+ samples with MC– samples. *p < 0.05; *p < 0.01 (paired Wilcoxon test)

relative content of LGC was also observed in MC+ samples compared to MC– samples (p = 0.03125). In the TN group, although there is a trend towards a decrease in the content of LGC in MC+ samples compared to MC– samples, this decrease did not reach statistical significance (p = 0.05974).

In order to assess the likely role of cytokines in stimulating BC cell differentiation under the influence of MC, a study was conducted on the effect of MC on cytokine production by BC samples when they were cultured in a medium containing MC. The indices of mitogen complex effect (IMCE) were calculated equal to the ratio of the concentration of the studied cytokines in the supernatants with MC to the concentrations of cytokines in the supernatants of BC samples cultured in a medium without MC. At the same time, the LGC effect index was calculated, equal to the ratio of the number of LGC in samples cultured in a medium without MC to the number of LGC in samples cultured in a medium containing MC. On the basis of neural network analysis methods, NNMs were formed and trained separately for each BC subtype, on the basis of which the NI of each input predictor of the independent variable – the index of the MC effect on the production of a cytokine – was calculated in predicting the target dependent variable, to which only two values were assigned – the presence of differentiation stimulation (1) or its absence (0). When the LGC IMCE value exceeded 1.1, the presence of a differentiation effect was noted, and when the value was less than or e ual to 1.1 – its absence. The validity of this choice of assessment was justified by the fact that, in the group comparison of the relative content of LGC in BC samples after culturing in a medium with MC and without MC, provided that their ratio exceeds 1.1, the differences (evaluated by the paired Wilcoxon test) were significant (Table 3).

Table 3

Results of Neural Network Analysis of the Role of IMCE of Various Cytokines in the Development of the Differentiation Effect of LGC BC under the Action of MC in BC of Various Molecular Subtypes

EICM	Molecular BC Subtype			
	Lum A	Lum B	Her2+	TN
	Normalized importance (NI), %			
IL-2	30.0	48.7	44.0	47.4
IL-4	44.3	18.7	36.5	56.7
IL-6	87.3	14.1	67.9	81.9
IL-8	74.3	70.9	52.3	32.5
IL-10	34.2	45.0	45.6	41.8
IL-17	51.0	32.1	91.9	67.6
IL-18	36.3	68.7	43.2	45.8
IL-1β	28.0	13.2	84.0	100.0
IL1-Ra	100.0	28.7	54.8	41.0
TNFα	69.7	17.7	21.5	47.0
IFNγ	80.9	12.2	100.0	63.9
G-CSF	40.8	30.9	58.2	45.7
GM-CSF	29.1	76.5	73.2	44.9
VEGF	69.6	21.9	42.4	42.7
MCP-1	57.8	100.0	96.4	35.7

The results of the neural network analysis of the association of IMCE with the production of various cytokines by BC samples and the LGC IMCE, depending on the molecular genetic subtype of BC, are presented in Table 3. It is shown that in Lum A BC, the IMCE IL-1Ra had a maximum NI value (100%), which indicates the leading role of IL-1Ra in the manifestation of the effect of differentiation of TC under the action of MC in tumor samples of this subtype. At the same time, high NI values of the IMCE were established for a number of other cytokines: IFNγ (NI = 80.9%), IL-6

(NI = 87.3%), TNFα (NI = 69.7%), and VEGF (NI = 69.6%). In Lum B, the largest value of NI was shown by MCP-1 (100%). A relatively high level of NI was also identified for cytokines: GM-CSF (NI = 76.5%), IL-8 (NI = 70.9%), and IL-18 (NI = 68.7%).

For HER2+ BC, other combinations of cytokines with large NI values were identified: IFNγ (NI = 100%), MCP-1 (NI = 96.4%), IL-17 (NI = 91.9%), and IL-1β (NI = 84.0%). In TN BC, the highest NI was shown by IL-1β (NI = 100%). At the same time, highly significant cytokines in the development of the effect of TC differentiation under the MC effect were IL-6 (NI = 81.9%), IL-17 (NI = 67.6%), and IFNγ (NI = 63.9%). Thus, the neural network analysis of the importance of the IMCE values of various cytokines in the development of the effect of BC LGC differentiation exposed to MC showed significant differences between the molecular BC subtypes.

DISCUSSION

Currently, the prospects of differentiation therapy as an effective treatment method and its application remain limited due to a lack of understanding of the possible pathways of TC differentiation in solid malignant neoplasms, including a lack of sufficient information about the immunological mechanisms that can regulate these processes [1–3]. This study was aimed at investigating the likely immune mechanisms of the MC effect on the differentiation of BC cells, and in particular, on the formation of the cytokine network underlying this process.

The MC effect on the differentiation of BC cells turned out to depend on the molecular subtype of the tumor. The most pronounced effect was observed in Lum A and Lum B, where MC caused a statistically significant decrease in the relative content of LGC. Similar trends were observed in the HER2-positive subtype, although the effect was less pronounced. It is noteworthy that in (TN BC), only a trend towards a decrease in LGC was observed, which did not reach statistical significance. These data are consistent with the understanding of the molecular heterogeneity of BC and differences in the mechanisms of regulation of differentiation in various BC subtypes. Presumably, MC affects specific signaling pathways that play a more important role in the differentiation of luminal and HER2-positive tumors than in TN BC. This may also be due to the heterogeneity of TN BC and its known resistance to various types of therapy [16].

The results of the neural network analysis showed significant differences in the NI of various cytokines depending on the molecular subtype of BC. Differences in the NI of cytokines between BC subtypes reflect the diversity of the tumor's immune microenvironment. Thus, for Lum A BC, IL-1Ra, IFNγ, IL-6, TNFα, and VEGF played a key role in the effect of stimulating the differentiation of BC cells. The role of IL-1Ra may be related to its ability to block the pro-inflammatory effect of IL-1β, thereby shifting the regulatory balance towards the differentiation of Lum A TC [17, 18]. Presumably, IFNγ can directly induce the expression of genes involved in the differentiation of BC cells [19], and IL-6 and TNFα can have both pro-inflammatory and differentiation effects depending on thebaseline level of their production [9, 20]. VEGF, as an angiogenesis factor, can affect the vascular microenvironment of the tumor and, indirectly, the differentiation of BC TC.

In BC Lum B, MCP-1, a chemokine involved in the recruitment and activation of macrophage cells [21], had the highest NI of IMCE. GM-CSF, IL-8, and IL-18 were also significant. As is known, GM-CSF stimulates the differentiation of myeloid cells [6], while IL-8 and IL-18 are involved in the regulation of inflammation and immune response [22, 23]. In HER2+, IFNγ had the highest IMCE NI, which may be due to its ability to activate a complex anti-tumor immune response and induce differentiation. At the same time, MCP-1, IL-17, and IL-1β may also play an important role in this process [24]. It should be noted, however, that due to the small sample of the HER2+ group, the results obtained for this HER2+ group by the neural network analysis method should be considered as preliminary and requiring confirmation with a larger sample.

In TN BC, the highest NI values were identified for the IMCE of IL-1β, IL-6, IL-17, and IFNγ, which may reflect the peculiarities of the immune microenvironment of this subtype. The fact that the IL-1β IMCE was of great importance for stimulating differentiation in triple-negative BC suggests that targeting the IL-1β signaling pathway may be a promising therapeutic approach for this particularly aggressive BC subtype.

The NI in neural network analysis is a tool for assessing the relative strength of the influence of variables on the expected effect, and not their share in the overall effect. Therefore, there is no universal, rigidly defined boundary for dividing predictors into "significant" and "insignificant" based on NI. Given that in our study, NI assesses the prognostic strength of each individual predictor (IMCE) in the

development of the TC differentiation effect, it is only possible to conditionally identify (for example, with NI > 65%) the most significant cytokines that affect the development of this effect in various BC subtypes: in Lum A – IL1-Ra, IL-6, IFNγ, IL-8, TNFα, and VEGF; in Lum B – MCP-1, GM-CSF, and IL-8; in HER2+ – IFNγ, MCP-1, IL-17, and IL-1β; in the TN subtype – IL-1β and IL-6.

Thus, the differences in the relationships we identified between the IMCE and the development of the differentiation effect under the action of MC may reflect complex interactions between various cells of the immune system that form the tumor microenvironment and the intratumoral cytokine network, which controls the processes that regulate the differentiation of BC cells. Some cytokines may directly affect differentiation, while others may modulate this process indirectly, through interaction with other components of the tumor microenvironment.

CONCLUSION

The results obtained are important for understanding the mechanisms underlying the effect of MC on the differentiation of BC cells. Identifying the key cytokines involved in this process can serve as the basis for developing new therapeutic strategies aimed at inducing the differentiation of tumor cells and increasing the effectiveness of BC treatment. The study results emphasize the importance of a personalized approach to the treatment of BC within the framework of differentiation therapy, taking into account the molecular subtype of the tumor.

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Features of the Proinflammatory Adipose Tissue Phenotype in Experimental Metabolic Syndrome

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ABSTRACT

Aim. To investigate the proinflammatory activity markers of adipose tissue in a rat model of metabolic syndrome (MetS).

Materials and methods. The experiments were performed on male Wistar rats fed with either a standard diet or a high-fat, high-carbohydrate diet (HFHCD). Physiological and biochemical parameters were assessed in the respective animal groups. The concentrations of adipokines (leptin and adiponectin) and cytokines: tumor necrosis factor alpha (TNFα), interleukins (IL-6 and IL-10) and monocyte chemoattractant protein-1 (MCP-1) secreted by adipose tissue cells were determined using enzyme-linked immunosorbent assay. The phenotypic profile of adipose tissue cells expressing the CD68 marker was examined by immunohistochemistry, and the level of *CD68* gene expression was additionally assessed using real-time polymerase chain reaction. A histological examination of visceral adipose tissue fragments was performed.

Results. It was found that in rats fed with a HFHCD, there was a significant correlation ($p < 0.05$) between an increase in adipose tissue mass and elevated serum levels of leptin ($r = 0.57$), glucose ($r = 0.62$), and insulin ($r = 0.61$). In the experimental group, the concentrations of proinflammatory cytokines (IL-6, TNFα, and MCP-1), and leptin produced by adipose tissue cells were higher than in the control group ($p < 0.05$). Additionally, we demonstrated that rats fed fed with a HFHCD exhibited a significant ($p < 0.05$) decrease in the secretion of the anti-inflammatory cytokine IL-10 and adiponectin. Histological examination of the experimental group revealed adipocyte hypertrophy and anisocytosis, venous vessel congestion, and thickening of the interlobular connective tissue layer. Furthermore, rats receiving a HFHCD exhibited an increased number of CD68-positive cells in visceral fat samples and elevated *CD68* gene expression.

Conclusion. Thus, in experimental diet-induced metabolic syndrome, adipose tissue acquires a proinflammatory phenotype due to adipocyte hypertrophy, infiltration by immune-competent cells (CD68+ macrophages), and increased secretion of proinflammatory chemo- and adipokines (TNFα, IL-6, MCP-1, and leptin).

Keywords: metabolic syndrome, obesity, inflammation, cytokines, macrophages

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Особенности провоспалительного фенотипа жировой ткани при экспериментальном метаболическом синдроме

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РЕЗЮМЕ

Цель. Изучение показателей провоспалительной активности жировой ткани в экспериментальной модели метаболического синдрома (МС) у крыс.

Материалы и методы. Эксперименты выполняли на самцах крыс линии Вистар, которых содержали на стандартном корме или высокожировой и высокоуглеводной диете (ВЖВУД). У животных из опытной и контрольной групп оценивали физиологические и биохимические показатели; определяли концентрацию секретируемых клетками жировой ткани адипоцитарных гормонов (лептина, адипонектина) и цитокинов: фактора некроза опухоли альфа (TNF-α), интерлейкинов (IL) (IL-6, IL-10), моноцитарного хемотаксического фактора-1 (MCP-1) методом иммуноферментного анализа; исследовали фенотипический профиль клеток жировой ткани по экспрессии маркера CD68 методом иммуногистохимии и уровень экспрессии гена *CD68* методом полимеразной цепной реакции. Для анализа и оценки выраженности морфологических изменений проводили гистологическое исследование фрагментов висцеральной жировой ткани.

Результаты. Установлено, что у крыс, получавших ВЖВУД, наблюдалась статистически значимая ($p < 0,05$) корреляция между увеличением массы жировой ткани и повышением концентрации лептина ($r = 0,57$), глюкозы ($r = 0,62$), инсулина ($r = 0,61$) в сыворотке крови. У животных опытной группы концентрация продуцируемых клетками жировой ткани провоспалительных цитокинов IL-6, TNF-α, MCP-1 и лептина превышала таковую в контроле ($p < 0,05$). Наряду с этим у крыс на фоне ВЖВУД было зарегистрировано статистически значимое ($p < 0,05$) снижение секреции противовоспалительного IL-10 и адипонектина. При гистологическом исследовании у животных опытной группы были выявлены гипертрофия и анизоцитоз адипоцитов, полнокровие венозных сосудов, утолщение прослойки междольковой соединительной ткани. Также у крыс, получавших ВЖВУД, наблюдалось увеличение числа CD68-позитивных клеток в образцах висцерального жира и повышение экспрессии гена *CD68*.

Заключение. При экспериментальном диет-индуцированном МС жировая ткань приобретает провоспалительную активность за счет гипертрофии адипоцитов, инфильтрации иммунокомпетентными клетками (CD68+-макрофагами) и повышенной секреции провоспалительных хемо- и адипокинов (TNF-α, IL-6, MCP-1, лептина).

Ключевые слова: метаболический синдром, ожирение, воспаление, цитокины, макрофаги

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INTRODUCTION

To date, the problem of metabolic syndrome (MetS) and obesity has become a global medical and social issue and remains relevant due to the rapid increase in the prevalence of these pathological conditions worldwide. Diet-induced obesity is the most common form of obesity and is of particular interest from a pathogenetic viewpoint, as excessive calorie intake leads not only to lipid accumulation and disruption of all types of metabolism but also to the activation of inflammatory reactions in adipocytes and cells of the stromal – vascular fraction of adipose tissue [1, 2].

The steady increase in the number of overweight and obese people requires close attention from the scientific community. Despite significant progress in studying the clinical and laboratory manifestations of metabolic disorders, some aspects of the pathogenesis of MetS and obesity require further research. In particular, the molecular genetic mechanisms underlying adipose tissue dysfunction and mediating its role in the initiation of systemic metabolic disorders and chronic inflammation are still not fully understood [3, 4]. A priority remains research on the molecular basis of intercellular communication in adipose tissue, including key signaling pathways involving adipokines, cytokines, and extracellular vesicles, along with the epigenetic mechanisms that regulate these processes [5, 6].

Since pathological remodeling of adipose tissue, characterized by both structural and functional impairments, is a key pathogenetic factor in obesity and its associated metabolic disorders and systemic complications, it is crucial to study the mechanisms underlying the dysfunction of adipose tissue cellular components to understand their role in the pathogenesis of meta-inflammation in obesity.

For this purpose, experimental animal models serve as an indispensable tool, as they allow not only for the recreation of the pathophysiological features of MetS and obesity under controlled conditions but also for a detailed investigation of the relationship between the metabolic and inflammatory processes developing in visceral adipose tissue [7, 8].

The aim of the study was to investigate the parameters of proinflammatory activity of adipose tissue in an experimental model of MetS in rats.

MATERIALS AND METHODS

The study was performed on 24 male Wistar rats. The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) (Minutes No. 1 dated March 6, 2024). The procedures for animal care, housing, euthanasia, and experimental manipulations complied with international guidelines for good laboratory practice. The method for modeling MetS was described in detail by us previously [9]. The control group rats (n = 12) received a standard diet (protein:fat:carbohydrate 24%:6%:44%) with free access to food and water. The experimental group rats (n = 12) were fed with a high-fat, high-carbohydrate diet (HFHCD) (protein:fat:carbohydrate 16%:21%:54%) and provided with a 20% fructose drinking solution for 12 weeks.

Animals were euthanized by CO_2 asphyxiation. The blood samples were collected from the heart to obtain serum, and fragments of the white adipose tissue (WAT) were extracted. The relative mass of WAT (tissue mass/100 g of rat body weight) was assessed. The serum concentrations of glucose, triacylglycerides (TAG), and total cholesterol (TC) (all assays from Olvex Diagnosticum, Russia) were determined by enzymatic methods. Insulin (Rat INS(Insulin) ELISA Kit, Elabscience, China) and leptin (Rat Leptin ELISA Kit, ELK Biotechnology, China) levels were measured by enzyme-linked immunosorbent assay (ELISA). The HOMA-IR index (Homeostasis model assessment of insulin resistance) was calculated as (serum insulin) × (serum glucose) / 22.5.

To assess the secretory activity of WAT cellular elements, fragments of visceral fat were cultured in a nutrient medium based on DMEM/F-12 (Servicebio, China) supplemented with a penicillin – streptomycin antibiotic mixture (Capricorn Scientific, Germany), 200 mM L-glutamine (Capricorn Scientific, Germany), and 2% bovine serum albumin (Sigma-Aldrich, USA). The dissection and preparation of adipose tissue samples were performed under sterile conditions; fat fragments weighing 150 mg were placed into the wells of a 24-well culture plate containing 1.5 ml of the nutrient medium. The samples were incubated for 24 hours in a CO_2 incubator at a temperature of 37 °C and 5% CO_2. Upon completion of the incubation, aliquots of the conditioned medium were taken and frozen at –70 °C. The concentration of adipocyte hormones (leptin and adiponectin) and cytokines: tumor necrosis factor-alpha (TNFα), interleukins (IL) (IL-6 and IL-10), and monocyte chemoattractant factor-1 (MCP-1) in the conditioned medium was determined by (ELISA) using the corresponding Rat ELISA Kit sets (ELK Biotechnology, China).

A pathomorphological study of WAT fragments was performed to analyze and assess the severity of morphological changes. For this purpose, during the sampling stage, the fat samples were fixed in 10% neutral buffered formalin (BioVitrum, Russia). To prepare histological specimens, the samples were washed for 24 hours to remove the fixative, then dehydrated in an isopropanol-based solution according to the manufacturer's protocol (IsoPrep, BioVitrum, Russia), and placed in an intermediate medium vaseline oil/liquid paraffin (MEDKHIM, Russia). The tissue samples were embedded in the paraffin medium Histomix (BioVitrum, Russia), and thin sections (5–6 μm) were made using an HM355 S automatic microtome (Thermo Scientific, USA). The sections were stained with hematoxylin and eosin (BioVitrum, Russia).

The presence of CD68+ cells in the adipose tissue was detected using an immunohistochemical method. Thin sections of the adipose tissue were deparaffinized in xylene and rinsed sequentially in 96% and 70% ethanol and then in distilled water. Subsequently, antigen retrieval was performed in a retrieval buffer (Target Retrieval Solution, pH 9.0, Dako, USA) using a water bath at 96ºC. After cooling, the sections were sequentially treated with a peroxidase block (Peroxidase-Blocking Reagent, Dako, USA) and incubated for 45 minutes with the primary antibody (PA5-78996, Invitrogen, Thermo Fisher Scientific, USA) at a 1:100 dilution under the conditions recommended by the manufacturer. The samples were then washed twice with phosphate buffer and incubated for 30 minutes with a secondary antibody (Rabbit IgG (H+L), HRP, 31460, Invitrogen, Thermo Fisher Scientific, USA). The EnVision FLEX system (Dako, USA) was used for visualization, with diaminobenzidine solution (Liquid DAB+, Dako, USA) employed as the chromogenic substrate; the nuclei were counterstained with hematoxylin (BioVitrum, Russia). Cell counting was performed in 30 fields of view for each sample, obtained using the EVOS M7000 system (Thermo Fisher Scientific, USA).

The level of *CD68* gene expression was determined. For this purpose, total mRNA was extracted from WAT fragments using the RNeasy Lipid Tissue Mini Kit (Qiagen, Germany), followed by quantitative mRNA analysis on the Agilent 4150 TapeStation automated gel electrophoresis system (Agilent Technologies, USA). Complementary DNA (cDNA) was then synthesized from the mRNA template using the M-MuLV-RH reverse transcriptase kit (Biolabmix, Russia). Quantitative real-time polymerase chain reaction (qPCR) was performed using the BioMaster HS-qPCR (2×) kit (Biolabmix, Russia). The analysis was carried out using the $2^{-\Delta\Delta Ct}$ method relative to the expression level of the housekeeping gene β-actin (*ACTB*). Primers and probes for the *ACTB* and *CD68* genes were synthesized by DNA-Sintez LLC (Russia) (Table 1).

Table 1

Sequences of Primers and Probes for the *ACTB* and *CD68* Genes

Gene	Primer sequences (F&R)	Probe sequences (FAM/BHQ1)
ACTB	5' GAGAAGATGACCCAGATCATGTT 3' 5' ATAGCACAGCCTGGATAGCAA 3'	5' AGACCTTCAACACCCCAGCCAT 3'
CD68	5' GACACTTCGGGCCATGCT 3' 5' TAACGCAGAAGGCAATGAG 3'	5' CCAATCTCTCTTGCTGCCTCTCATC 3'

Statistical analysis was performed using SPSS Statistics 23 software. Data consistent with a normal distribution are described as mean and standard deviation ($M \pm SD$), while data not consistent with a normal distribution were presented as median and interquartile range ($Me\ (Q_{25};Q_{75})$). Difference analysis was conducted using the Student's t-test or the Mann–Whitney *U*-test. Differences were considered statistically significant at $p < 0.05$. To assess the relationship between parameters, the Spearman's rank correlation coefficient (r) was determined.

RESULTS

It was found that the animals in the experimental group exhibited an increase in body weight (485.1 ± 33.8 g vs. 430.3 ± 25.4 g in the control group ($p = 0.005$)), as well as in the relative mass of visceral fat, which was 4.6 ± 0.5 g in the experimental group and 2.1 ± 0.3 g in the control group ($p = 0.002$).

In the experimental group of rats subjected to the 12-week HFHCD, statistically significant changes in blood biochemical parameters were observed

compared to the corresponding values in the control group animals. Specifically, there was an increase in the concentration of glucose and TAG (on average, 1.4-fold, $p < 0.05$) and cholesterol of more than 1.6-fold ($p = 0.009$). These detected changes were accompanied by a marked increase in blood levels of insulin and leptin (Table 2). It was found that in rats fed with the HFHCD, a positive correlation was established between the increase in adipose tissue mass and the increase in serum concentrations of leptin ($r = 0.57$, $p = 0.005$), glucose ($r = 0.62$, $p < 0.001$), and insulin ($r = 0.61$, $p < 0.001$).

Table 2

Biochemical Parameters of Rats fed a Standard diet or HFHCD, *M* ± *SD*

Parameter	Control group ($n = 12$)	Experimental group ($n = 12$)	p
Glucose, mM	5.1±0.5	6.6±0.2	0.005
Insulin, pM	12.9±0.7	22.3±5.9	0.002
Leptin, ng/ml	2.2±0.2	6.9±1.3	0.003
HOMA-IR	2.9±0.2	6.5±0.4	0.012
Triacylglycerols, mM	1.8±0.3	2.6±0.4	0.014
Total cholesterol, mM	2.9±0.3	4.8±0.4	0.009

Here and in Tables 3, 4: p – level of statistical significance of the differences.

According to ELISA of conditioned medium samples from cultured WAT fragments, it was found that in the experimental group of rats, the concentration of proinflammatory cytokines IL-6, TNFα, and MCP-1 exceeded that in the control group, whereas the level of anti-inflammatory IL-10, on the contrary, was lower than the control values (Table 3). Along with this, the samples from the experimental animal group showed a statistically significant increase in leptin concentration (on average 3-fold, $p = 0.004$), but a decrease in adiponectin concentration (on average 1.4-fold, $p = 0.018$).

Histological examination of WAT sections revealed that in the control group rats, the WAT consisted of large lobules composed of large adipocytes of regular rounded shape and approximately uniform size; the connective tissue interlobular septa were poorly developed, contained blood vessels of normal structure, and showed no signs of congestion (Fig. 1, A). In the animals of the experimental group, the adipose tissue also has a lobular structure. However, the lobules were smaller than those in the control group animals, were irregularly rounded in shape, and the adipocyte size varied significantly. The interlobular connective tissue septa were more pronounced, with areas of vascular congestion (engorgement of blood vessels) observed within them (Fig. 1, B). Thus, the adipose tissue of the rats in the experimental group exhibited pathomorphological signs of impaired circulation, hypertrophy, and hyperplasia of fat cells.

According to the immunohistochemical analysis results (Fig. 2, A), it was found that the experimental group showed an increase in the number of cells with a CD68 phenotype compared to the control: 158.6 (142.3; 178.1) vs. 52.4 (45; 62.5) ($p = 0.001$).

A comparative assessment of the *CD68* gene expression level by cellular elements of the adipose tissue (Fig. 2, B) revealed an approximately 2-fold increase in its level in the samples from the experimental group animals compared to the control samples: 1.6 (0.9; 2.5) vs. 4.6 (2.7; 5.8) relative units ($p = 0.001$).

Correlation analysis revealed a statistically significant positive association between the concentrations of cytokines IL-6, TNFα, MCP-1 and the level of *CD68* gene expression in rat adipose tissue (Table 4).

Along with this, it was established that an increase in the concentration of leptin from adipose tissue samples in the conditioned medium positively correlated with the level of produced pro-inflammatory cytokines; whereas for adiponectin, an inverse correlation with the same cytokines was revealed (Table 4). A positive relationship was also found between an increase in the *CD68* gene expression level and the concentration of leptin ($r = 0.58$, $p = 0.001$) and serum insulin in rats with MetS ($r = 0.65$, $p = 0.001$).

Table 3

Concentration of Adipocyte Hormones and Cytokines in the Conditioned Medium of WAT, *Me* (Q_{25}; Q_{75})

Parameter	Control group ($n = 12$)	Experimental group ($n = 12$)	p
Leptin, ng/ml	0.3 (0.1;0.5)	0.9 (0.7;1.1)	0.024
Adiponectin, ng/ml	8.4 (7.9;8.8)	6.1 (5.8;6.9)	0.018
IL-6, pg/ml	107.5 (100.4;129.7)	169.57 (148.4;198.2)	0.017
IL-10, pg/ml	119.8 (90.7;135.8)	84.5 (69.2;100.6)	0.021
TNFα, pg/ml	0.9 (0.5;1.2)	2.3 (1.9;2.8)	0.003
MCP-1, pg/ml	135.9 (110.6;176.6)	272.7 (161.6;309.1)	0.005

Table 4

Spearman's Rank Correlation Coefficients (R) between WAT Secretory Activity Parameters and *CD68* Gene Expression Level in Rat adipose Tissue

Parameters	Concentration of cytokines, pg/ml			
	IL-6	IL-10	TNF-α	MCP-1
Concentration of leptin, ng/ml	0.61, $p = 0.001$	–0.32, $p = 0.006$	0.58, $p = 0.001$	0.55, $p = 0.001$
Concentration of adiponectin, ng/ml	–0.41, $p = 0.003$	0.51, $p = 0.001$	–0.48, $p = 0.002$	–0.53, $p = 0.001$
CD68 gene mRNA expression, r.u.	0.65, $p = 0.001$	–0.29, $p = 0.008$	0.51, $p = 0.002$	0.64, $p = 0.001$

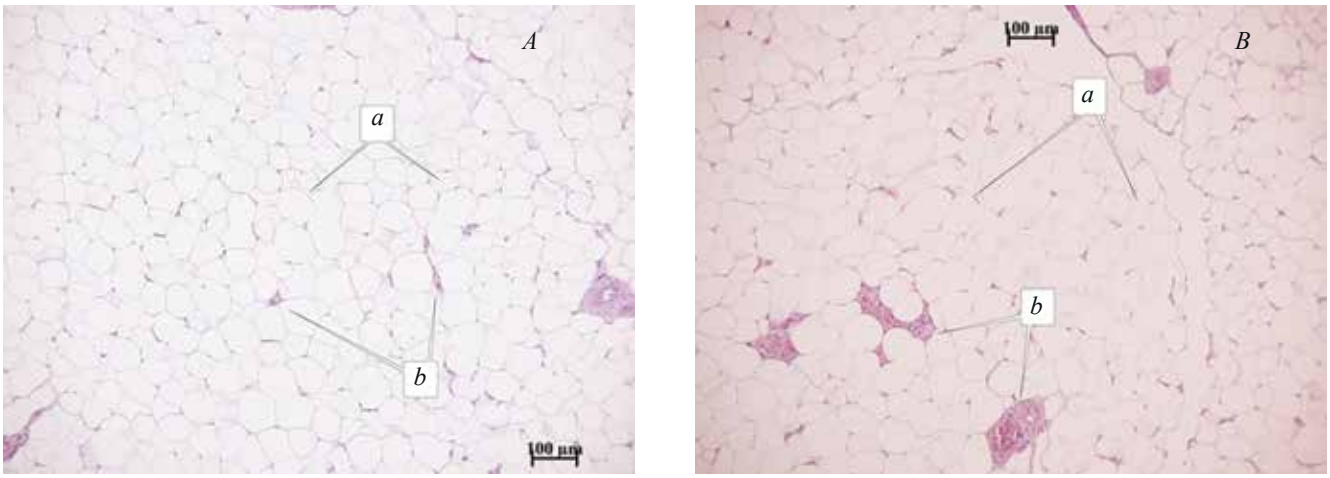
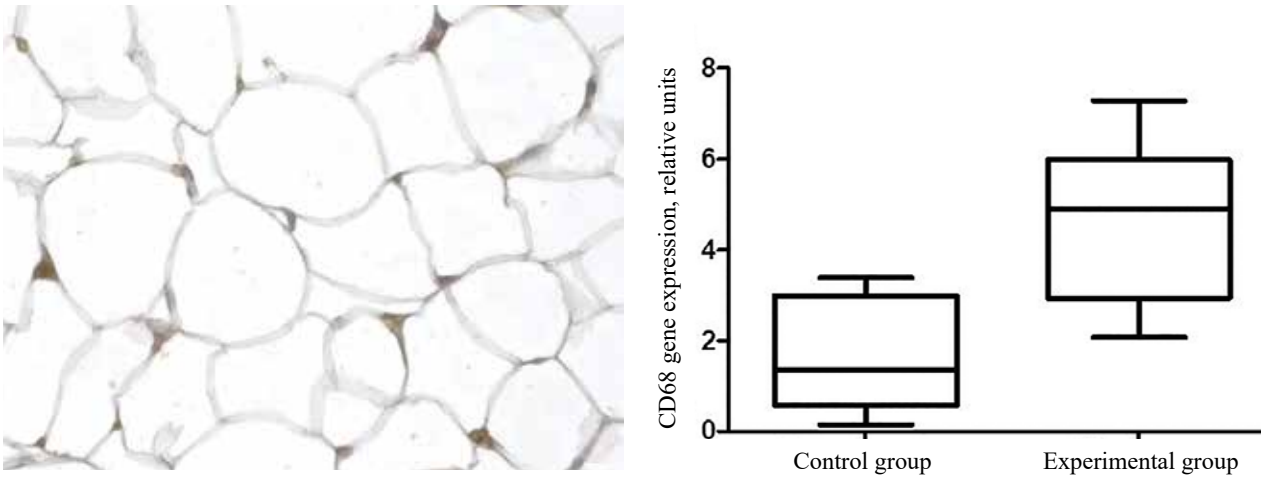


Fig. 1. Morphological features of visceral adipose tissue: *A* – fragment of white adipose tissue from a rat in the control group. Adipocytes (*a*) and blood vessels (*b*), ×200. *B* – fragment of white adipose tissue from a rat in the experimental group. Anisocytosis of adipocytes (*a*) and venous vessel congestion (*b*), ×200.



a *b*

Fig. 2. Evaluation of CD68 expression by adipose tissue cells: *a* – immunohistochemical reaction for CD68-positive cells in a white adipose tissue sample of a rat from the experimental group. Diaminobenzidine and hematoxylin counterstain. ×400; *b* – Relative *CD68* mRNA expression in control and experimental samples of rat adipose tissue; *p* – level of statistical significance of differences.

DISCUSSION

A number of studies have established that lipid overload in adipose tissue leads to structural and functional alterations. These are characterized not only by adipocyte hypertrophy but also by excessive accumulation of the extracellular matrix due to increased activity of collagen-producing fibroblasts. This results in a significant reduction in tissue elasticity, impaired blood supply, and the development of hypoxia and oxidative stress reactions [10, 11]. The results of our own pathomorphological study of adipose

tissue samples confirm these findings. Specifically, the animals in the experimental group exhibited adipocyte hypertrophy and anisocytosis, impaired blood supply, engorgement of venous vessels, and thickening of the interlobular connective tissue layer.

Excessive secretion of pro-inflammatory adipocyte hormones (leptin, visfatin, and chemerin) and chemokines (MCP-1, chemokine (C-X-C motif) ligand 12 (CXCL12)) by hypertrophied adipocytes contributes to the initiation and maintenance of inflammation [3, 12]. Our studies have shown that the increase in adipose tissue mass in animals fed with a HFHCD for 12 weeks correlates with elevated serum concentrations of leptin and insulin. Furthermore, the leptin level was statistically significantly increased in the conditioned medium of the rat WAT from the experimental group. Leptin, which is overproduced by hypertrophied adipocytes, is known to be actively involved in the pathogenesis of insulin resistance through the activation of pro-inflammatory cytokine (TNFα and IL-6) production, the enhancement of oxidative stress reactions, and a direct effect on insulin-secreting cells [13]. Thus, a vicious cycle emerges – leptin resistance and insulin resistance mutually reinforce each other, contributing to the progression of metabolic disorders.

The inflammation of adipose tissue developing in this context is characterized by increased infiltration by immune cells (macrophages and lymphocytes) and the secretion of inflammatory mediators – reactive oxygen species, cytokines, chemokines, non-enzymatic cationic proteins, etc. [14]. Our study demonstrated that in the experimental group of rats, the production of pro-inflammatory cytokines IL-6, TNFα, and MCP-1 by adipose tissue cells increased, while the level of the anti-inflammatory cytokine IL-10 decreased.

The shift in the functional profile of resident and recruited macrophages from the M2 to the M1 phenotype, which is characterized by increased secretion of the pro-inflammatory cytokines TNFα, IL-6, and IL-1β, contributes to the enhancement of pro-inflammatory activity in adipose tissue [15]. It is noted that macrophages expressing the CD68 surface marker play a key role as the primary cellular component of adipose tissue, regulating the inflammatory response and modulating the secretion and differentiation of various cytokines [16, 17]. Moreover, their quantity is positively correlated with the insulin resistance index. At the same time, a study of the adipocyte microenvironment in obesity revealed a “crown-like structure” zone containing M1-polarized CD68+ macrophages and CD8+ T-lymphocytes [18]. We demonstrated that the animals in the experimental group exhibited an increase in the number of CD68-positive cells in the visceral adipose tissue samples, which is supported by the upregulation of the *CD68* gene. Furthermore, the active production of pro-inflammatory cytokines (IL-6, TNFα, and MCP-1) and leptin by visceral adipose tissue cells in rats with MetS directly correlates with the level of *CD68* gene expression. As established, IL-6 stimulates monocyte migration into adipose tissue by increasing the expression of adhesion molecules (VCAM-1/ICAM-1) and enhances macrophage polarization towards the M1 phenotype [19], while MCP-1 is a key chemokine that recruits monocytes through the activation of the CCR2 receptor [20]. Leptin, acting through the Ob-Rb receptor and the JAK2/STAT3 signaling pathway, directly stimulates the proliferation of CD68+ cells in visceral fat [21]. Our study also revealed a positive correlation between the increased *CD68* gene expression level and the rising concentrations of leptin and insulin in the serum of rats with MetS.

As mentioned, T-lymphocytes are also actively involved in the inflammatory response, particularly cytotoxic cells (CD8+) and T-helper 17 cells (Th17), whereas the tissue pool of regulatory T-lymphocytes (Treg), on the contrary, becomes depleted [22, 23]. One of the mechanisms driving the progression of inflammation in adipose tissue is the activation of the NLRP3 inflammasome complex (NLR family pyrin domain containing 3) in macrophages. This activation mediates the increased production of pro-inflammatory cytokines IL-1β and IL-18, stimulates pyroptosis of adipose tissue cells, and enhances fibrosis due to increased production of transforming growth factor beta (TGFβ) [24]. Studying the transition of macrophages from the anti-inflammatory phenotype (M2) to the proinflammatory phenotype (M1) in the context of adipose tissue inflammation, particularly through the modulation of cytokine secretion alongside the activation of the JNK/NF-κB signaling pathway and the NLRP3 inflammasome complex, is crucial for understanding the pathogenesis mechanisms of MetS and obesity.

CONCLUSION

Thus, the study found that in experimental diet-induced MetS, adipose tissue acquires pro-inflammatory activity through adipocyte hypertrophy, infiltration by CD68+ macrophages, and increased secretion of proinflammatory chemokines and

adipokines (TNFα, IL-6, MCP-1, and leptin). All of this contributes to the development and maintenance of chronic systemic inflammation, insulin resistance, and exacerbates metabolic disorders. Undoubtedly, a detailed study of the molecular and cellular mechanisms of adipose tissue dysfunction in MetS and obesity is of key importance for understanding the pathogenesis of these conditions and for developing new diagnostic methods and personalized treatment approaches for their associated complications.

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Signaling Pathway MEK1/2–ERK1/2 is Involved in the Cardioprotective Effect of Probiotic Strains in the Systemic Inflammatory Response in Rats

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ABSTRACT

Aim. To experimentally test the hypothesis of the participation of MEK1/2 and ERK1/2 kinases in the mechanism of the probiotic cardioprotection in the implementation of the signaling stage of the cardioprotective response to the administration of probiotic strains in the systemic inflammatory response in rats.

Materials and methods. The experiments were performed on male Wistar rats using a model of systemic inflammatory response syndrome, which includes obesity and chemically induced colitis. To provide probiotic cardioprotective effects, the animals were administered probiotic strains LA-5 and BB-12 orally. An inhibitor of MEK1/2 kinase and its associated ERK1/2 kinase PD98059 at a dose of 0.3 mg/kg were administered intravenously 20 minutes before the start of Langendorff perfusion of an isolated heart. The size of the necrosis zone (SNZ) was histochemically determined after 30 minutes of global ischemia and 90 minutes of reperfusion were simulated. Markers of the systemic inflammatory response (SIR) were detected in the blood.

Results. In the group of rats with a model of SIR in comparison with the control, a significant increase in the number of leukocytes and an increase in the level of proinflammatory cytokines in the blood, as well as a significant increase in SNZ were found (by 39% in relation to CTR, $p < 0.05$). In the group with probiotic correction, a significantly smaller SNZ was noted in relation to SIR, whereas in rats with the introduction of probiotics and the substance PD98059, SNZ was significantly bigger, i.e. the cancellation of the cardioprotective effect of probiotic therapy occurred.

Conclusion. Based on the conclusion that the cardioprotective effect was abolished by PD98059 administration, it can be assumed that the probiotic effect is provided by the MEK1/2 and ERK1/2 kinase pathways.

Keywords: myocardium, ischemia-reperfusion, cardioprotection, systemic inflammatory response, probiotics, MEK1/2 and ERK1/2 kinases, PD 98059

Conflict of interest. The authors declare the absence of obvious or potential conflicts of interest related to the publication of this article.

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Внутриклеточный сигнальный путь MEK1/2–ERK1/2 участвует в реализации кардиопротективного эффекта пробиотических штаммов при системном воспалительном ответе у крыс

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РЕЗЮМЕ

Цель. Экспериментально проверить гипотезу об участии киназ MEK1/2 и ERK1/2 в реализации сигнального этапа кардиопротективного ответа на введение смеси пробиотических штаммов *Lactobacillus acidophilus* (LA-5) и *Bifidobacterium animalis* subsp. *lactis* (BB-12) при системном воспалительном ответе у крыс.

Материалы и методы. Эксперименты выполнены на самцах крыс стока Вистар на модели синдрома системного воспалительного ответа, включающей ожирение и химически индуцированный колит. Для обеспечения пробиотической кардиопротекции животным внутрижелудочно вводили пробиотические штаммы LA-5 и BB-12. Ингибитор MEK1/2 киназы и сопряженной с ней ERK1/2 киназы PD98059 в дозе 0,3 мг/кг вводили внутрибрюшинно за 20 мин до начала перфузии изолированного сердца по Лангендорфу. Моделировали 30 мин глобальной ишемии и 90 мин реперфузии, после чего гистохимически определяли размер зоны некроза (РЗН). В крови определяли маркеры системного воспалительного ответа (СВО).

Результаты. В группе крыс на модели СВО по отношению к контролю отмечено значимое увеличение числа лейкоцитов и повышение уровня провоспалительных цитокинов в крови, а также значимое увеличение РЗН (на 39% по отношению к КТР, $p < 0,05$). В группе с пробиотичекой коррекцией отмечен значимо меньший РЗН по отношению к СВО, тогда как у крыс с введением пробиотиков и вещества PD98059 РЗН был значимо выше, т.е. произошла отмена кардиопротективного эффекта пробиотической терапии.

Заключение. На модели СВО пробиотик-индуцированная кардиопротекция обеспечивается при участии сигнального пути киназ, предотвращающих реперфузионное повреждение, включая MEK1/2 и ERK1/2 киназы.

Ключевые слова: миокард, ишемия-реперфузия, кардиопротекция, системный воспалительный ответ, пробиотики, киназы MEK1/2 И ERK1/2, PD 98059

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией данной статьи.

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INTRODUCTION

According to the Fourth Universal Definition of Myocardial Infarction (MI), most cases of the disease belong to type 1, which occurs spontaneously as a result of destabilization of an atherosclerotic plaque, the development of thrombosis, and atherothrombotic and atherothrombotic occlusion of a coronary artery. Early myocardial revascularization in MI is associated with a significant improvement in prognosis; however, restoration of blood flow through the infarct-related artery leads to the myocardial reperfusion injury. Under specific circumstances, reperfusion injury may be irreversible and result in a twofold increase in infarct size compared to the volume observed at the time of ischemia cessation [1]. The mechanisms of early ischemia – reperfusion injury (IRI) of the myocardium include oxidative stress, hypercontracture of cardiomyocytes, calcium overload, and opening of the mitochondrial permeability transition pore (mPTP). Experimental studies have demonstrated the effectiveness of numerous pharmacological and non-pharmacological interventions that reduce myocardial IRI. Nevertheless, the results of randomized clinical trials of different types of myocardial conditioning, as well as pharmacological cardioprotectors reproducing the effects of brief ischemia – reperfusion cycles, have been less convincing [2]. These facts encourage the ongoing search for new non-invasive and safe methods to induce a cardioprotective response. One such approach is targeted modulation of the intestinal microbiota composition – a new direction in non-pharmacological cardioprotection that has emerged over the past 10–15 years [3]. One possible explanation for the insufficiently effective transfer of experimental data on cardioprotection into clinical practice is the reduced efficacy of cardioprotective interventions with age and in the presence of comorbidities. In our previous studies, administration of the probiotic strains *Lactobacillus acidophilus* (LA-5) and *Bifidobacterium animalis subsp. lactis* (BB-12) to animals with systemic inflammatory response (SIR) led to a reduction in infarct size, which was associated with specific qualitative changes in the intestinal microbiota composition and decreased plasma concentrations of proinflammatory cytokines [4]. Other authors have shown that administration of *Bifidobacterium animalis subsp. lactis 420* (B420) to mice with dysbiosis induced by a high-fat diet resulted in a significant decrease in infarct size caused by 30-minute coronary artery occlusion followed by reperfusion *in vivo* [5]. Investigation of the molecular mechanisms of probiotic-induced cardioprotection is at an early stage. It has been suggested that the reduction in MI size after probiotic administration may be due to decreased intestinal epithelial permeability accompanied by reduced microbial translocation, altered production of several gut microbial metabolites – primarily short-chain fatty acids – and increased bile acid levels [3]. At the same time, the similarity between the molecular mechanisms of intracellular protective signaling in the myocardium during classical cardioprotection (e.g., ischemic conditioning) and those during probiotic-induced protection remains poorly studied. It is known that classical cardioprotective stimuli activate the reperfusion injury salvage kinase (RISK) pathway, which includes phosphatidylinositol-3-OH kinase (PI3K) and extracellular signal-regulated kinases 1/2 (ERK1/2), as well as the survivor activating factor enhancement (SAFE) pathway [6]. Activated kinase cascades affect end effectors, such as ATP-sensitive potassium channels and the mitochondrial pore, thereby directly reducing IRI [7].

The present study is aimed at examining the dependence of the infarct-limiting effect of probiotic cardioprotection, induced by LA-5 and BB-12 administration to rats with SIR, on activation of one branch of the RISK signaling pathway – specifically, the MEK1/2 - ERK1/2 kinases. To achieve this goal, pharmacological inhibition of the interaction between phosphorylated MEK1/2 and inactive ERK1/2 was performed using the noncompetitive cyclic inhibitor PD98059 containing an amino group. The ERK1/2 inhibitor PD98059 was administered to animals exhibiting a previously formed probiotic cardioprotective response immediately before the start of isolated heart perfusion and simulation of global myocardial ischemia – reperfusion.

MATERIALS AND METHODS

The study was conducted on male Wistar rats obtained from the Nursery for Laboratory Animals, branch of the Institute of Bioorganic Chemistry, Russian Academy of Sciences (Pushchino, Russia), in compliance with the principles of humane treatment of laboratory animals. The protocol was approved by the Animal Care and Use Committee of the Almazov National Medical Research Center, Ministry of Healthcare of the Russian Federation (Minutes No. PZ23_9_V2 dated September 6, 2023). The animals were randomly divided into four groups: 1) control group (CTR, $n = 9$): rats kept under standard vivarium conditions with a regular laboratory diet

and free access to drinking water; 2) systemic inflammatory response (SIR, n = 9): after modeling of SIR [8], animals received 1 mL of normal saline orally once daily for 7 days. Twenty minutes before heart removal, 0.2 mL of water for injection was administered intravenously; 3) SIR + probiotic correction (SIR+PRC, n = 9) – after SIR modeling, the rats were intragastrically administered a a mixture of probiotic strains Lactobacillus acidophilus (LA-5) and Bifidobacterium animalis subsp. lactis (BB-12) at a dose of 10^8 CFU per animal for 7 days. Intravenous injection of 0.2 mL water was performed according to the previous protocol; 4) SIR + probiotic correction + PD98059 inhibitor (SIR+PRC+IPD, n = 9) – animals underwent the same procedures as the SIR+PRC group but received an intravenous injection of the MEK1/2 and ERK1/2 kinase inhibitor PD98059 at a dose of 0.3 mg/kg 20 minutes before heart removal in 0.2 mL of water for injection [9].

Global ischemia – reperfusion of the isolated heart was modeled using the Langendorff technique of isolated heart perfusion. One day before the completion of the experiment, under short-term anesthesia, 1.5 mL of whole blood was collected from the large subcutaneous vein for hematological and immunological analyses. A complete blood count was performed using an automated veterinary three-differential hematology analyzer (URIT-3000 Vet Plus, URIT Medical Electronic, China). The levels of tumor necrosis factor-alpha (TNFα), interleukin (IL)-1β, IL-6, and interferon-gamma (IFNγ) were determined by enzyme-linked immunosorbent assay (ELISA) (MR-96A, Mindray, China). Using the PhysExp hardware-software system (Cardioprotect LLC, Russia), the following parameters were recorded after 15 minutes of stabilization and at 15, 30, 45, 60, 75, and 90 minutes of reperfusion: left ventricular systolic pressure (LVSP, mm Hg), heart rate (HR, bpmin), and coronary flow (CF, mL/min). Infarct size was determined planimetrically after staining heart slices with 1% 2,3,5-triphenyltetrazolium chloride (TTC) for 15 minutes at 37 °C. The 1.5-2.0-mm-thick slices were photographed from both sides. The unstained (TTC-negative) area was calculated as a percentage of the total slice area. The mean size of necrotic zone (SNZ) for each heart was expressed as a percentage of the total analyzed area.

Statistical analysis was performed using the STATISTICA 12.0 software package. The Shapiro–Wilk and Kolmogorov–Smirnov tests were used to assess normality. To address the issue of multiple comparisons, three initial groups were formed at the experimental planning stage: CTR (n = 9), SIR (n = 9), and SIR+PRC (n = 18). On the final day, after administration of water or the inhibitor, the SIR+PRC (n = 9) and SIR+PRC+IPD (n = 9) groups were formed. Considering the small sample size and the lack of normal distribution for several variables, blood parameters were analyzed using the nonparametric Kruskal–Wallis ANOVA by ranks, followed by post-hoc multiple comparisons (Kruskal–Wallis ANOVA and median test). The size of the necrosis zone (SNZ, %) was analyzed using the same statistical approach as for blood parameters. Data in tables and text were presented as median (Me) with interquartile range (25%; 75%). For hemodynamic measurements, Repeated Measures ANOVA followed by Tukey's post-hoc test was applied. Hemodynamic data were presented as mean ± standard error of the mean (SEM). A p-value < 0.05 was considered statistically significant.

RESULTS

Animals in the SIR group had significantly lower body weight at the end of observation compared with the control group (326 ± 19 g vs. 350 ± 8 g, $p < 0.01$). The changes in body weight did not differ between the SIR, SIR+PRC (323 ± 13 g), and SIR+PRC+IPD (318 ± 12 g) groups. In the SIR group, the total leukocyte count was significantly higher than in the control group – by 43%, with lymphocytes increased by 46%, monocytes – by 56%, and granulocytes – by 39% ($p < 0.05$). In the SIR+PRC and SIR+PRC+IPD groups, no significant differences were observed compared with CTR and SIR, except for a reduction in total leukocyte count in the SIR+PRC+IPD group relative to SIR ($p < 0.05$).

In the SIR group compared with CTR, levels of TNFα increased by 48%, IL-1β – by 507%, IL-6 – by 75%, and IFNγ – by 342% ($p < 0.05$). In the SIR+PRC and SIR+PRC+IPD groups, cytokine levels were close to control values, showing significant decreases relative to SIR, except for persistently elevated IL-1β by 350% and 407%, respectively ($p < 0.05$, Table 2).

Hemodynamic parameters did not differ significantly between groups at baseline. During the entire reperfusion period, the SIR group showed a pronounced increase in LVSP and CF by the end of observation. In the probiotic-treated groups, LVSP remained at the control level. At the end of reperfusion, CF was higher in the SIR+PRC group relative to CTR, while in SIR+PRC+IPD, it was reduced compared with SIR (Table 3).

Table 1

Hematological Parameters, *Me* [25; 75]				
Group	CTR	SIR	SIR+PRC	SIR+PRC+IPD
Leukocytes, 10^9/L	12(7;13)	21(19;23)*	13(10;17)	13(9;14)#
Lymphocytes, 10^9/L	2.7(1.5;3.2)	5.0(4.1;5.5)*	3.7(2.6;4.1)	3.2(2.4;4.4)
Monocytes, 10^9/L	1.1(0.8;1.4)	2.5(1.5;5.7)*	2.1(1.1;2.4)	1.4(1.1;1.9)
Granulocytes, 10^9/L	7.3(5.9;8.0)	11.9(11.5;13.1)*	8.3(6.8;10.6)	8.1(6.3;8.3)
Erythrocytes, 10^{12}/L	4.0(3.7;4.3)	4.2(4.0;4.3)	4.2(3.6;4.4)	3.2(3.2;3.3)
Platelets, 10^9/L	305(218;373)	310(286;400)	433(238;469)	415(358;463)

* $p < 0.05$ vs. CTR; # $p < 0.05$ vs. SIR (Mann–Whitney *U* test).

Table 2

Levels of TNFα, IL-1β, IL-6, and IFNγ, *Me* [25; 75]				
Group Analyte	*CTR*	*SIR*	*SIR+PRC*	*SIR+PRC+IPD*
TNFα	9.8(8.1;11.3)	19.0(17.0;20.4)*	12.6(10.1;14.0)#	13.1(12.7;13.5)#
IL-1β	14(11;20)	85(57;119)*	63(30;81)*	76(48;85)*
IL-6	6.9(6.8;7.8)	12.1(8.3;14.0)*	7.8(7.3;9.0)#	6.3(5.7;6.6)#
IFNγ	6.1(5.3;9.8)	27(25;48) *	7.0(4.8;32)#	6.8(5.3;35.0)#

* $p < 0.05$ vs. CTR; # $p < 0.05$ vs. SIR (Mann–Whitney *U* test).

Table 3

Hemodynamic Parameters, $M \pm SEM$								
Group	Hemodynamic parameters	Basel.	reperfusion					
			15 min.	30 min.	45 min.	60 min.	75 min.	90 min.
CTR	LVSP	129±24	93±3	84±4	79±2	77±2	75±2	73±1.3
	HR (bpm)	291±10	394±39	377±22	367±11	290±19	304±37	371±23
	CF (mL/min)	11.0±1.6	6.0±0.9	5.0±0.5	4.0±0.1	3.5±0.6	3.1±0.4	2.3±0.3
SIR	LVSP	140±13	158±27*	149±25*	143±24*	145±21*	144±23*	141±23*
	HR (bpm)	244±43	280±29	274±23	320±32	310±27	318±15	345±29
	CF (mL/min)	8.6±0.6	4.9±0.5	4.6±0.5	4.4±0.4	4.1±0.4	3.8±0.3	3.4±0.3*
SIR+ PRC	LVSP	128±15	89±3#	83±3.5#	78±4#	75±3#	74±3#	72±3#
	HR (bpm)	232±20	461±51	214±38	353±31	341±22	338±24	313±17
	CF (mL/min)	10.1±1.2	5.4±0.5	4.9±0.4	5.0±0.5	4.4±0.4	4.4±0.4*	3.8±0.4*
SIR+ PRC+ IPD	LVSP	132±10	95±4#	88±5#	85±4#	83±5#	82±5#	79±5#
	HR (bpm)	259±25	314±51	373±22	343±27	356±27	344±21	337±24
	CF (mL/min)	9.3±0.9	4.8±0.5	3.6±0.4	3.2±0.2	2.3±0.5#	2.4±0.4#	2.3±0.4#

* $p < 0.05$ vs. CTR; # $p < 0.05$ vs. SIR (Tukey's post-hoc test).

In the CTR, SIR, SIR+PRC, and SIR+PRC+IPD groups, the necrotic zone occupied 37(37;45)%, 61(57;64)%, 49(45;53)%, and 56(53;60)% of the total slice area, respectively. This indicates a 39% increase in SNZ in the SIR group compared with CTR ($p < 0.05$) and a 20% reduction in the SIR+PRC group compared with SIR ($p < 0.05$).

DISCUSSION

In this study, a model of systemic inflammation was used as a comorbid background for the analysis of cardioprotection. This model was based on an increase in visceral adipose tissue mass due to feeding the animals with a high-fat diet in combination with acute inflammation of the large intestine, induced by chemical injury to the mucosa. In fact, such a combination of low-grade and acute inflammation is accompanied by the development of systemic inflammatory response syndrome (SIRS), the presence of which was confirmed by an increased leukocyte count in peripheral blood, negative body weight dynamics, and a marked elevation of proinflammatory cytokines (TNFα, IL-1β, IL-6, and IFNγ) in the blood. Our findings of more pronounced myocardial

damage during global ischemia – reperfusion under SIRS conditions are generally consistent with the literature. Thus, in a clinical study by J. Odeberg et al. (2016), it was shown that pre-existing inflammation, verified by elevated C-reactive protein levels and leukocyte count, was associated with higher incidence of myocardial infarction in patients with unstable angina and with a less favorable clinical course [10]. Experimental studies also indicate that the severity of myocardial IRI increases in the presence of systemic inflammation, for example in a model of dextran sulfate-induced inflammatory bowel disease in mice [11]. There is no doubt that a key role in reducing myocardial resistance to IRI under SIRS conditions belongs to the effects of proinflammatory cytokines and chemokines on cardiomyocytes. It is known that cytokines such as TNFα and IL-1β mediate receptor-dependent damaging effects on cardiomyocytes, which include the activation of programmed cell death pathways, enhanced production of reactive oxygen species, and other mechanisms [12]. This is supported by the fact that genetic or pharmacological blockade of proinflammatory cytokines is accompanied by a reduction in infarct size and leukocyte infiltration, as well as attenuation of left ventricular dilatation and dysfunction [13].

Probiotic therapy with *Lactobacillus acidophilus* (LA-5) and *Bifidobacterium animalis* subsp. *lactis* (BB-12) administered for seven days to animals with SIRS was associated with a reduction in infarct size. At the same time, the use of probiotics led to a decrease in TNFα and IL-1β concentrations. The pathways by which the protective changes in gut microbiota composition are transferre from the intestine to the heart during IRI are of particular interest. In addition to attenuating the deleterious effects of proinflammatory cytokines, current literature considers mechanisms of direct influence of substances secreted by the intestinal microbiota and entering the circulation. Such humoral molecular signals may include short-chain fatty acids acting on free fatty acid receptor 3 (FFAR3), as well as bile acids, which affect target cells through the nuclear farnesoid X receptor (FXR) and the G protein-coupled bile acid receptor 1 (TGR5) [14]. A neurogenic pathway of cardioprotective signal transmission resulting from activation of the microbiota – gut – brain axis is also not excluded [15]. However, in the context of the present work, the main focus was not on the mechanisms of signal transfer from the intestine with an altered microbiota composition to the heart or on the receptor systems of cardiomyocytes that perceive these stimuli, but rather on the intracellular signaling systems responsible for increasing cardiomyocyte resistance to IRI. Traditionally, the molecular mechanisms of cardioprotection are considered in terms of three successive stages: 1) the trigger stage, associated with receptor and non-receptor actions of signaling molecules on molecular targets in the cardiomyocyte; 2) the mediator stage, which includes activation of several intracellular signaling kinase cascades; and 3) the effector stage, involving changes in the activity of several terminal effectors of cardioprotection, such as mitochondrial and sarcolemmal ATP-sensitive potassium channels and the mitochondrial permeability transition pore. A pivotal role in the description of intracellular cardioprotective signaling pathways was played by the discovery of the RISK pathway by D.M. Yellon et al. [16]. This pathway is activated by many endogenous ligands, including adipokines, growth factors, hormones, and other biologically active substances. The circulating levels of some of these mediators may change upon probiotic administration or other interventions targeting gut microbiota composition, such as microbiota transplantation or metabolic surgery. The RISK signaling pathway is also activated during ischemic preconditioning of the myocardium; its activation persists throughout the ischemic phase of injury and exerts a cardioprotective effect in the reperfusion phase by limiting opening of the mitochondrial permeability transition pore [17]. This leads to reduced apoptosis, attenuation of oxidative stress, decreased mitochondrial calcium overload, and other effects that contribute to the mitigation of myocardial IRI [18]. The RISK pathway has two branches that converge on p70S6 kinase, phosphorylating and activating it; activated p70S6 kinase in turn suppresses glycogen synthase kinase-3β (GSK-3β) activity. One branch of the RISK pathway is represented by phosphatidylinositol-3-kinase (PI3K) and protein kinase B (Akt), and the other – by the mitogen-activated protein kinases MEK1/2 and ERK1/2. There is a reciprocal relationship between these two branches, since inhibition of one cascade leads to activation of the other, and vice versa [19]. As active GSK-3β promotes opening of the mitochondrial permeability transition pore, suppression of its activity as a result of RISK pathway activation has pronounced cardioprotective consequences [20]. In recent years, it has been shown that inhibition of GSK-3β can elicit cardioprotection not only by suppressing opening of the mitochondrial pore but

also through other mechanisms, such as modulation of autophagy [21].

In the present study, we tested the hypothesis that the cardioprotective action of altered intestinal microbiota composition resulting from regular administration of probiotic strains is mediated at the level of intracellular signaling involving components of the RISK pathway, specifically through its cascade associated with MEK1/2 and ERK1/2 kinases. Blockade of signaling at the MEK1/2 - ERK1/2 level using PD98059 led to the loss of the infarct-limiting effect of probiotic cardioprotection under systemic inflammation, which indicates the key role of this signaling cascade in mediating the effects of probiotics on the heart. Apparently, the RISK pathway represents a non-specific, common terminal cell signaling pathway aimed at increasing myocardial resistance to IRI.

CONCLUSION

This study demonstrated that repeated gastric administration of probiotic bacterial strains to rats with systemic inflammatory response produced a cardioprotective effect manifested by a reduction in infarct size. Pharmacological inhibition of the MEK1/2 - ERK1/2 signaling pathway, belonging to the broader RISK pathway, abolished the probiotic-induced cardioprotection. These findings indicate that maintaining ERK1/2 activity during reperfusion is essential for protection against ischemia – reperfusion injury induced by gut microbiota modulation.

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The Expression Levels of SIRT1 Splicing Isoforms and Genes Regulating Mitochondrial Homeostasis in the Liver of Patients with Type 2 Diabetes Mellitus and Obesity

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ABSTRACT

Aim. To evaluate the association between the expression levels of sirtuin 1 (*SIRT1)* splicing isoforms and the genes associated with mitochondrial homeostasis (*PGC-1a, PPAR-γ, PPAR-α, TFAM, MFN2, OPA1,* and *DRP1*) in the liver of patients with type 2 diabetes mellitus (T2DM).

Material and methods. The study included 59 patients who were divided into two groups: 1) control group, body mass index (BMI) < 30 kg/m^2, without cardio-metabolic disorders; 2) patients with T2DM, BMI > 30 kg/m^2. A biochemical analysis of the patients' blood parameters was performed, and the expression level of the genes of interest in the liver tissue was studied using quantitative RT-PCR.

Results. It was found that the *SIRT1* splicing isoforms *V1, V2*, and *V3* were stably expressed in the liver of patients with T2DM. *SIRT1* isoforms occur not only individually, but also in various combinations. The expression of the *SIRT1 V3* isoform was significantly increased in the group of patients, while the remaining analytes did not significantly differ between the groups. The *SIRT1 V3* isoform positively correlated with glucose levels. It is worth noting that the total *SIRT1* did not show significant correlations with the genes of interest and biochemical parameters, which only confirms the need to study the expression of isoforms separately.

Conclusion. *SIRT1* isoforms were stably expressed in the liver, and the expression level of *SIRT1 V3* isoform was significantly higher in patients with T2DM. The results of this work can serve as a basis for further studies of interactions between *SIRT1* splicing isoforms and mitochondrial homeostasis proteins at the post-translational level.

Keywords: type 2 diabetes mellitus, alternate splicing, mitochondria, mitochondrial fission and fusion, liver

Conflict of interest. The authors declare the absence of obvious or potential conflicts of interest related to the publication of this article.

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Conformity with the principles of ethics. The study was conducted in accordance with the Declaration of Helsinki of the World Medical Association (2000) and the Protocol to the Convention on Human Rights and Biomedicine (1999). The study was approved by the local Ethics Committee of Immanuel Kant Baltic Federal University (Minutes No. 40 dated June 26, 2023). All participants signed a consent form to participate in the study.

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Исследование уровней экспрессии сплайсинговых изоформ SIRT1 и генов – регуляторов митохондриального гомеостаза в печени больных сахарным диабетом 2-го типа и ожирением

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РЕЗЮМЕ

Цель. Оценка ассоциации между уровнем экспрессии изоформ сиртуина 1 (*SIRT1*) и генами белков, связанных с митохондриальным гомеостазом (*PGC-1α*, *PPAR-γ*, *PPAR-α*, *TFAM, MFN2, OPA1, DRP1*) в печени больных сахарным диабетом второго типа (СД2).

Материалы и методы. В исследование включено 59 пациентов, которые были разделены на две группы: 1) контрольная группа, индекс массы тела (ИМТ) менее 30 кг/м^2, без кардиометаболических нарушений; 2) пациенты с СД2, ИМТ более 30 кг/м^2. Выполнялся биохимический анализ показателей крови пациентов, а уровень экспрессии генов интереса в печеночной ткани изучали с помощью количественной полимеразной цепной реакцией с обратной транскрипцией.

Результаты. Обнаружено, что сплайсинговые изоформы *SIRT1 V1, V2* и *V3* стабильно экспрессировались в печени у больных СД2. Выявлено, что изоформы *SIRT1* встречаются не только по отдельности, но и в различных сочетаниях. Экспрессия изоформы *SIRT1 V3* значимо повышалась в группе больных, в то время как остальные аналиты значимо не различались между группами. Изоформа *SIRT1 V3* положительно коррелировала с уровнем глюкозы. Стоит отметить, что общий *SIRT1* не показал значимых корреляций с генами интереса и биохимическими показателями, что только подтверждает необходимость изучения экспрессии изоформ отдельно.

Заключение. Изоформы *SIRT1* стабильно экспрессировались в печени, уровень экспрессии изоформы *SIRT1 V3* был значимо выше у больных СД2. Результаты работы могут послужить основой для дальнейших, более точечных исследований взаимодействий между сплайсинговыми изоформами *SIRT1* с белками митохондриального гомеостаза на посттрансляционном уровне.

Ключевые слова: сахарный диабет 2-го типа, альтернативный сплайсинг, изоформы сиртуина 1, митохондриальный гомеостаз, митохондриальная динамика

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Исследование выполнено при поддержке гранта государственного задания FZWM-2024-0012.

Соответствие принципам этики. Все пациенты подписали информированное согласие на участие в исследовании. Исследование одобрено локальным этическим комитетом Балтийского федерального университета им. И. Канта (заключение № 40 от 26.06.2023).

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INTRODUCTION

The incidence of type 2 diabetes mellitus (T2DM) has increased alarmingly over the last 40 years [1]. The liver is particularly affected by T2DM, recent meta-analyses confirm that patients with T2DM develop steatosis, steatohepatitis, and fibrosis of the liver tissue in around 60% of cases [2]. Insulin resistance combined with adipose tissue dysfunction is considered to be the main cause of the development of comorbid liver pathology in T2DM [3]. Active lipolysis, which occurs as a result of adipose tissue dysfunction [3], leads to an increase in the amount of free fatty acids (FFA) in the bloodstream [4].

The liver actively accumulates FFA, but their excess is associated not only with steatosis but also with the development of oxidative stress. Thus, the increased β-oxidation of FFA leads to the accumulation of reactive oxygen species (ROS) in hepatocytes [5]. Oxidative stress is an integral part of T2DM [6] and has a detrimental effect on the cell. Oxidative stress leads to a violation of mitochondrial homeostasis [7] and the development of mitochondrial dysfunction in the form of dysregulation of mitophagy [8] and mitochondrial dynamics (MD) [9], a decrease in the copy number of mitochondrial DNA (mtDNA) [10], and mitochondrial biogenesis [11].

It is well known that mitochondrial dysfunction plays a key role in the pathogenesis of T2DM, as insulin resistance and hyperglycemia eventually lead to dysregulation of energy substrate consumption in all body tissues [12]. This not only destabilizes energy homeostasis, but subsequently also impairs the formation of ROS, MD, and apoptosis: an imbalance of these processes in T2DM subsequently leads to a lack of vital functions, including disruption of hepatocytes and cardiac muscle cells, hindered production of insulin by beta cells, impaired homeostasis of neurons, etc. [13].

Mitochondrial homeostasis is a complex process controlled at many molecular levels: by MD (mitochondrial fusion/division), mitophagy, mitochondrial dissociation, and antioxidant systems [12]. Certain other processes also have recently become the focus of research: mtDNA heteroplasmy, the non-coding RNAs, and their epigenetic regulation of the mitochondrial genome and transcriptome [13]. From this perspective, the phenomenon of mitochondrial hormesis (a slight increase in ROS concentration leading to an improvement in mitochondrial homeostasis) is also interesting. It has been shown that mitochondrial hormesis can activate *AMPK* and *PGC-1a* through *SIRT1/3*, thus restoring normal mitochondrial homeostasis [14]. Therefore, investigating the molecular mechanisms of mitochondrial homeostasis maintenance/disruption is an important task in the context of investigating the pathogenesis of T2DM comorbidities.

Mitochondrial homeostasis in the cell is regulated by both the nuclear and mitochondrial genomes [7]. Thus, the main players are: 1) transcription factors – *TFAM, NRF-1*, and *NRF-2*, which control the expression of mtDNA genes [7]; 2) receptors activated by peroxisome proliferators (*PPARs*), which belong to the nuclear hormone receptor superfamily [7, 15]. As a transcription factor, PPARα regulates the expression of genes encoding enzymes that metabolize fatty acids and the activity of mitochondrial β-oxidation in the liver [16]. PPARγ, in turn, stimulates *de novo* lipogenesis [17]; 3) *PGC-1* family transcriptional coactivators (*PGC-1a, PGC-1b,* and *PRC*) that contribute to the maintenance of mitochondrial biogenesis [18], particularly *PGC-1a* [19]; 4) proteins associated with mitochondrial division (DRP1) and fusion [20] (*MFN1, MFN2*, and *OPA1*).

Many researchers are now focusing on the obvious aspects of impaired mitochondrial homeostasis in the liver, such as the role of transcription factors associated with mitochondrial biogenesis [21] or the expression of genes that regulate oxidative phosphorylation [22]. Of course, these are important links in the pathogenesis of mitochondrial homeostasis disorders, but it is equally important to investigate regulators at a higher level, in particular the histone deacetylase sirtuin 1 (*SIRT1*).

SIRT1 has been extensively studied in the context of T2DM and its comorbidities [23, 24]. *SIRT1* is known to regulate the work of many proteins associated with mitochondrial homeostasis at the post-translational level [25]. Deacetylation increases the activity of the transcriptional coactivator *PGC-1a* [26, 27] and the transcription factor *PPARα* [28] and represses the activity of *PPARγ* [29, 30]. Deacetylation, also by *SIRT* family proteins (*SIRT1* and *SIRT3*), is associated with the regulation of balance in the MD. It is known that deacetylation of *DRP1* leads to a reduction in the activity of this protein, suppressing mitochondrial division [31], while deacetylation of *MFN2* [32] and *OPA1* [33], on the contrary, stimulates their active fusion. Furthermore, it has been shown that *SIRT1* splicing isoforms may be involved in the regulation of oxidative phosphorylation and ATP formation by deacetylation of mitochondrial respiratory chain complex 1 [34].

Indeed, particular attention should be paid to the fact that the *SIRT1* gene is a multi-exon gene that undergoes alternative splicing [35]. Three isoforms of *SIRT1* are distinguished (Fig. 1). The first isoform (*V1*, transcript identifier ENST00000212015.11) is canonical and consists of nine exons (ex1–9) and has a length of 747 amino acid (AA) residues. This isoform contains two nuclear localization signal sites (NLS), encoded in ex1 and ex3, and two nuclear export signal sites (NES)in ex1 and ex7, respectively. Neither the second (*V2*) nor the third isoform (*V3*) has the first or third exon. These isoforms are therefore exclusively cytoplasmic, which has also been confirmed [35].

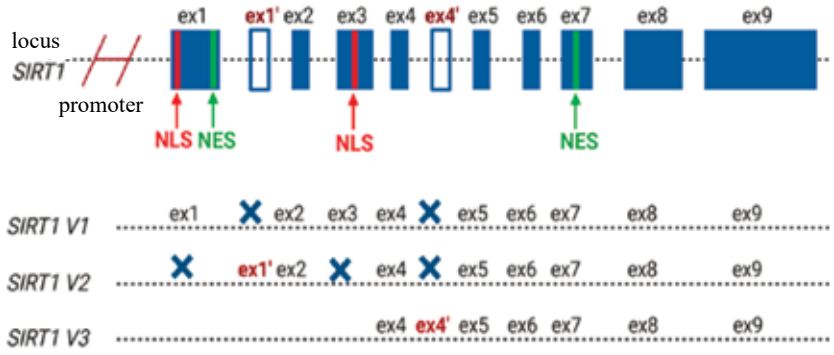

Fig. 1. Structure of SIRT1 isoforms [35]. Abbreviations: NLS – nuclear localization signal, NES – nuclear export signal

The second isoform (ENST00000432464.5) has a shortened N-terminus and consists of 452 AA residues. The transcript encoding *V2* has 8 exons: 7 exons similar to those of *V1* (ex2 and ex4–9) and one exon unique to this isoform (ex1'). However, since the reading frame covers the range from ex4 to ex9, *V2* differs from *V1* only by the shortened N-terminus and has no unique segments.

The third isoform (ENST00000406900.5) is the shortest with a length of 444 AA. *V3* has a unique sequence of 11 AA residues at the N-terminus. This is due to the fact that *V3*, like *V2*, has a variable exon, but unlike the second isoform, this exon (ex4') is included in the reading frame.

It is assumed [34] that these differences influence not only the localization of the isoforms but also their functional activity. However, this question has not yet been considered in the context of human liver tissue. Therefore, **the aim** of our study was to investigate the relationship between the expression level of *SIRT1* isoforms and the genes of proteins associated with mitochondrial homeostasis (*PGC-1a, PPARγ, PPARα, TFAM, MFN2, OPA1,* and *DRP1*) in the liver of patients with T2DM.

MATERIALS AND METHODS

The study participants were diagnosed by the assigned healthcare providers and admitted to Kaliningrad Regional Clinical Hospital. Before the elective abdominal surgery, which was performed under general anesthesia, the participants underwent a standard dietary adjustment. All obese patients stopped taking medication that affects carbohydrate and lipid metabolism 36 hours before the surgery. On the day of the surgery, venous blood samples were taken in the morning following overnight fasting. During surgery, liver samples up to 0.5 cm^3 in volume were collected for RNA isolation (biopsies were preserved in 600 µl RNAlater solution (Ambion, Waltham, Massachusetts, USA)). The study was conducted in accordance with the Declaration of Helsinki of the World Medical Association (2000) and the Protocol to the Convention on Human Rights and Biomedicine (1999). The study was approved by the local Ethics Committee of Immanuel Kant Baltic Federal University (Decision of the Ethics Committee of IKBFU No. 40 dated June 26, 2023). All participants signed a consent form to participate in the study.

Inclusion criteria were as follows: individuals over 21 years of age referred for elective abdominal surgery for various indications: hernioplasty, gastric resection, and pathology of the gallbladder and biliary tract (cholelithiasis, cholecystitis, polyposis, cyst, etc.). Participants had to confirm their willingness to participate in the study by providing a written informed consent.

Exclusion criteria were the following: infectious liver diseases, concurrent somatic-symptom and infectious diseases in the acute inflammatory stage, a known infection with the human immunodeficiency virus or any malignant or benign neoplasms. Individuals under the age of 21 or those who refused to undergo medical and laboratory examinations during the study or to sign a consent form were also excluded.

The study enrolled 59 patients with a mean age of 49.15 ± 10.96 years; 23 men and 36 women), who were divided into two groups: 1) control group (n = 28: 13 men and 15 women) with body mass index (BMI) less than 30 kg/m^2 and without cardio-metabolic disorders; 2) patients with type 2 diabetes mellitus (n = 31: 10 men and 21 women) with BMI exceeding 30 kg/m^2.

The material for the biochemical tests was blood obtained the morning before surgery following overnight fasting by puncturing the ulnar vein in vacuum tubes with a clot-forming activator. The biochemical parameters of the blood were analyzed with a Furuno CA-180 analyzer (Furuno Electric Company, Japan) using DiaSys test systems (DiaSys Diagnostic Systems, Holzheim, Germany).

Biopsies of liver tissue were used as material for analyzing the expression of genes of interest. Total RNA was isolated from a liver biopsy with a volume of approximately 100 µl using ExtractRNA (Evrogen, Russia) according to the manufacturer's protocol. The isolated total RNA was eluted in 50 µl of RNase-free water. The RNA concentration was measured immediately after isolation using an Implen NanoPhotometer N (Implen, Germany). Samples were stored at –80°C until reverse transcription, followed by real-time polymerase chain reaction (RT-PCR).

Universal reverse transcription was performed using the MMLV RT kit (Eurogen, Moscow, Russia) with the addition of RiboCare RNase inhibitor (Eurogen, Moscow, Russia) according to the manufacturer's protocol. HS-SYBR PCR (Eurogen, Moscow, Russia) was used for quantitative PCR. The primer sequences are listed in the text below, the annealing temperature of all primers was 62°C. PCR results were amplified and read using a CFX96 thermal cycler (Bio-Rad, Hercules, CA, USA). After amplification, the melting curves were analyzed to verify the specificity of the reactions.

To normalize the gene expression data, the reference gene *RPLP0* was used as an internal control. The following primer sequences were used:

SIRT1-F: AGGAGCAGATTAGTAGGCGGC,
SIRT1-R: TGGACTCTGGCATGTCCCAC,
V1-F: AGGGCGAGGAGGAGGAAGAG,
V1-R: GTCCAGTCACTAGAGCTTGCA,
V2-F: TTCGCTCTTTTCCTCCGTCC,
V2-R: ACAGAAGGTTATCTGGCTGCT,
V3-F: CTGTGCAGTGGAAGGAAAACA,
V3-R: GATTCCCGCAACCTGTTCCA,
PPAR-γ-F: GATGACAGCGACTTGGCAATA,
PPAR-γ-R: GGCTTGTAGCAGGTTGTCTT,
PPAR-α-F: GCCCTGTCTGCTCTGTGGA,
PPAR-α-R: GCCGAGCTCCAAGCTACTCTT,
PGC-1α-F: TGCTCGGAGCTTCTCAAATATC,
PGC-1α-R: CCCAAGGGTAGCTCAGTTTATC,
MFN2-F: CCAGCGTCCCATCCCTCT,
MFN2-R: TCCACACCACTCCTCCAACA,
DRP1-F: TCTGGAGGTGGTGGGGTTG,
DRP1-R: TGGGTTTTGATTTTTCTTCTGCTAAT,
OPA1-F: ATCTGTGGATGCTGAACGCA,
OPA1-R: GAATCCTGCTTGGACTGGCT,
PRKAA1-F: ACAGAGATCGGGATCAGTTAG,
*PRKAA1-R:*GAGGTCACAGATGAGGTAAGA,
TFAM-F: CGCTCCCCCTTCAGTTTTGT,
TFAM-R: TACCTGCCACTCCGCCCTAT,
RPLP0-F: GGCGACCTGGAAGTCCAACT,
RPLP0-R: CCATCAGCACCACAGCCTTC.

Transcripts cycle of thresholds (Ct) were converted to relative expression values using the $2^{-\Delta Ct}$ method and then transformed to Log_{10} for normalization. Outliers were identified and excluded using the ROUT method (Q=1%). The normality of the data distribution was assessed using the Shapiro–Wilk test. If the data conformed to a normal distribution, the hypothesis of equality of sample means was tested using the Welch's t-test, otherwise the non-parametric Mann–Whitney test was used. The difference in the occurrence of transcript combinations of *SIRT1* isoforms between the groups was assessed using the chi-square test (χ^2 test). Correlations were determined using the Spearman's rank correlation coefficient. Differences were considered significant at $p < 0.05$. Statistical data processing was performed using GraphPad Prism 9.3.1 software.

Primers were designed for the *SIRT1* isoforms and *SIRT1* total (Fig. 2) so that they were only annealed at isoform-specific sites.

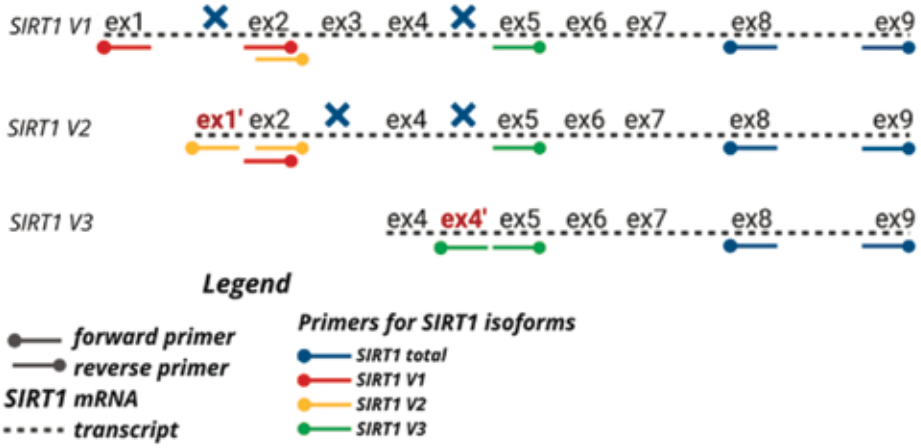

Fig. 2. Design of the primers for the *SIRT1* isoforms. *SIRT1 V1-V3* – *SIRT1* isoforms and specific primers for them; *SIRT1 total* – primers annealed to all *SIRT1* isoforms; ex1–9 – exons of the *SIRT1* gene

RESULTS

A comparative analysis of the clinical and biochemical parameters of the groups involved in the study is shown in Table 1. The control group differed significantly from the study group in terms of BMI, fasting glucose, triglycerides, and HDL. The groups did not differ significantly in other anthropometric and biochemical parameters.

The expression level of the genes of interest in liver biopsies was investigated. We found no significant differences in the mRNA expression of the transcription factors *PGC-1a* (p = 0.1275), *PPARγ* (p = 0.8047), and *PPARα* (p = 0.7927) (Figure 3, a). The expression level of genes associated with MD *TFAM* (p = 0.4188), *MFN2* (p = 0.6295), *OPA1* (p = 0.5149), and *DRP1* (p = 0.7507) also did not differ significantly (Fig. 3, a). The expression level of the AMPK subunit *PRKAA1* (p = 0.1430) and of *SIRT1 total* (p = 0.5609) and isoforms *V1* (p = 0.3166) and *V2* (p = 0.2254) did not change significantly, but the expression of isoform *V3* was significantly increased in patients with T2DM compared to the control group (1.3-fold change; p = 0.0009) (Fig. 3, b).

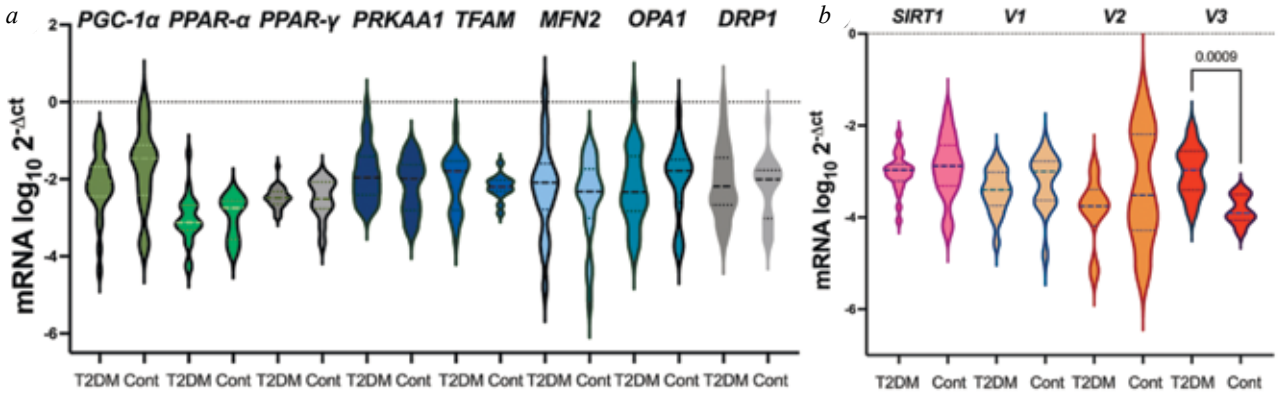

Fig. 3. Expression level of genes of interest: *a* – Genes related to mitochondrial homeostasis *PGC-1a, PPARα, PPARγ,* and *PRKAA1* and MD *TFAM, MFN2, OPA1,* and *DRP1*; *b* – *SIRT1* and its isoforms. Statistical analysis was performed using the Shapiro-Wilk test, the unpaired Mann–Whitney test and the Welch's t-test. Abbreviations: Cont – conditionally healthy donors, T2DM – patients with type 2 diabetes mellitus.

Table 1

Clinical and Kiochemical Parameters of the Studied Groups, *M* ± *SD*

	Control group, n = 28	T2DM, n = 31	p-value
BMI, kg/m^2	24.12 ± 3.78	49.49 ± 10.61	<0.0001**
Age, years	51.07 ± 13.43	47.22 ± 7.52	0.2009**
Sex (men / women)	13 / 15	10 / 21	0.2965***
Fasting glucose, mmol/l	4.56 ± 0.54	7.45 ± 1.98	<0.0001*
Total cholesterol (TC), mmol/l	5.27 ± 1.10	5.30 ± 0.91	0.9066**
Triglycerides (TG), mmol/l	1.17 ± 0.44	2.01 ± 1.09	<0.0001*
High-density lipoproteins (HDL), mmol/l	1.44 ± 0.37	1.23 ± 0.73	0.0036*
Low-density lipoproteins (LDL), mmol/l	3.18 ± 0.84	2.97 ± 0.71	0.5544*
Alanine aminotransferase (ALT), mmol/l	17.85 ± 13.87	22.45 ± 15.07	0.2010*
Aspartate aminotransferase (AST), mmol/l	24.27 ± 15.23	21.00 ± 8.36	0.2040*

* the analysis was performed using the unpair ed Mann–Whitney test, ** the analysis was performed using the Welch's t-test, *** the analysis was performed using the Fisher's exact test

When investigating the expression of *SIRT1* isoforms, it was found that *SIRT1* isoforms occurred not only individually, but also in various combinations. The occurrence of transcript combinations of *SIRT1* isoforms was analyzed in different groups (Fig. 4).

It was found that the *V2* isoform did not occur individually in patients with T2DM, while, in contrast, the *V3* isoform alone was only found in patients with T2DM, and the combination of *V2* + *V3* isoforms was not found in either patients or healthy individuals.

None of the *SIRT1* isoforms was detected in 15 individuals. The combination of isoforms differed significantly between patients with T2DM and the control group ($p = 0.0047$) (Fig. 4). The processes that control the expression patterns of the *SIRT1* isoforms have not yet been investigated.

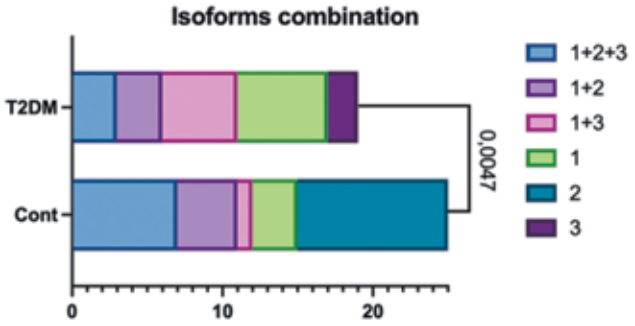

Fig. 4. Analysis of the combination of isoforms depending on the study group. The statistical analysis was performed using the χ^2 test.

The results of the correlation analysis are shown in Figure 5. It is worth noting that *SIRT1 total* did not show significant correlations with the genes of interest (*PGC-1a, PPARγ, PPARα, TFAM, MFN2, OPA1,* and *DRP1*), which only confirms the need to study the expression of the isoforms separately. In contrast to the *SIRT1 total*, *SIRT1* splicing isoforms correlated differently with genes related to mitochondrial homeostasis.

The expression level of the *V1* isoform correlated significantly with the expression level of *PGC-1a, PPARα,* and *PPARγ* ($r = 0.73$, $r = 0.73$, and $r = 0.45$, respectively; $p < 0.05$) and with *PRKAA1* ($r = 0.50$, $p < 0.05$) as well as with the expression level of the MD genes *MFN2, OPA1,* and *DRP1* ($r = 0.45$, $r = 0.50$, and $r = 0.45$, respectively; $p < 0.05$). The expression level of the *V2* isoform correlated positively with the expression level of *PGC-1a* ($r = 0.69$, $p < 0.05$), as well as with *PRKAA1* ($r = 0.58$, $p < 0.05$) and the MD genes *MFN2, OPA1,* and *DRP1* ($r = 0.74$, $r = 0.65$, and $r = 0.57$, respectively; $p < 0.05$). In contrast, the expression level of the *V3* correlated significantly only with the expression level of *MFN2* ($r = 0.53$, $p < 0.05$). In addition, a strong positive correlation was found between *V3* and FBG levels ($r = 0.68$, $p < 0.05$).

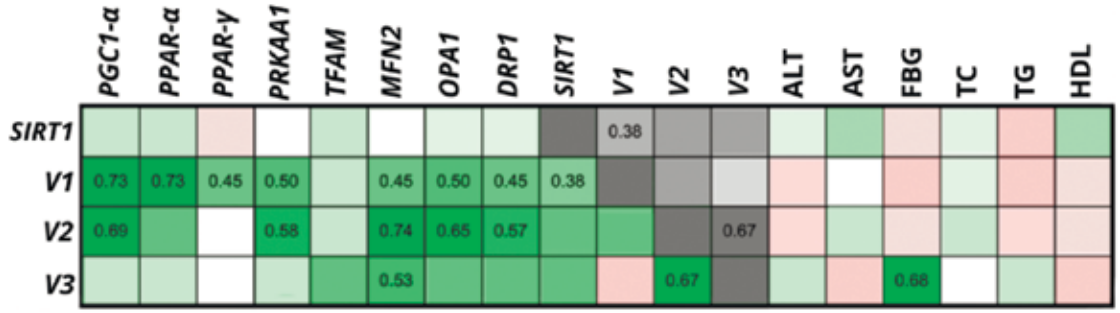

Fig. 5. Correlation matrix. Spearman's rank correlation coefficient, only significant correlations are shown ($p < 0.05$). Abbreviations: ALT – alanine aminotransferase; AST – aspartate aminotransferase; FBG – fasting blood glucose; TC – total cholesterol; HDL – high-density lipoproteins; TG – triglycerides

DISCUSSION

In this work, the expression of the histone deacetylase *SIRT1* in liver samples from patients with T2DM and obesity and apparently healthy donors was comprehensively investigated for the first time, taking into account the different *SIRT1* splicing isoforms and their relationship to the expression of the main signaling-associated genes.

The transcription factors *PGC-1a, PPARγ, PPARα,* and *TFAM*, which are associated with mitochondrial biogenesis and ATP synthesis, as well as the MD genes *MFN2, DRP1*, and *OPA1* were studied in detail in the context of T2DM and its comorbidities. For example, it was shown that the expression of *PGC-1a* was suppressed in the skeletal muscle of patients with T2DM [36, 37].

In mouse models of hepatic steatosis, it was shown that the level of *PGC-1a* expression and production in the liver decreased significantly, resulting in suppression of the expression of the mitochondrial transcription factor *TFAM* [21], and the level of *PPARα* expression also reduced significantly, while the level of *PPARγ* expression increased [38].

Using a cellular model of hepatic steatosis (HepG2 + oleic acid) *in vitro*, it was found that the production of *SIRT1* and *PGC-1a* decreased significantly, while a decrease in *SIRT1* levels directly suppressed mitochondrial division due to a decrease in MFF protein levels [39]. It was found that in the oxidative stress model

(HepG2 + tret-butylhydroperoxide), the expression of *TFAM, DRP1*, and *MFN2* was significantly reduced [40]. Consistent with these results, the expression and production of *MFN2* in the liver were reduced in a rat model of insulin resistance [41].

Our study showed that the expression level of all listed genes remained unchanged in the liver of patients with T2DM and obesity.

There are few such data on studies performed on human liver tissue (for T2DM or its comorbidities, especially steatosis and steatohepatitis).

Thus, in the English-language literature, no information was found on the nature of the expression of the *PRKAA1, TFAM, MFN2, DRP1,* and *OPA1* genes in the human liver in T2DM. At the same time, the expression of *PGC-1a* was found to be significantly decreased in the liver tissue of patients with obesity and T2DM [42]. The same pattern of expression changes was observed in hepatic steatosis [43], which was associated with an increase in the degree of methylation of the promoter of this gene. In another study, *PPARα* levels in human liver tissue negatively correlated with the degree of steatosis and the presence of steatohepatitis and positively correlated with adiponectin level [44]. In contrast, *PPARγ* levels were elevated in individuals with NAFLD [45]. The reasons why the expression of these genes did not change in our study are not entirely clear and require further investigation.

However, unique results were obtained for *SIRT1*. Previously, it was shown in animal models that *SIRT1* expression significantly decreased in T2DM or its concomitant diseases [46, 47]. In general, the role of *SIRT1* in insulin resistance in the liver is defined as protective as it enhances the regulation of gluconeogenesis and mitochondrial biogenesis [48]. Data on *SIRT1* expression in human liver are limited, but suggest that the pattern of *SIRT1* expression is generally similar to cellular and mouse models of T2DM, with a decreasing trend [49, 50]. We have shown that the expression of *SIRT1* in the liver did not change in patients with T2DM and obesity compared to the control group. However, this does not apply to the individual *SIRT1* isoforms. Thus, *V3* expression was significantly increased in patients with T2DM ($p = 0.0009$). In addition, *V3* expression level correlated positively with FBG levels ($r = 0.68, p < 0.05$), indicating a fundamental, completely unexplored relationship of this subunit with the pathogenesis of T2DM.

Differences in the expression patterns of *SIRT1* transcripts were found between the study groups. In both groups, a combination of all three isoforms was most frequently observed. However, the *V2* isoform was only expressed alone in the control group, while the *V3* isoform was only found individually in patients with T2DM. In general, the occurrence of transcript combinations of the *SIRT1* isoforms differed significantly between the control group and the study group (χ^2 test, $p = 0.0047$).

The correlation analysis data showed that *SIRT1 total* did not correlate significantly with the genes of interest. This is an unexpected and interesting result that only confirms the need to study the *SIRT1* splicing isoforms separately. At the same time, the individual *SIRT1* isoforms showed positive correlations with many genes of interest. Most importantly, the *SIRT1* isoforms had slightly different correlation signatures. Nuclear *V1* and cytoplasmic *V2* isoforms were similar in their association with MD gene expression levels (positively correlated with *MFN2, DRP1,* and *OPA1*) and with *PGC-1a* and *PRKAA1*, but differed in their association with the transcription factors *PPARγ* and *PPARα*: *V1* significantly correlated with them, whereas *V2* did not. At the same time, *V3* showed a correlation only with *MFN2*, which further underlines the presumed special function of *V3* in the liver.

Thus, the *SIRT1* isoforms may represent novel links in the regulation of mitochondrial homeostasis in T2DM. It is particularly important to study the isoforms separately as they have different expression patterns and probably regulate metabolic processes in the cell in different ways, including mitochondrial homeostasis.

CONCLUSION

SIRT1 V1, *V2*, and *V3* splicing isoforms were stably expressed in the liver of patients with T2DM, and the expression level of *SIRT1 V3* was significantly increased in the liver of patients with T2DM. Studying the *SIRT1* splicing isoforms thoroughly is necessary in the context of the molecular mechanisms of disruption/maintenance of mitochondrial homeostasis as it is likely that *SIRT1* isoforms regulate this process in different ways. The resulting correlation map can be used for further selective studies of interactions between *SIRT1* isoforms and mitochondrial homeostasis proteins.

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Prevalence of Iron Deficiency in Patients with Acute Decompensated Heart Failure

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ABSTRACT

Aim. The research aims to systematize current data on the prevalence, diagnosis, and clinical significance of iron deficiency (ID) in patients with acute decompensated heart failure (ADHF).

Materials and methods. A systematic analysis of studies from 2019 to 2024 in PubMed and eLibrary databases was conducted, including data from 6,500 patients with ADHF. Selection criteria were as follows: confirmed ADHF diagnosis, assessment of iron status using standard parameters (ferritin and transferrin saturation (TSAT)), and availability of clinical outcome data.

Results. To differentiate the type of iron deficiency, optimal diagnostics of simultaneous assessment of ferritin and TSAT levels requires: ferritin < 100 μg/L – absolute ID; ferritin 100–299 μg/L in combination with TSAT < 20% – functional ID. ID was found in 45–89% of ADHF patients and was associated with: more severe disease progression (functional class III-IV according to the New York Heart Association system in 68% of cases), elevated NT-proBNP levels (35% higher compared to non-ID patients), reduced exercise tolerance (six-minute walk test: 278±45 m vs 342±38 m in non-ID group).

Conclusion. Iron deficiency is an independent prognostic factor in ADHF. Early diagnosis and correction, particularly through intravenous ferric carboxymaltose administration, may improve clinical outcomes and reduce hospital readmission rates.

Keywords: heart failure, acute decompensated heart failure, iron deficiency, prevalence, clinical outcomes, prognosis

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Распространенность дефицита железа у больных с острой декомпенсацией сердечной недостаточности

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РЕЗЮМЕ

Цель. Систематизировать современные данные о распространенности, диагностике и клиническом значении дефицита железа (ДЖ) у пациентов с острой декомпенсацией сердечной недостаточности (ОДСН).

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Материалы и методы. Проведен систематический анализ исследований за период с 2019 по 2024 г. в базах PubMed и eLIBRARY, включающий данные 6 500 пациентов с ОДСН. Критерии отбора: подтвержденный диагноз ОДСН, оценка статуса железа по стандартным параметрам (ферритин, коэффициент насыщения трансферрина железом (КНТЖ)), наличие данных о клинических исходах.

Результаты. Для разграничения типа дефицита железа оптимальная диагностика требует одновременной оценки уровня ферритина и КНТЖ: ферритин менее 100 мкг/л – абсолютный ДЖ; ферритин 100–299 мкг/л в комплексе с КНТЖ менее 20% – функциональный ДЖ. Установлено, что ДЖ встречается у 45–89% пациентов с ОДСН и ассоциирован с более тяжелым течением заболевания (функциональный класс III–IV по классификации Нью-Йоркской кардиологической ассоциации в 68% случаев), повышением уровня N-концевого пропептида натрийуретического пептида типа B (в среднем на 35% по сравнению с пациентами без ДЖ), снижением толерантности к физической нагрузке (тест шестиминутной ходьбы: 278 ± 45 м против 342 ± 38 м в группе без ДЖ).

Заключение. Дефицит железа – независимый прогностический фактор при ОДСН. Его ранняя диагностика и коррекция, в частности внутривенным введением железа карбоксимальтозата, могут улучшить прогноз и снизить частоту повторных госпитализаций.

Ключевые слова: сердечная недостаточность, острая декомпенсация сердечной недостаточности, дефицит железа, распространенность, клинические исходы, прогноз

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Авторы заявляют об отсутствии финансирования при проведении исследования.

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INTRODUCTION

Heart failure (HF) represents one of the most significant challenges in modern cardiology. Its global prevalence ranges from 1 to 2% among the adult population, increasing with age: from less than 1% in individuals under 55 years to over 10% in those over 70 years. The true prevalence of HF is believed to be significantly higher, especially among patients with heart failure with preserved ejection fraction (HFpEF) [1].

In the Russian Federation, epidemiological studies indicate that the prevalence of chronic heart failure (CHF) in the general population reaches 7% (12.35 million people), including 4.5% of cases with clinically manifested CHF [2]. Particular attention is paid to patients with acute decompensated heart failure (ADHF), who have an extremely poor prognosis, high rates of readmission, and, consequently, impose significant financial burdens – making them a critical public health issue. The 5-year mortality rate following an ADHF episode can be as high as 75% [3, 4].

Numerous studies support the concept that hospitalization for ADHF often signifies a sharp turn in the natural history of HF. The rate of rehospitalization or death reaches 50% within 6 months after the initial ADHF event, which is considerably higher than the event rates observed after acute myocardial infarction [5, 6]. By 2030, the annual number of patients hospitalized with ADHF is projected to rise from 1.5 million to 8 million, with associated financial costs steadily increasing by over 50% compared to previous periods [7]. According to the independent Russian ORACUL-RF registry, following an ADHF hospitalization episode, the 30-day readmission rate was 31%, and all-cause mortality was 13%, increasing to 43% at one year of follow-up [8].

Iron deficiency (ID) and anemia are among the most common comorbidities in CHF, associated with an unfavorable prognosis, reduced exercise tolerance, and impaired quality of life [9]. ID is significantly more prevalent in HF patients than anemia, being diagnosed in 60% of outpatients and 80% of inpatients with HF. Literature data suggest that ID occurs in over 80% of hospitalized patients in Russia [10]. Recently, ID has come to be viewed not merely as a concomitant disease but as an integral component involved in the pathophysiology of HF

development and progression [11–13]. Growing evidence supports a key role for ID in adverse long-term outcomes in HF patients.

Optimal HF therapy, including a comprehensive pharmacological approach, contributes to a reduction in the frequency of ID, even without direct iron supplementation. However, despite a substantial number of conducted studies, the significance of ID in the post-ADHF patient population remains uncertain, posing a challenge for the scientific community to continue optimizing algorithms for timely diagnosis and effective correction strategies [14].

The aim of this review is to systematize current data on the prevalence of ID in patients with ADHF and to assess its impact on the disease course and outcomes. The article will review key studies conducted in this field and present data on the frequency of ID in this patient population.

MATERIALS AND METHODS

A systematic approach was chosen to review the literature on ID in patients with ADHF. The methodology included the following steps:

Formulating research questions: defining key questions the review should answer, including the prevalence of ID and its impact on patient status.

Study selection criteria: inclusion of studies published in Russian and English over the last 5 years (2019 to 2024). Works lacking empirical data or not relevant to the review topic were excluded.

Source search: utilizing medical databases, such as PubMed and eLibrary with specialized search queries, including keywords like "iron deficiency" and "acute decompensated heart failure."

Data collection and analysis: reviewing and synthesizing data from selected studies to identify common trends and differences in methodologies and results.

Results synthesis: integrating the obtained data into a unified analytical review reflecting the current state of knowledge on the problem.

IRON DEFICIENCY AND ITS SIGNIFICANCE IN HEART FAILURE

Iron is one of the most essential micronutrients, an integral component of many enzymes and proteins playing a central role in functions, such as cellular respiration, cell proliferation, biosynthesis of oxygen-transporting molecules, synthesis and repair of nucleic acids, and as a cofactor in numerous other enzymatic reactions [15, 16].

Iron deficiency is defined as "a health-related condition in which iron availability is insufficient to meet the body's needs and which can be present with or without anemia." In most clinical situations, diagnosing ID requires the determination of two parameters: serum ferritin level and transferrin saturation (TSAT) [17].

In patients with CHF, iron deficiency is defined as a serum ferritin level < 100 μg/L (absolute ID) or a ferritin level in the range of 100–299 μg/L (functional ID) in combination with transferrin saturation < 20%, alongside a decrease in serum iron to a level less than 13 μmol/L with TSAT < 20% [19]. According to the Russian Society of Cardiology Guidelines for CHF, all HF patients should undergo regular screening for anemia and ID with a complete blood count, measurement of serum ferritin concentration, and TSAT (Class of recommendation I, Level of evidence C) [2].

It is known that in the absence of inflammation or chronic disease, serum ferritin correlates with body iron stores, and a serum ferritin level of 100 μg/L corresponds to approximately 1g of tissue iron. In healthy individuals, a ferritin level below 30 μg/L and TSAT below 16% define iron deficiency [9]. In inflammation, including HF, the ferritin level is non-specifically elevated as an acute-phase reactant, making the identification of absolute or functional ID challenging [20]. For this reason, various clinical trials on ID correction in HF patients have used ferritin levels < 100 μg/L or < 300 μg/L if TSAT < 20% to identify patients with absolute and functional ID [21, 22].

Ferritin is the primary storage protein for iron in tissues. There is a direct correlation between iron stores in the reticuloendothelial system and the serum ferritin level, allowing the latter to be used as a marker of iron reserves. A serum ferritin level < 30 μg/L indicates low iron stores and is diagnostic for ID in patients without chronic inflammatory or infectious diseases [20]. However, the properties of ferritin as an acute-phase protein mean its level increases in inflammatory conditions, such as chronic kidney disease, HF, liver disease, and cancer. Consequently, higher threshold ferritin concentrations are used to define ID in HF patients

compared to healthy individuals. Under these conditions, ferritin levels within the normal range differ from those mentioned above (Table 1).

Table 1

Diagnostic Criteria for Iron Deficiency [20]

Condition	Serum ferritin, μg/L	TSAT, %
Preoperative Iron Deficiency	CRP <5 mg/L: <30 CRP >5 mg/L: <100	<20
Iron Deficiency in HF	<100	<20 (if serum ferritin is 100–299 μg/L)
Iron Deficiency in CKD	CKD Stage C3–C5: ≤100 CKD Stage C5D: ≤200	≤20

Note. TSAT – transferrin saturation, HF – heart failure, CKD – chronic kidney disease, CRP – C-reactive protein

In chronic inflammatory or infectious diseases, measuring serum ferritin alone may not always suffice to diagnose ID. Under these circumstances, calculating TSAT is also necessary. Transferrin saturation represents the ratio of serum iron to total iron-binding capacity, expressed as a percentage, and reflects the proportion of active sites on serum transferrin occupied by iron atoms [27]. TSAT values < 20% are accepted as thresholds for diagnosing ID in chronic somatic-symptom diseases, such as HF or chronic kidney disease (Table 1).

Some studies emphasize the more significant role of TSAT compared to ferritin as a key indicator of ID and adverse outcomes [14, 23]. For instance, the 2021 study by P. Palau et al. assessed the association between potential markers of ID – TSAT and serum ferritin – and the risk of readmission (RA) within 30 days or death in patients with ADHF. Over 30 days, 177 events (10.4%) were recorded (95 deaths and 85 HF-related readmissions). After multivariate adjustment, lower TSAT was associated with an increased risk of short-term events (p = 0.009), while no such association was found for ferritin levels (HR 1.00; 95% CI 0.99 1.00, p = 0.347) [23].

These diagnostic criteria for ID in HF patients are presented in the 2021 European guidelines, which is consistent with the opinion of Russian experts [20]. It is important to remember that serum iron concentration can exhibit significant diurnal variations in HF patients; therefore, this laboratory parameter cannot be used independently to diagnose ID in this patient category.

Another proposed marker, suggested as an accessible and cost-effective tool for identifying ID in ADHF patients, is reticulocyte hemoglobin content (Ret-He) [24]. Reticulocytes now offer a rapid way to assess iron status. Unlike mature erythrocytes, which have a lifespan of 120 days, reticulocytes are renewed in the bone marrow every 2–4 days. This feature provides current data on the quality of the cellular pool and iron availability at the time of testing, unlike a standard complete blood count, which reflects the state of hematopoiesis with a significant time delay. Unlike traditional parameters (serum iron, ferritin, and transferrin), Ret-He levels are not influenced by inflammatory processes, making it a reliable indicator of current iron bioavailability.

Dynamic monitoring of this parameter allows for an objective assessment of the effectiveness of therapy for ID states: an increase in Ret-He values indicates a positive response to treatment [25]. A unified Ret-He threshold value of 32.4 pg for ID screening, based on two criteria (TSAT < 20% and serum ferritin <100 μg/L), underscores its potential as a universal indicator for diagnosing ID in this patient population. This approach may improve clinical outcomes in patients hospitalized with ADHF by enabling faster, more accurate, and accessible determination of ID, both in the inpatient setting and during subsequent outpatient visits [24].

PREVALENCE OF IRON DEFICIENCY AMONG ADHF PATIENTS

The first studies exploring the relationship between ID and HF appeared in the scientific literature in the late 20th century. However, a deeper understanding of this link and its clinical significance became a subject of active investigation only in the early 21st century. Since 2010, following large-scale clinical trials and meta-analyses, the topic of ID in HF has seen significant development. These works have provided a better understanding of the mechanisms through which ID affects myocardial function and its role in HF progression [26–28].

One of the key studies addressing ID in ADHF patients was work by international researchers published in 2019. In this study conducted at a leading medical center, ID was diagnosed based

on a serum ferritin level < 100 μg/L or TSAT < 20% with a ferritin level of 100–299 μg/L. Among the 503 included patients, 270 (55%) had heart failure with preserved ejection fraction (HFpEF), 160 (33%) had heart failure with reduced ejection fraction (HFrEF), and 57 (12%) had HFmrEF. ID was identified in 54% of patients with HFrEF and 56% of patients with HFpEF. The authors emphasized the high prevalence of ID among ADHF patients, regardless of HF type [29].

In 2023, the study "Ferric Carboxymaltose in Patients with Acute Decompensated Heart Failure and Iron Deficiency: A Real-Life Study" investigated the efficacy of ID correction using ferric carboxymaltose in ADHF patients. Among 104 hospitalized patients (mean age 84 years, 53.5% with HFpEF), 90 underwent a complete iron status assessment. ID was diagnosed in 73 (81.1%) patients [30].

In 2022, data covering the period from January 2013 to December 2018 were published, showing that among 1,863 patients hospitalized with ADHF (both HFrEF and HFpEF), 840 (45%) had laboratory signs of ID (absolute or functional), meeting the inclusion criteria [31].

In the study by D.H.Van Dalen et al. (2022), the prevalence and dynamics of ID in ADHF patients were examined. At hospitalization (T0), ID was detected in 71.8% of patients (44.1% had absolute ID, 27.7% – functional ID). After clinical stabilization prior to discharge (T1) and 10 ± 6 weeks post-discharge (T2), ID persisted in 56.4% and 50.3% of patients, respectively. Absolute ID persisted from T0 to T2 in 66% of patients, whereas functional ID resolved in 56% of patients. Ferritin, transferrin saturation, and serum iron levels increased significantly from T0 to T1 and from T1 to T2, even without iron supplementation. The authors concluded that ID was highly prevalent in ADHF patients but may resolve spontaneously during treatment in some. Absolute ID resolved more often, while functional ID frequently resolved with ADHF therapy [32].

In the study by K.AlAayedi (2023), current practices for diagnosing and treating ID in hospitalized ADHF patients with HFpEF were evaluated. Among 111 patients, 74% (82) had their iron status analyzed, and ID was diagnosed in 63% (52) of them according to European Society of Cardiology (ESC) criteria. Among patients with ID, 54% (28) also had anemia. ID correction was prescribed to 34 out of 52 patients (65%), indicating insufficient clinician awareness of the importance of treating ID [36].

In the retrospective multicenter study "Iron Deficiency and Short-term Adverse Events in Patients with Decompensated Heart Failure" (P. Palau et al., 2021), which included 1,701 patients, ID was identified in 1,246 (73.3%) patients according to the ESC definition [23].

The research conducted by V.Yu.Mareev et al. demonstrated a high prevalence of ID among HF patients. In an extensive analysis encompassing 498 patients (198 women and 300 men), ID was detected in 83.1% of those examined. Patients with ID were older and had more pronounced functional myocardial changes: median age was 70.0 [63.0; 79.0] years compared to 66.0 [57.0; 75.2] years in the non-ID group (p = 0.009). Notably, anemia was diagnosed in only 43.5% of patients with ID, highlighting the independence of these two conditions [33]. In the study by Smirnova et al., the prevalence of iron deficiency was analyzed among 294 patients with chronic heart failure (mean age 71.3±0.4 years) hospitalized due to decompensation in coronary artery disease and/or arterial hypertension, and worsening HF symptoms. The results demonstrated a progressive decrease in serum iron levels and transferrin saturation with increasing NYHA functional class. Iron deficiency was diagnosed in 72% of examined patients (n = 213), with a pronounced gender imbalance (78% of cases in women vs. 22% in men). The combination of ID and anemia was observed in 25% of cases, whereas isolated anemia without ID was found in 16% [34].

In the study by E.A.Smirnova et al., data from 80 patients with ADHF revealed a high prevalence of ID – 80% of cases. Absolute ID was diagnosed in 82.8% of patients, while functional ID was observed in only 17.2% of cases. Concomitant anemia of varying severity was detected in 35% of those examined: mild degree was registered in 64.3%, moderate and severe – in 25 and 10.7% of patients, respectively. Importantly, in 89.3% of cases, anemia was iron-deficient, while anemia of chronic disease occurred in only 10.7% of cases. The combination of ID and anemia was noted in

31.2% of patients, while normal iron metabolism and hemoglobin levels were recorded in only 16.3% of patients [10].

The study conducted by Zh.D. Kobalava et al. investigated the prevalence and prognostic significance of ID in patients with ADHF. The results showed that the frequency of ID ranged from 70% to 89% depending on the diagnostic criteria used. Using Criterion A (ferritin level < 100 μg/L or ferritin 100–299 μg/L in combination with TSAT < 20%), ID was detected in 89% of patients, including absolute ID in 153 (69%) and functional ID in 46 (20%). Criterion B (TSAT < 20% and serum iron level < 13 μmol/L) showed ID in 70% of patients [35].

Analysis of data obtained from 223 ADHF patients demonstrated that according to Criterion A, ID without anemia was observed in 106 (47%) patients, ID with anemia – in 93 (42%), and anemia without ID – in 9 patients (4%). Fifteen (7%) patients had normal hemoglobin and serum iron metabolism markers. Using Criterion B, ID without anemia was identified in 77 (35%) patients, ID with anemia – in 79 patients (35%), and anemia without ID – in 24 cases (11%). Forty-three (19%) patients had no abnormalities in hemoglobin content or serum iron metabolism markers. The study underscores the high prevalence of ID among ADHF patients and its independence from the presence of anemia. The authors also noted that the choice of diagnostic criteria significantly affected ID detection rates, highlighting the need for standardized diagnostic approaches [14, 35].

Studies show that ID is a common condition among patients with ADHF. Across various studies, the prevalence of ID ranges from 45% to 89% (Table 2).

IMPACT OF IRON DEFICIENCY ON CLINICAL PARAMETERS

According to the available data, patients with ADHF and concomitant ID have distinct clinical characteristics indicating a more severe disease course and a less favorable prognosis.

1. Patients with ID cover a shorter distance in the six-minute walk test (6MWT) compared to patients without ID, indicating worse physical endurance.

Data from the Russian multicenter cross-sectional screening study (2023) determined that the mean 6MWT distance in CHF patients with concomitant ID was 155.9 ± 84.0 m versus 239.6 ± 82.7 m in the group without iron metabolism disorders (p = 0.01) [10]. These results are corroborated by the study of V.Yu.Mareev et al. (2022), which also found a significant reduction in the 6MWT distance in ID (250 [170;320] m vs. 299 [210;358] m in the non-ID group, $p < 0.001$) [33].

2. Patients with ID have lower scores on the visual analog scale (VAS), indicating a lower quality of life.

As shown by the study by E.A. Smirnova et al., organized by the Russian Heart Failure Society, the mean VAS score in the ID patient group was 36.4 ± 16.3 versus 46.3 ± 20.7 in the group without iron metabolism disorders (p = 0.036), indicating a substantial deterioration in quality of life associated with concomitant ID [10].

3. The level of N-terminal pro-B-type natriuretic peptide (NT-proBNP) is significantly higher in patients with ID, which may indicate more pronounced heart failure symptoms.

In the study led by V.Yu. Mareev et al. (2022), it was shown that each 100 pg/mL increase in NT-proBNP level was associated with an increased likelihood of having ID (OR 1.006 [1.002–1.011], p = 0.0152) [33]. These data correlate with the results of E.A. Smirnova et al., where patients with ID had significantly higher NT-proBNP levels (5,155.5 [3,267.3;9,786.3] pg/mL) compared to the group without ID (2,055.5 [708.8; 2,839] pg/mL, $p < 0.001$). Interestingly, the presence of concomitant anemia did not have an additional impact on NT-proBNP levels in patients with ID (5,683.0 [3,494.5; 7,863.5] pg/mL in ID with anemia vs. 5,110.0 [2,779.0; 10,140.0] pg/mL in isolated ID, p = 0.799) [10].

Additional evidence of the link between ID and HF progression and myocardial fibrosis was obtained in the study by Zh.D. Kobalava et al. (2022). In this work, patients with ID had significantly higher levels of C-reactive protein (15.1 mg/L vs. 6.2 mg/L in the control group, $p < 0.001$), NT-proBNP (5,422 pg/mL vs. 2,380 pg/mL, $p < 0.001$), and soluble suppression of tumorigenicity 2 receptor (sST2) (59.6 ng/mL vs. 42 ng/mL, p = 0.02). The observed elevation in sST2, a specific marker of myocardial fibrosis, is of particular significance. The obtained data suggest that patients with ID

Table 2

Prevalence of Iron Deficiency Among Heart Failure Patients

Study	Number of Patients	Age (years)	ID Prevalence, %	Anemia Prevalence, %	ID & Anemia Co-prevalence, %
Factors Associated with Iron Deficiency in Patients with Chronic Heart Failure.M.P. Smirnova et al. (2023) [34]	213 (Women – 166 (78%), Men – 47 (22%))	71.3±0.4	72	22.4	25
Prevalence of Iron Deficiency in Patients with Chronic Heart Failure in the Russian Federation. Data from an Observational Cross-sectional Study. Failure.M.P. et al. (2022) [33]	510	70 [63; 79]	83.1	40.4	43.5
Prognostic Significance of Different Iron deficiency Criteria in Patients with Decompensated Heart Failure. Zh.D.Kobalava et al. (2022) [35]	223	73 [65; 82]	Criterion (1) ferritin < 100 ng/mL or 100–299 ng/mL with TSAT < 20%* Criterion (2) TSAT < 20% and serum iron < 13 μmol/L By Criterion (1): 89% (Absolute ID – 69%, Functional ID – 20%) By Criterion (2): 70%	By Criterion (1): 4% By Criterion (2): 11%	By Criterion (1): 42% By Criterion (2): 35%
Prevalence and Clinical Significance of Iron Deficiency in Patients with Acute Decompensated Heart Failure. E.A.Smirnova et al. (2023) [10]	80 (62.5% men)	68.4±11.1	80	35	31.2
Iron Deficiency in Acute Decompensated Heart Failure. A.Beale et al. (2019) [29]	503 (43% women) HFpEF (55%) HFrEF (33%) HFmrEF (12%)	78 ± 11	Overall ID –57% (of which 54% HFrEF, 56% HFpEF) *Absolute ID –38%* *Functional ID – 18%*	–	–
Ferric Carboxymaltose in Patients with Acute Decompensated Heart Failure and Iron Deficiency: A Real-Life Study. F. Capone et al. (2023) [30]	90 HFpEF – 53.5%	84	81.1	66.7	69.9
IV Sodium Ferric Gluconate Complex in Patients Hospitalized Due to Acute Decompensated Heart Failure and Iron Deficiency". I. Borreda et al. (2022) [31]	1,863	74.28 [65.42; 81.55]	45	–	–
Iron deficiency and short-term adverse events in patients with decompensated heart failure. P. Palau et al. (2021) [23]	1,701	76 [68; 82]	73.3	–	–
The Burden of Iron Deficiency in Heart Failure: Therapeutic Approach. D.H.Van Dalen et al. (2022) [32]	692	78 [70; 84]	At hospitalization (T0): 71.8% At discharge (T1): 56.4% 10 ± 6 weeks post-discharge (T2): 50.3% Absolute ID persisted in 66% from T0 to T2. Functional ID resolved in 56%	–	–
An Audit of Iron Deficiency in Hospitalized Heart Failure Patients: A Commonly Neglected Comorbidity K.AlAayedi (2023) [36]	82	>18	63	–	54

Note. SAT – transferrin saturation, ID – iron deficiency, ADHF – acute decompensated heart failure, HFrEF – heart failure with reduced ejection fraction, HFmrEF – heart failure with mid-range ejection fraction, HFpEF – heart failure with preserved ejection fraction.

exhibit more pronounced myocardial remodeling compared to patients without iron metabolism disorders. These results align with the general trend observed in the study by T.B. Pecherina et al., where sST2 and NT-proBNP demonstrated high prognostic value for assessing the risk of myocardial fibrosis [18, 35].

4. Significantly more patients with ID have NYHA functional class IV heart failure, indicating a more severe disease course.

Studies show that ID correlates with CHF functional class. Research by Zh.D.Kobalava et al. (2022) and E.A. Smirnova et al. (2023) found that patients with ID had higher NYHA functional classes [10, 35]. M. Smirnova and P. Chizhov identified ID in 68% of patients with NYHA functional class III-IV CHF and in 58% of patients with stage IIB–III CHF according to the Strazhesko-Vasilenko classification [34].

5. According to the EUROQOL GROUP EQ-5D questionnaire, patients with ID more frequently experience difficulties with mobility and daily activities and report significant discomfort.

Within the Russian multicenter screening study, analysis of the EUROQOL EQ-5D questionnaire data revealed that patients with ID experienced significantly more pronounced limitations: 41.3% reported an inability to walk independently (vs. 6.3% in the non-ID group, $p < 0.001$) and 49.2% reported significant difficulties in daily activities (vs. 18.8%, $p < 0.01$). The presence of ID was associated with more severe clinical manifestations of CHF, reduced exercise tolerance, and a substantial deterioration in quality of life indicators [10].

6. Patients with ID more frequently present with edema, pulmonary rales, hydrothorax, and other manifestations of heart failure.

According to data from the study by E.A. Smirnova, patients with ID had significantly more frequent signs of congestion in the pulmonary circulation (46.9% of cases) manifesting as cardiac asthma (43.8%) and pulmonary edema (6.3%), as well as pronounced symptoms of systemic congestion (96.9% of cases), including lower extremity edema (84.4%), hydrothorax (65.6% vs. 31.3% in the non-ID group; $p = 0.012$), ascites (29.7%), hydropericardium (18.8%), and anasarca (15.6%, with a complete absence of this symptom in patients without ID) [10].

7. Patients with ID receive higher initial doses of intravenous diuretics, which may be associated with a more severe condition.

In the study conducted by E.A. Smirnova et al., ID was more common in patients with frailty, which also required the prescription of higher initial doses of intravenous diuretics. These data underscore the need for an individualized treatment approach for this patient category, with special attention to ID correction, to improve treatment outcomes and quality of life [10].

8. The length of hospital stay (LOS) for patients with ID is slightly higher, but the difference is insignificant and comparable to patients without ID.

A 2023 Russian study assessed the prognostic significance of ID in patients suffering from ADHF. ID was associated with an increased risk of readmission and death, once again emphasizing the importance of considering this parameter [14]. In a 2009 study by international colleagues (published in 2019), the problem of ID in ADHF patients was examined. The study aimed to characterize ID and determine its association with dyspnea class, length of hospitalization, biomarker levels, and echocardiographic parameters of diastolic function in patients with reduced and preserved left ventricular ejection fraction.

Among the 503 enrolled patients, 270 (55%) had HFpEF, 160 (33%) had HFrEF, and 57 (12%) had HFmrEF. ID was identified in 54% of HFrEF patients and 56% of HFpEF patients. In the HFpEF patient group, ID was associated with increased LOS (11 ± 7.7 days vs. 9 ± 6 days in patients without ID, $*p* = 0.036$) and remained an independent predictor of increased LOS after adjusting for comorbidities, age, and ID status. The finding of increased LOS specifically in HFpEF patients, which was not observed in HFrEF patients, is particularly significant. This may indicate a more pronounced role of ID in the pathogenesis of HFpEF [29].

The work by K. AlAayedi also evaluated the significance of ID in hospitalized ADHF patients with HFpEF: the mean length of hospitalization for patients with ID was 13.8 days versus 11.2 days for patients without ID [36]. These findings highlight the need for further study of ID as a potential therapeutic target in the treatment of ADHF, especially in patients with HFpEF, and

could significantly impact clinical practice and the direction of future research in cardiology.

CONCLUSION

The conducted analysis has shown that ID is an extremely prevalent (45–89% of cases) and clinically significant condition in patients with ADHF. Differentiating between absolute and functional iron deficiency against the background of chronic inflammation characteristic of HF poses a particular diagnostic challenge, necessitating the use of comprehensive criteria that include not only traditional parameters (ferritin and transferrin saturation) but also modern markers, such as soluble transferrin receptors, hepcidin, and reticulocyte hemoglobin.

Intravenous iron administration, particularly ferric carboxymaltose, has demonstrated efficacy in reducing the risk of hospital readmissions and improving patients' functional capacity. However, questions remain regarding the long-term impact of ID correction on survival, optimal treatment regimens for different HF phenotypes, and the role of new biomarkers in predicting structural and functional myocardial changes. Promising directions for future research include investigating the mechanisms of ID influence on remodeling processes, developing personalized diagnostic and treatment algorithms considering individual HF course characteristics, and assessing the cost-effectiveness of ID screening in routine clinical practice.

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The Role of IL-1β and RANK-L in the Pathogenesis of Chronic Periodontitis

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ABSTRACT

Aim. To establish the role of interleukin 1β (IL-1β) and receptor activator of nuclear factor κB ligand (RANK-L) in combination with microbial invasion in chronic periodontitis.

Materials and methods. The clinical material was the gingival fluid of patients with chronic periodontitis (60 people) and with an intact periodontium (28 people). The content of IL-1β and RANK-L was determined using enzyme-linked immunosorbent assay (ELISA). Markers of periodontopathogenic bacteria were isolated during real-time polymerase chain reaction (PCR). Statistical data processing was carried out using the STATA v.14 software package.

Results. In the group of patients with chronic periodontitis, the levels of IL-1β and RANK-L were significantly higher than in individuals with intact periodontium (median 37.1 [32.9; 41.3] pg/ml versus 2.5 [1.9; 3.4], $p < 0.001$) and median 6.3 [4.2; 10.4] pg/ml versus 0.0 [0.0; 0.7], $p < 0.001$), respectively). In patients with chronic periodontitis, periodontopathogens were detected in 100.0% of the cases (*A. actinomycetemcomitans* – 81.7%, *P. gingivalis* – 76.7%, *T. forsythia* – 70.0%, associations – 60.0%), while in the group with intact periodontium, periodontopathogenic bacteria were isolated in only 32.1%. In the group of patients with periodontitis, the quantitative content of IL-1β and RANK ligand positively correlated with all periodontopathogens of the first order, while the strongest correlations were found with an average degree of destruction of periodontal tissues.

Conclusion. The presence of relationships between *A. actinomycetemcomitans, P. gingivalis,* and *T. forsythia* with an increased content of the proinflammatory cytokine IL-1β and the immune mediator RANK-L and the severity of bone tissue destruction may indicate a key synergistic effect of these cytokines in the inflammatory and bone-plastic events of the pathogenesis of chronic periodontitis.

Keywords: cytokines; periodontopathogenic bacteria; chronic periodontitis

Conflict of interest. The authors declare the absence of obvious and potential conflict of interest related to the publication of this article.

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Conformity with the principles of ethics. All patients signed a voluntary informed consent to participate in the study. The study was approved by the local Ethics Committee at Federal State Budgetary Educational Institution of Higher Education Northern State Medical University (Arkhangelsk) of the Ministry of Healthcare of the Russian Federation (Minutes No. 8/11 dated November 28, 2018).

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Роль IL-1β и RANK-L в патогенезе хронического пародонтита

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РЕЗЮМЕ

Цель. Установить роль интерлейкина 1β (IL-1β) и лиганда активатора рецептора ядерного фактора κB (RANK-L) на фоне микробной инвазии в патогенезе хронического пародонтита.

Материалы и методы. Клиническим материалом послужила десневая жидкость пациентов с хроническим пародонтитом (60 человек) и с интактным пародонтом (28 человек). С помощью иммуноферментного анализа определяли содержание IL-1β и RANK-L. Маркеры пародонтопатогенных бактерий выделяли в ходе полимеразной цепной реакции в режиме реального времени. Статистическая обработка данных проведена с помощью пакета программ STATA v. 14.

Результаты. В группе пациентов с хроническим пародонтитом уровни IL-1β и RANK-L были значительно выше, чем у лиц с интактным пародонтом (37,1 [32,9; 41,3] пг/мл против 2,5 [1,9; 3,4], $p < 0,001$) и 6,3 [4,2; 10,4] пг/мл против 0,0 [0,0; 0,7], $p < 0,001$) соответственно). У пациентов с хроническим пародонтитом частота выявления пародонтопатогенов составила 100,0% (*A. actinomycetemcomitans* – 81,7%, *P. gingivalis* – 76,7%, *T. forsythia* – 70,0%, ассоциации – 60,0%), тогда как в группе с интактным пародонтом пародонтопатогенные бактерии выделялись лишь у 32,1%. В группе пациентов с пародонтитом количественное содержание IL-1β и лиганда RANK положительно коррелировало со всеми пародонтопатогенами I порядка, при этом наиболее сильные корреляции были выявлены при средней степени деструкции тканей пародонта.

Заключение. Наличие взаимосвязей между выделением пародонтопатогенов *A. actinomycetemcomitans, P. gingivalis, T. forsythia* с повышенным содержанием провоспалительного цитокина IL-1β и иммунного медиатора RANK-L, а также выраженностью степени деструкции костной ткани может свидетельствовать о ключевом синергидном эффекте данных цитокинов в воспалительных и деструктивных процессах патогенеза хронического пародонтита.

Ключевые слова: цитокины, пародонтопатогенные бактерии, хронический пародонтит

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INTRODUCTION

The multifactorial etiology of periodontal diseases determines the complexity of their pathogenesis and requires a systematic approach to study the mechanisms driving pathology development. A key link in the pathogenic chain is an imbalance in the immune response system, which leads to an inappropriate inflammatory response to the invasion of periodontopathogenic microbiota [1]. Cytokines play a central role in the pathogenesis of periodontal diseases, acting as the main mediators of intercellular interactions and the activation of immune and stromal cells, leading to local inflammation and tissue damage, including the destruction of periodontal ligaments, gingiva, and alveolar bone resorption [2].

Immunopathological mechanisms of periodontitis development are characterized by a response to microbial invasion by periodontopathogenic microorganisms (mainly *P. gingivalis, A. actinomycetemcomitans,* and *T. denticola*) and by an imbalance between pro- and anti-inflammatory cytokines, which leads to the development of chronic inflammation [3]. A number of studies have demonstrated that interleukin 1β (IL-1β), IL-6, and tumor necrosis factor-alpha (TNFα) may play a key role in the mechanisms driving periodontitis development [4, 5].

Chronic periodontitis is characterized by alveolar bone resorption caused by the proliferation of immature osteoclast precursors and their differentiation into mature osteoclasts, which promotes the degradation of organic and inorganic bone components. Osteoclast differentiation is primarily regulated by the receptor activator of nuclear factor κB (RANK), RANK ligand (RANK-L), and osteoprotegerin. RANK-L, also known as osteoclast differentiation factor, osteoprotegerin ligand, and TNF-associated activating cytokine, is the most potent known inducer of osteoclastogenesis [6].

Although IL-1β was one of the first cytokines proposed as a relevant biomarker for the early diagnosis of periodontitis [2], its relationship with RANK-L, periodontopathogenic microflora, and the degree of periodontal tissue destruction remains poorly understood. Therefore, the aim of this study was to establish the role of IL-1β and RANK-L in the pathogenesis of chronic periodontitis caused by microbial invasion.

MATERIALS AND METHODS

The analysis included data from clinical and laboratory examinations of 88 patients (men and women aged 18–45 years) who visited a periodontist at the Severodvinsk Dental Polyclinic, State Autonomous Healthcare Institution of the Arkhangelsk Region. These included 60 patients with a confirmed diagnosis of chronic periodontitis (according to ICD-10 – K05.3) and 28 patients with clinically healthy periodontium. The study was cross-sectional in design.

In accordance with the World Medical Association Declaration of Helsinki (last revision at WMA General Assembly, Fortaleza, Brazil, October 2013), each patient provided a written voluntary informed consent to participate in the study. A positive conclusion was received from the local Ethics Committee of Northern State Medical University (Arkhangelsk) (Minutes No. 8/11dated November 28, 2018).

The study inclusion criteria were as follows: a signed written informed consent, "young age" according to the WHO, a diagnosis of "K05.3 – chronic periodontitis," and no antimicrobial use in the past six months. Exclusion criteria were lack of a signed informed consent, age under 18 or over 45 years, and orthodontic treatment at the time of the study. Participants were excluded from the study if they had other inflammatory oral diseases, any concomitant somatic-symptom pathology in the decompensation stage, pregnancy or lactation, or received antibacterial therapy in the past six months.

The clinical material consisted of periodontal pocket fluid collected using a paper absorbent point during a dental examination. The resulting samples were centrifuged at 1,500 rpm for 20 minutes. Aliquots of the samples were frozen and stored at –80°C until molecular genetic and immunological testing were conducted.

The Russell's Periodontal Index (PI, 1956) was used to assess the presence and depth of the periodontal pocket, the degree of tooth mobility, and the severity of gum inflammation. The technique included the assessment of each tooth using a periodontal probe. The result was entered into the periodontogram as a score: 0 points – no inflammation; 1 point – mild degree, inflammation does not surround the entire tooth; 2 points – inflammation surrounds the tooth, without damage to the epithelial attachment; 4 points – initial degree of resorption of the apices of the interdental septa; 6 points – presence of a periodontal pocket, the tooth is stable; 8 points – severe destruction of periodontal tissues, the tooth is mobile. The index is calculated using the formula: PI = sum of teeth / n; where n is the number of examined teeth. The index value of 0.1–1.5 points is stage 1 of the disease, 1.5–4.0 points is stage 2 and 4.0–8.0 points is stage 3.

Bone density was assessed using the Fuchs Index (FI). By analyzing orthopantomograms, the root of each tooth was assessed, divided into three parts, and a score was assigned according to the following formula: 0 points – the tooth is outside the bone or was removed due to periodontal disease; 1 point – bone loss exceeding two-thirds of the root length; 2 points – from one-third to two-thirds of the root length; 3 points – up to one-third of the root length; and 4 points – no bone loss detected, or the tooth was removed due to complicated caries. The result was calculated using the formula: sum of the scores / n * 4, where n is the number of teeth in the oral cavity. An FI value of 0 points indicates bone tissue resorption up to the root

tips, 0.25–0.5 points corresponds to resorption at 2/3 of the root length, 0.5–0.75 implies resorption at half of the root length, > 0.75 corresponds to resorption at 1/3 of the root length, and 1 point indicates normal bone tissue condition.

The RANK-L and IL-1β levels in gingival fluid were determined using enzyme-linked immunosorbent assay (ELISA) in thawed samples according to the kit instructions provided by Wuhan Fine Biotech Co., Ltd. (China). The optical density of the wells was measured and recorded using a Multiscan EX photometer (Thermo Fisher Scientific, USA). The results were evaluated according to the kit instructions using calibration curves constructed based on standard measurements. Molecular genetic methods included the determination of marker periodontal pathogens Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, and Tannerella forsythia (periodontopathogens of the first order), Treponema denticola and Prevotella intermedia (periodontopathogens of the second order), and Candida albicans fungi using the real-time polymerase chain reaction (RT-PCR) in accordance with the instructions for the manufacturer's kits (ParodontoScreen, DNA-Technology LLC, Russia).

Statistical processing of the obtained results, assessment of the distribution of indicators, and comparative analysis of samples were performed using the STATA v.14 software package for statistical data processing (College Station, TX: StataCorp LP., USA). The normality of distribution was assessed using the Shapiro–Wilk test. Quantitative data for normal distribution were presented as the arithmetic mean and standard deviation $M \pm SD$, and in the case of abnormal distribution, as the median and interquartile range Me $[Q_{25};Q_{75}]$. For statistical comparison of RANK-L and IL-1β levels in two independent groups, the nonparametric Mann–Whitney test was chosen. Spearman's rank correlation coefficient was used to search for intragroup relationships between the levels of individual parameters. The critical level of significance at all stages of the statistical analysis was taken as $p \leq 0.05$.

RESULTS

In a study of gingival fluid samples from patients with chronic periodontitis and a control group with intact periodontium, the levels of the proinflammatory cytokine IL-1β and the receptor activator of nuclear factor kappa-B ligand were determined (Table 1). Thus, in patients with chronic periodontitis, a significant increase in IL-1β content was found compared to those in the control group (median – 37.1 [32.9; 41.3] pg/ml versus 2.5 [1.9; 3.4], $p < 0.001$). The concentration of RANK-L in gingival fluid was also higher in patients with chronic periodontitis (median 6.3 [4.2; 10.4] pg/ml versus 0.0 [0.0; 0.7], $p < 0.001$).

In a group of patients with chronic periodontitis, the levels of bone destruction varied among individuals. The average FI was 0.83 ± 0.03 in patients with mild chronic periodontitis and 0.71 ± 0.05 in those with moderate chronic periodontitis.

Analysis of IL-1β concentrations in patients with chronic periodontitis showed that in the subgroup with a moderate degree of bone tissue destruction, its content was 3.7 ($p = 0.042$) times higher than in the subgroup of patients with a mild degree. Similarly, the RANK-L indicator in patients with a moderate degree of destruction was 2.5 times higher than in patients with a mild degree ($p = 0.037$). Correlations were established between the content of IL-1β and RANK-L with the degree of bone tissue destruction: $r = 0.562$ ($p = 0.025$) and $r = 0{,}408$ ($p = 0.033$), respectively.

Table 1

The Levels of IL-1β and RANK-L (pg/ml) in Individuals with Chronic Periodontitis and Intact Periodontium, Me $[Q_{25}; Q_{75}]$

Parameter	Chronic periodontitis	Intact periodontium
IL-1β	37.1 [32.9; 41.3]	2.5 [1.9; 3.4]
RANK-L	6.3 [4.2; 10.4]	0.0 [0.0;0.7]

$p < 0.001$

A study of the frequency of periodontopathogenic microbiota in gingival fluid samples obtained from examined people revealed significant group differences. Thus, in patients with chronic periodontitis, the frequency of detection of one or more periodontopathogens was 100.0% (60 individuals), whereas in the group of subjects with an intact periodontium, periodontopathogenic bacteria were isolated in only 32.1% (9 individuals). In the chronic periodontitis group, the following first-order periodontopathogens were isolated with the highest frequency: *A. actinomycetemcomitans* (81.7%), *P. gingivalis* (76.7%), *and T. forsythia* (70.0%), and associations of periodontopathogens (60.0%). Periodontopathogenic species of the second order were isolated with a lower frequency: *T. denticola* (63.3%), *P. intermedia* (56.7%), and *C. albicans* (30.0%). While periodontopathogens of the first order were not isolated in the examined individuals of the control group, periodontopathogenic bacteria of the

second order predominated: *T. denticola* – 17.8% and *P. intermedia* – 10.7%. The fungi *C. albicans* were also isolated in one examined individual.

In order to identify the relationships between the gingival microbiota and cytokines, a correlation analysis was performed, with statistically significant correlations being found only in the group with chronic periodontitis (Table 2).

The quantitative content of both proinflammatory cytokines IL-1β and RANK ligand positively correlated with all representatives of the group of periodontopathogenic bacteria of the first order, with the strongest correlations being found at the moderate degree of periodontal tissue destruction. For periodontopathogenic bacteria of the second order, direct correlations of weak strength were revealed, with the exception of a correlation between *T. denticola* and RANK-L (r = 0.452, p = 0.029) at the moderate degree of periodontal tissue destruction. Associations of periodontopathogens positively correlated with the content of both cytokines at both degrees of periodontal tissue destruction, but the moderate strength of correlations was established at the moderate degree of destructive changes.

Table 2

Correlation Matrix of Cytokines IL-1β, RANK-L and Periodontopathogenic Bacteria of Gingival Fluid in Patients with Chronic Periodontitis with Varying Degrees of Bone Tissue Destruction

Parameter	IL-1β		RANK-L	
	Mild degree of destruction (Fuchs index)	Moderate degree of destruction	Mild degree of destruction	Moderate degree of destruction
P. gingivalis	r = 0.548* (p = 0.003)	r = 0.618* (p = 0.008)	r = 0.232 (p = 0.04)	r = 0.612* (p = 0.016)
A. actinomycetemcomitans	r = 0.485* (p = 0.032)	r = 0.539* (p = 0.002)	r = 0.342 (p = 0.028)	r = 0.553* (p = 0.042)
T. forsythia	r = 0.188 (p = 0.052)	r = 0.423* (p = 0.037)	r = 0.118 (p = 0.048)	r = 0.618* (p = 0.006)
T. denticola	r = 0.267 (p=0.035)	r = 0.231 (p = 0.04)	r = 0.152 (p = 0.029)	r = 0.452* (p = 0.029)
P. intermedia	r = 0.278 (p = 0.047)	r = 0.134 (p = 0.026)	r = 0.243 (p = 0.0212	r = 0.243 (p = 0.034)
C. albicans	r = 0.218 (p = 0.48)	r = 0.175 (p = 0.041)	r = 0.134 (p = 0.016)	r = 0.168 (p = 0.013)
Periodontopathogenic associations	r = 0.318 (p = 0.006)	r = 0.452* (p = 0.042)	r = 0.288 (p = 0.034)	r = 0.589* (p = 0.002)

*moderate strength of correlation r at $p < 0.05$

DISCUSSION

Destruction of periodontal tissues is caused by a non-specific inflammatory response, leading to a shift in the dynamic balance of inflammatory mediators, among which cytokines play a leading role [7].

IL-1β belongs to the family of proinflammatory cytokines and has potent immunoregulatory functions in chronic periodontitis [8, 9]. Released during periodontal cell damage and immune cell activation, IL-1β is involved in innate immunity mechanisms, inflammasome activation processes, and the T-cell-mediated immune response mechanism. This cytokine ensures control of the spread of inflammation to deeper areas of connective tissue; high levels of IL-1β are associated with loss of connective tissue attachment, osteoclast activation, and subsequent alveolar bone loss [8].

Our study revealed an increased content of the cytokine IL-1β in the gingival fluid of patients with chronic periodontitis, as compared to those in the control group with an intact periodontium, which may indicate increased activity of immunocompetent cells and a shift in the immune homeostasis of periodontal tissues with a predominant production of proinflammatory cytokines, which is consistent with data from previous studies [10, 11].

RANK-L, a member of the TNF family of cytokines, plays a key role in bone resorption: when it binds to the NF-κB receptor activator, a membrane receptor primarily produced by osteoclasts and their progenitor cells, it both induces the differentiation of progenitor cells into osteoclasts and stimulates the activity of mature osteoclasts.

Previous studies have shown that RANK-L levels are the highest in patients with severe periodontitis compared with those with moderate to mild periodontitis or with an intact periodontium [1]. In our study, RANK-L concentrations in gingival fluid were also higher in patients with chronic periodontitis compared to those with an intact periodontium (median 6.3 [4.2;10.4] pg/ml vs. 0.0 [0.0; 0.7], $p <$ 0.001). It is worth noting the correlations identified in our study: IL-1β and RANK-L positively correlated with the degree of bone tissue destruction in patients with chronic periodontitis. These cytokines likely control key pleiotropic pathways that are crucial for the homeostasis of bone and connective tissue in the periodontium.

However, the microbial component, namely the imbalance of the gingival microbiota with a predominance of periodontopathogenic flora, may be a key link in the development of the initial nonspecific inflammatory process, which deepens into the gingival sulcus, subsequently forming a periodontal pocket [2, 12]. In our study, in patients with chronic periodontitis, the frequency of detection of one or more periodontopathogens was 100.0%, whereas in the group of subjects with an intact periodontium, periodontopathogenic bacteria were isolated in only 32.1%.

In the chronic periodontitis group, periodontopathogens of the first order were isolated with the highest frequency: *A. actinomycetemcomitans, P. gingivalis, T. forsythia,* and associations of periodontopathogens. The periodontopathogenic bacteria isolated by us exhibit the greatest bone-resorptive activity. Thus, *P. gingivalis* is one of the key pathogens that activates osteoclasts via the TLR4 system and enhances the production of proinflammatory cytokines, including IL-1β and RANK-L, as well as TNFα and IL-6. *T. forsythia* promotes increased osteoclast activation and suppression of bone formation, and *A. actinomycetemcomitans* stimulates the production of matrix metalloproteinases and proinflammatory factors, which also leads to bone resorption [13].

The presence in our study of a large number of positive correlations of moderate strength between the content of both the proinflammatory cytokine IL-1β and the RANK ligand with a group of periodontopathogenic bacteria of the first order, identified in the group of patients with a moderate degree of bone tissue destruction, may indicate that these bacteria trigger a cascade of inflammatory reactions leading to increased secretion of proinflammatory cytokines, activation of osteoclastogenesis, suppression of osteogenesis, and increased destruction of bone tissue [14].

These periodontopathogens probably do not act in isolation, but as part of a biofilm, where their combined effect significantly enhances the destructive potential, and the presence of correlations between the isolated associations of periodontopathogens with cytokines confirms this assumption. The presence of these correlations may also indicate an imbalance between bone formation and resorption in favor of the latter, which leads to progressive bone loss in periodontitis. Also, the identified correlation of the second-order periodontopathogen *T. denticola* with RANK-L of moderate strength ($r = 0.452$, $p = 0.029$) with an average degree of periodontal tissue destruction may reflect the presence of many virulence factors in this bacterium, such as the production of proteolytic enzymes (oligopeptidase, dentipain, dentilisin, etc.), which also have a destructive effect on periodontal tissues.

CONCLUSION

The pathogenesis of chronic periodontitis is determined by a number of factors, with a key role played by the interaction of periodontopathogenic bacteria and their aggressive factors with gingival epithelial cells, which leads to the stimulation of mediator production in the inflammatory zone. Mediators detected in gingival crevicular fluid are primarily proinflammatory cytokines, including IL-1β and the immune mediator RANK-L, which are central to soft tissue destruction and periodontal bone resorption.

These mediators likely acting in synergy participate in the cytokine cascade, promoting the degradation of collagen and other extracellular matrix components enhancing osteoclast activation and differentiation, and binding to the RANK receptor on the osteoclast surface, thereby leading to increased alveolar bone resorption and disrupting bone remodeling processes. Conversely, proteolytic bacterial enzymes (oligopeptidases, dentipains, dentilisins) and endotoxins can directly disrupt periodontal tissue homeostasis by suppressing periodontal ligament cell function and inducing nitric oxide secretion by macrophages, which promotes bone resorption.

Thus, the associations between the periodontopathogenic bacteria *A. actinomycetemcomitans, P. gingivalis*, and *T. forsythia*, and their associations with elevated cytokine levels and the severity of bone tissue destruction, may indicate a key joint role of the proinflammatory cytokine IL-1β and the immune mediator RANK-L in the inflammatory and bone-destructive processes in the pathogenesis of chronic periodontitis. RANK-L in combination with IL-1β may also serve as a valuable diagnostic biomarker for periodontitis, reflecting the progression of the pathological process.

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Author Contribution

Davidovich N.V., Solovieva N.V., and Bazhukova T.A. – conception and design. Sabanaev M.A. and Galieva A.S. – collection and processing of material.Sabanaev M.A.. – figure preparation. Galieva A.S. and Sabanaev M.A. – statistical processing of data. Davidovich N.V. and Sabanaev M.A. – drafting of the manuscript. Solovieva N.V. and Bazhukova T.A. – editing of the manuscript. All coauthors – final approval of the manuscript, responsibility for the integrity of all parts of the manuscript .

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Assessment of Ovarian Function in Infertile Women with Compensated Autoimmune Thyroiditis and Premature Ovarian Insufficiency without Clinical Definitions

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ABSTRACT

Aim. To assess the ovarian reserve and ovarian response to controlled ovarian hyperstimulation (COS) in patients in assisted reproductive technology (ART) programs with compensated chronic autoimmune thyroiditis (CAIT) and with predicted premature ovarian insufficiency (POI).

Materials and methods. We performed a retrospective study of 166 patients with infertility and compensated CAIT (group I, $n = 44$), predicted signs of POI (group II, $n = 62$), tubal infertility factor (group III, $n = 66$), including a comparative analysis of clinical and patient history data, indicators of ovarian reserve, and the effectiveness of COS (number of oocytes retrieved). The groups were divided by age into subgroups: patients younger than 35 years of age and 35 years and older (Ia, Ib, IIb, IIb, IIIa, and IIIb, respectively).

Results. Significant differences in the values of follicle-stimulating hormone (FSH) anti-mullerian hormone (AMH) were revealed in the study groups under 35 years of age: FSH (7.24 (6.0–9.63) mIU/l and 10.35 (10.13–11.01) mIU/L, respectively; $p < 0.001$) and AMH (3,2 (1.48–6.80) ng/ml and 0.68 (0.44–2.91 ng/ml), respectively; $p = 0.015$). The poor ovarian response in COS programs is most often obtained in patients under of 35 years of age with infertility with occult POI. In the age groups 35 years and older, the poor ovarian response to COS was equally common in groups I and II. Estradiol levels in the groups of 35 years of age and older were lower in patients with occult POI.

Conclusion. In groups of patients under 35 years of age with compensated CAIT and with tubal infertility factor, higher rates of ovarian reserve and the effectiveness of COS were recorded than in the groups with occult POI. A decrease in estradiol in patients aged 35 years and older with occult POI and older indirectly indicates a lesion of the ovarian somatic cells, probably as a result of autoimmune aggression.

Keywords: chronic autoimmune thyroiditis, autoimmune oophoritis, premature ovarian insufficiency, "poor ovarian response", assisted reproductive technologies, ovarian reserve

Conflict of interest. The authors declare the absence of obvious or potential conflict of interest related to the publication of this article.

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Оценка функции яичников у женщин с бесплодием при компенсированном аутоиммунном тиреоидите и преждевременной недостаточности яичников без клинических дефиниций

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РЕЗЮМЕ

Цель. Оценка овариального резерва и ответа яичников на контролируемую индукцию суперовуляции (КИСО) у пациентов в программах вспомогательных репродуктивных технологий (ВРТ) с компенсированным хроническим аутоиммунным тиреоидитом (ХАИТ) и при прогнозируемой преждевременной недостаточности яичников (ПНЯ).

Материалы и методы. Ретроспективное исследование 166 пациентов с бесплодием и компенсированным ХАИТ (группа I, n = 44), «оккультной» формой ПНЯ (группа II, n = 62), трубным фактором бесплодия (группа III, n = 66), включающее сравнительный анализ клинико-анамнестических данных, показателей овариального резерва, результативности КИСО (количество полученных ооцитов). Группы разделены по возрасту на подгруппы: до 35 лет и 35 лет и старше (Ia, Ib, IIb, IIb, IIIa, IIIb соответственно).

Результаты. Выявлены достоверные отличия в значениях фолликулостимулирующего (ФСГ) и антимюллерового (АМГ) гормонов в группах исследования до 35 лет: ФСГ (7,24 (6,0–9,63) мМЕ/л и 10,35 (10,13–11,01) мМЕ/л соответственно; p < 0,001) и АМГ (3,2 (1,48–6,80) нг/мл и 0,68 (0,44–2,91 нг/мл) соответственно; p = 0,015). «Бедный ответ» в программах КИСО наиболее часто получен у пациенток до 35 лет при бесплодии с «оккультной» формой ПНЯ. В возрастных группах 35 лет и старше «бедный ответ» КИСО встречался одинаково часто в I и II группах. Эстрадиол у пациентов 35 лет и старше был ниже у пациентов с формирующимся ПНЯ.

Заключение. В группах пациентов до 35 лет с компенсированным ХАИТ и с трубным фактором бесплодия зафиксированы более высокие показатели овариального резерва и эффективность КИСО, чем в группе женщин с «оккультной» формой ПНЯ. Снижение эстрадиола у пациентов 35 лет с «оккультной» формой ПНЯ и старше косвенно свидетельствует о поражении овосоматического гистиона, вероятно, в результате аутоиммунной агрессии.

Ключевые слова: хронический аутоиммунный тиреоидит, аутоиммунный оофорит, преждевременная недостаточность яичников, «бедный ответ» яичников, вспомогательные репродуктивные технологии, овариальный резерв

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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Соответствие принципам этики. Все пациенты подписали информированное согласие на участие в исследовании. Исследование одобрено локальным этическим комитетом СибГМУ (протокол № 9308 от 15.12.2022).

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INTRODUCTION

The outcome of the autoimmune inflammatory process in the gonads is endocrine and reproductive dysfunction of the ovarian follicles and premature ovarian insufficiency (POI), which leads to infertility and complications associated with hormone deficiency. Autoimmune POI in the population is much more common in women with other autoimmune diseases [1], including autoimmune thyroid diseases (14–32.7%) [2–4].

Chronic autoimmune thyroiditis (CAIT) is the most common cause of thyroid dysfunction in women of reproductive age [5]. It is known that thyroid dysfunction can exacerbate a decrease in ovarian reserve [3], which reduces the effectiveness of assisted reproductive technology (ART) programs. Y.T. Hsieh and J.Y.T.Ho published data on a high risk of POI development in patients with autoimmune thyroiditis [6]. A condition for overcoming infertility is a hormone-compensated CAIT. Euthyroidism in the absence of other detectable causes of infertility is not a guarantee of successful controlled ovarian hyperstimulation (COH) in ART programs. Since 2006–2008, Nelson and Welt have proposed to use the term occult (i.e. latent) form without clinical definitions. Currently, signs of early onset of POI without typical clinical manifestations have not been established. The problems of idiopathic infertility and poor ovarian response to COH remain very relevant in reproductive medicine, in which autoimmune gonadal damage is possible.

The aim of the study was to assess ovarian reserve and ovarian response to COH in patients in assisted reproductive technology (ART) programs with compensated CAIT and with occult POI.

MATERIALS AND METHODS

The present work is a retrospective study. The medical records of patients with infertility who were treated at the ART center of Siberian State Medical University from 2017 to 2022 were studied. The research work was approved by the local Ethics Committee of Siberian State Medical University (Minutes No. 9308 dated December 15, 2022).

The study included 166 patients. Main group I consisted of patients aged 18–40 years with infertility with a history of CAIT compensated with hormone therapy ($n = 44$), who were divided into 2 age-adjusted subgroups: IA – patients under 35 years of age ($n = 22$), IB – patients 35 years of age and older ($n = 44$). Main group II consisted of patients with infertility, in whom hormonal examination revealed signs of occult POI (concentration of follicle-stimulating hormone (FSH) in blood serum was 10–12 mIU/l) without established thyroid pathology ($n = 62$) (IIA is a subgroup of patients under aged 35 years ($n = 28$), IIB is a subgroup of patients 35 years of age and older ($n = 34$)) [6]. The comparison group consisted of patients with tubal infertility (bilateral salpingectomy) ($n = 60$) (IIIA is a subgroup of patients younger than 35 years of age ($n = 33$), IIIB is a subgroup of patients aged 35 years and older ($n = 27$)). The division by age was performed to make the study groups uniform, taking into account the age-related features of the hormonal functioning of the ovaries, which are described in the POSEIDON stratification [7].

Inclusion criteria for the study were as follows: 1) age from 18 to 40 years; 2) tubal factor infertility. Exclusion criteria were: 1) non-compliance with the inclusion criteria; 2) CAIT without hormonal compensation; 3) uterine factor infertility; 4) history of ovarian surgery; 5) endocrine diseases (hyperprolactinemia, diabetes mellitus, and obesity of any degree); 6) gynecological diseases requiring surgical treatment; 7) endometriosis of any localization; 8) precancerous and malignant diseases; 9) any extragenital pathology associated with immune and endocrine manifestations; 10) the presence of contraindications for treatment as part of *in vitro* fertilization (IVF) (Order of the Ministry of Health of the Russian Federation dated July 31, 2020 No. 803n "On the Procedure for the Use of Assisted Reproductive Technologies, Contraindications and Restrictions to Their Use"). According to the patient management protocols, superovulation was stimulated in a fixed gonadotropin-releasing hormone antagonist protocol (recombinant and menopausal gonadotropins).

Statistical processing was performed using the computer program SPSS® 26.0. The normality of the data distribution in each of the groups was checked using the Kolmogorov–Smirnov and Shapiro–Wilk tests. Quantitative data that did not obey the law of normal distribution were presented as the median and the interquartile range Me (Q_1–Q_3). The significance of the differences was calculated using a nonparametric method for three or more independent groups using the Kruskal–Wallis test (H-test), and post hoc comparison was performed using the Mann–Whitney test. To solve the problem of multiple comparisons, the Bonferroni correction was applied, the significance level was set according to the formula $p = 1–0.95^{1/n}$», where n is the number of comparisons. Qualitative variables

were analyzed using Pearson's chi-squared test, and the results were described using the criterion value, significance level, relative risk (RR), and confidence interval (CI).

RESULTS

The clinical and patient history characteristics of the analyzed parameters of patients with infertility of the main groups and the comparison group are presented in Table 1. In statistical calculations, the body mass index (BMI) and age of menarche did not differ between the groups (Table 1). The average age of women in the main group I was 34.5 (30.0–38.0) years, in the main group II–36.0 (33.0–38,0) years old, and in the comparison group – 33.5 (29.0–36.0) years. When patients were divided into subgroups, their age distribution remained uniform across all subgroups..

The duration of infertility was longer in main group II than in the comparison group (p = 0.011). The number of IVF attempts in the history of women in main group I and main group II was higher than in the comparison group.

Table 1

Comparative Analysis of Clinical and Patient History Data of the Main Groups and Comparison Group, *Me* (Q_1–Q_3), Mann–Whitney Test

Parameters	Main group I	*p*, main group I and comparison group	Main group II	*p*, main group II and comparison group	Comparison group	*p*, main group I and comparison group
Age, years	34.5 (30.0–38.0)	0.774	36.0 (33.0–38.0)	0.024	33.5 (29.0–36.0)	0.827
BMI	21.60 (20.06–25.96)	0.589	23.10 (20.08–25.65)	0.402	23.70 (20.28–27.30)	0.286
Age at menarche, years	13.0 (13.0–14.0)	0.776	14.0 (12.0–14.0)	0.294	13.0 (12.0–14.0)	0.477
Duration of infertility, years	4.5 (2.0–9.3)	0.118	8.0 (2.0–13.0)	0.011	4.0 (2.0–7.0)	0.510
IVF in the anamnesis	1.0 (1.0–1.25)	0.052	2.0 (1.0–3.0)	<0.001	0	<0.001

Note. BMI is body mass index; IVF is *in vitro* fertilization.

The analysis revealed the following infertility profile: 9.6% of patients (16 out of 166) with combined infertility (male factor) and 7.2% of patients (12 out of 166) with combined infertility (anovulation). Primary and secondary infertility was recorded in 45.8% of patients (76 out of 166) and 54.2% of patients (90 out of 166), respectively. In the statistical analysis, the main groups and the comparison group were uniform according to the above criteria.

We compared the average concentrations of FSH, estradiol, anti-Müllerian hormone (AMH), the antral follicles count (AFC), as well as the number of oocytes obtained to calculate the poor ovarian response. The results are presented in Tables 2 and 3.

Table 2

Hormonal and Ultrasound Status of Women in the Main Groups and Comparison Group, the Number of Oocytes Obtained after Ovarian Puncture in the ART Program, *Me* (Q_1–Q_3)

Parameters	Main group I		Main group II		Comparison group	
	IA	IB	IIA	IIB	IIIA	IIIB
FSH, mME/ml	7.24 (6.0–9.63)	8.28 (6.7–15.7)	10.35 (10.13–11.01)	11.1 (10.29–11.63)	8.1 (5.49–11.15	7.57 (3.93–12.6)
Estradiol, pmol/l	108.0 (50.0–190.0)	118.5 (68.25–234.4)	132.5 (89.51–195.09)	119.0 (65.78–218.89)	140 (85.0–200,0)	215.0 (138.0–275.0)
AMH, ng/ml	3.2 (1.48–6.80)	1.19 (0.72–2.23)	0.68 (0.44–2.91)	1.14 (0.65–1.84)	4.07 (1.56–5.10)	2.41 (0.83–3.91)
AFC	14.0 (11.0–17.0)	9.0 (5.0–14.0)	9.0 (4.5–13.5)	6.0 (5.0–16.0)	11.0 (9.0–17.5)	9.0 (6.0–15.0)
Oocytes	10.0 (4.0–15.0)	4.0 (2.0–8.0)	1.0 (2.5–10.0)	3.0 (2.0–5.0)	5.0 (4.5–9.0)	5.0 (4.0–8.0)

Note. Here and in Table 3: FSH – follicle-stimulating hormone, AMH – anti-Müllerian hormone, AFC – antral follicle count.

Table 3

Post Hoc Comparisons of Hormonal and Ultrasound Status of Patients in the Main Groups and Comparison Group, the Number of Oocytes Obtained after Ovarian Puncture in the ART Program Using the Mann–Whitney Test

	FSH, mME/ml	Estradiol, pmol/l	AMH, ng/ml	AFC	Oocytes
p, IA and IB	0.243	0.654	0.023	0.193	0.116
p, IIA and IIB	0.118	0.728	0.544	0.934	1.0
p, IIIA and IIIB	0.672	0.009	0.025	0.248	0.231
p, IA and IIA	<0.001	0.501	0.015	0.072	0.044
p, IA and IIIA	0.380	0.641	0.946	0.063	0.450
p, IB and IIB	0.091	1.0	0.711	0.926	0.329
p, IIA and IIIA	0.010	0.968	0.002	0.056	0.137
p, IB and IIIB	0.293	0.068	0.123	0.465	0.546
p, IIB and IIIB	0.099	0.025	0.014	0.270	0.031

The concentration of FSH in patients of the IA subgroup was within the limits of the reference laboratory values for the follicular phase in women of reproductive age. This indicator was significantly higher in patients of subgroup IIA ($p < 0.001$) (Tables 2, 3). Also, in patients of the IIA subgroup with occult POI, it was significantly higher than in the comparison group of IIIA patients with tubal factor infertility ($p = 0.010$). There were no statistically significant differences in FSH in patients aged 35 and older in the main groups and the comparison group (Table 3).

The concentration of estradiol was comparable between the subgroups of main group 1 with CAIT and main group 2 with occult POI. In the comparison group, significant differences were found between the subgroups in patients after salpingectomy, estradiol prevailed in patients aged 35 years and older ($p = 0.009$). It was noted that the level of estradiol in group IIB with an occult POI tended to decrease compared to group IIIB with tubal infertility factor ($p = 0.024$) (Tables 2, 3).

The median AMH of the IB subgroup was lower than in the IA subgroup, probably due to age characteristics ($p = 0.023$) (Table 2). In the main group of patients with occult POI, this indicator had equally low values both in patients under the age of 35 and in those aged 35 years and older, which indicates a small ovarian reserve. The level of AMH in patients under 35 in the comparison group with occult POI (subgroup IIA) was significantly lower than that in patients in the main group with compensated hypertension (subgroup IA) ($p = 0.015$), whereas in patients aged 35 and older with compensated CAIT (subgroup IB) and with occult POI (subgroup IIB), there were no significant differences ($p = 0.711$). It was noted that in patients under 35 and those aged 35 years and older, the level of AMH in the main group with an occult POI was significantly lower than in the comparison group with tubal infertility factor.

Thus, the greatest decrease in the level of AMH was determined in the main group of patients with occult POI. Post hoc comparisons for AFC are provided for review, as the Kruskal–Wallis test showed insignificant results (Table 3). However, the highest value of AFC was noted in patients under 35 years of age in the main group with compensated CAIT.

The analysis of the effectiveness of COH in the main group with CAIT between the subgroups did not reveal statistically significant differences in the number of oocytes obtained, as well as between the subgroups in the main group with the occult POI and the comparison group (Tables 2, 3). In the main group with CAIT, the number of oocytes obtained tended to increase compared with the main group with occult POI ($p = 0.044$). In addition, there was a trend towards a decrease in the number of oocytes obtained in patients aged 35 and older in the main group with an occult POI, in contrast to the comparison group with tubal factor infertility ($p = 0.031$).

The proportion of poor ovarian response, as measured by the number of oocytes obtained after puncture in the main group with CAIT was 32% (14 out of 44), in the main group with occult POI – 58% (36 out of 62), in the comparison group with tubal factor infertility – only 10% (6 out of 60).

The proportion of poor ovarian response in women under 35 years of age in the main group (IA subgroup) was 18.2% (4 out of 22), in the comparison group with occult POI (IIA subgroup) – 57% (16 out of 28), (3.896, $p = 0.048$ with the main group; RR = 0.32, 95% CI: 0.12–0.82) and in the comparison group with tubal factor infertility (IIIA subgroup) – 6% (2 out of 33) (1.995, $p = 0.158$

with the main group; RR = 3.0 95% CI: 0.6–15.0). In patients aged 35 and older, the proportion of poor ovarian response in the main group (IB subgroup) was 46% (10 out of 22), in the comparison group with occult POI (IIB subgroup) – 59% (20 out of 34) (0.480, $p = 0.700$ with the main group; RR = 0.773, 95% CI: 0.45–1.32), and in the comparison group with tubal factor infertility (IIIB subgroup) – 15% (4 out of 27) (5.576, $p = 0.018$ with the main group; RR = 3.07 95% CI: 1.11–8.46). Thus, our data indicate a decrease in the effectiveness of superovulation stimulation in patients under 35 years with occult POI, whereas the most effective COH was in the comparison group with tubal factor infertility.

DISCUSSION

The question of the effect of CAIT on the ovarian reserve and reproductive function remains open. The relationship between thyroid and ovarian function is complex and interrelated [8]. Thyroid hormones are involved in the functioning of granulosa cells and the growth of follicles. With inflammatory changes in the thyroid gland of a low degree of activity, as in CAIT, the microenvironment of the ovarian somatic cells is disrupted, which leads to a decrease in ovarian function. Unambiguous studies indicating a close relationship between CAIT and ovarian insufficiency have not been published, however, there is evidence indicating lower AMH values in subclinical hypothyroidism and CAIT [9] and a decrease in ovarian reserve in CAIT [10–13].

A limitation in the work is the fact that the ART program does not include patients without thyroid function compensation. Thus, the main group consisted of patients receiving hormone replacement therapy and having an euthyroid condition at the time of the COH. Not all patients had medical history data on the duration of CAIT and hormone therapy, which did not allow us to assess the duration of the inflammatory process in the thyroid gland. However, compensation of thyroid function does not affect the activity of the autoimmune process.

The results of a comparative analysis of ovarian reserve and ovarian response to COH are of interest in groups of patients under 35 years of age. The ovarian reserve in the main group of patients with compensated CAIT was higher than in the main group with occult POI and did not differ from that in the comparison group with tubal factor infertility. The ovarian response to COH was lower in the main group with occult POI compared to the main group with compensated CAIT and the comparison group with tubal infertility.

Thus, the most significant indicator for predicting the ovarian response to stimulation according to the results of this study is not the presence of antibodies to thyroid tissue, but transitional FSH values (10–12 mIU/ml). It can be assumed that under the age of 35, the compensation of CAIT and the short-term effect of the autoimmune process of low activity do not achieve a significant effect on ovarian function.

It was found that the presence of CAIT compensated by hormonal therapy or occult POI in patients aged 35 years and older was not associated with diminished ovarian reserve. The decrease in ovarian reserve at the age of 35 years and older is due to the age-related features of folliculogenesis. Also, in the group of patients aged 35 years and older, no significant differences were found in the indicators of ovarian reserve in the main group with CAIT and the comparison group with tubal factor infertility. The most interesting fact was the differences in hormonal profile indicators in patients of main group II and the comparison group aged 35 years and older. The levels of estradiol and AMH were significantly higher in the comparison group with tubal factor infertility. At the same time, the number of oocytes obtained was smaller in patients with occult POI (main group I). Thus, in patients with occult POI aged 35 years and older with FSH levels of 10–12 mIU/l, the ovarian response to stimulation was diminished due to a decrease in ovarian reserve, which was indirectly confirmed by a decrease in estradiol levels compared to the group of patients after salpingectomy. The median estradiol index in the main group with CAIT is comparable to that in the main group in patients with occult POI. Estradiol is not a reliable marker of the assessment of ovarian reserve; however, its relative decrease may be an early marker of impaired functioning of ovarian somatic cells in autoimmune ovarian damage.

According to the two-cell theory of ovarian steroidogenesis, the synthesis of androgens and estrogens is compartmentalized. It is known that in autoimmune oophoritis, the aggression of immunocompetent cells in the early stages is aimed at damaging the theca cells. So, subsequently, the destruction of the internal theca leads to a decrease in the synthesis of estradiol in granulosa cells. According to the data of this study, the trend towards a decrease in the level of estradiol in main group I aged 35 years and older may be a consequence of the described pathogenetic mechanism, while an increase in FSH

concentration is secondary. Further prospective clinical studies are needed to confirm the data.

The results of this study are the closest to the results of a single-center cross-sectional study conducted in 2024 by M. Pan et al. (China), which studied the association of thyroid peroxidase autoantibodies and antithyroglobulin autoantibodies with infertility and AMH levels. Thus, the authors identified the association of a high titer of thyroid peroxidase autoantibodies with anovulatory infertility, but did not establish a significant correlation with the level of AMH. On the contrary, a high titer of antithyroglobulin autoantibodies was not associated with anovulatory infertility and with the level of AMH [14].

Analyzing the results of this study and the existing understanding of ovarian function in women with CAIT, it can be concluded that compromised ovarian function of unclear etiology with FSH values above the reference reduces the results of COH. It is of interest to study markers of autoimmune oophoritis in patients with occult POI, since the preservation of estradiol synthesis in women under 35 and a decrease in estradiol levels in women aged 35 years and older may be the basis for the hypothesis of gradual destruction of follicles as a result of partial damage to the internal theca, as is possible with autoimmune oophoritis. It is possible to confirm the hypothesis in a prospective clinical trial with the study of markers of autoimmune oophoritis both in blood serum and in histological examination of ovarian biopsies as a more reliable diagnostic method.

CONCLUSION

In the group of patients under 35 years of age with compensated CAIT and with tubal factor infertility, higher rates of ovarian reserve and the effectiveness of COH were recorded than in the group of women with occult POI. In patients aged 35 years and older with compensated CAIT and occult POI, in the absence of significant differences in FSH, there was a downward trend in the concentration of estradiol, which may indicate damage to ovarian somatic cells, probably as a result of autoimmune aggression. The data obtained will have an applied value in reproductology, but require further research.

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Author Contribution

Dmitrieva M.L., Tikhonovskaya O.A., and Petrov I.A. – conception and design. Dmitrieva M.L., Petrov I.A., Timofeeva O.S., Lasukova T.V., Akbasheva O.E., and Mikheenko G.A. – analysis and interpretation of data. Dmitrieva M.L., Tikhonovskaya O.A., Petrov I.A., and Logvinov S.V. – justification of the manuscript. Tikhonovskaya O.A. and Logvinov S.V. – final approval of the manuscript for publication.

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Peculiarities of Proliferative Activity of Non-Small Cell Lung Cancer in Case of Tumor Spread through Air Spaces

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ABSTRACT

Aim. To study the proliferative activity of non-small cell lung cancer with tumor spread through air spaces.

Materials and methods. The study included 88 patients with stage IA-IIIA non-small cell lung cancer. The operative material was evaluated. Histologic and immunohistochemical examination was performed according to the standard technique. Antibodies to Ki67 (clone SP6, Cell Marque) were used. On digitized preparations using automated system Pannoramic MIDI with software Slide Viver V2.8. and multi-module platform Quant Center (3D HISTECH, Hungary) we counted the percentage of cells with positive nuclear expression of Ki67 in the tumor as a whole and in the area of spreading. Statistical processing was performed using the STATISTICA 10.0 software package. Differences at $p < 0.05$ were considered statistically significant.

Results. The percentage of Ki67 expression in the tissue of the primary tumor node was higher in cases with the presence of tumor spread through air spaces, including stage IA lung adenocarcinoma. The percentage of Ki67 expression in the area of air space spread of the tumor was higher compared to the percentage of expression in the tumor as a whole. Higher proliferative activity in the zone of tumor air space spread was observed in cases with extensive spread compared to cases with a limited variant of air space spread.

Conclusion. The results obtained complement the existing data on the mechanisms of tumor spread through air spaces as a form of invasive growth and one of the variants of tumor progression characteristic of lung cancer.

Keywords: lung cancer, spread through air spaces, proliferative activity, Ki67

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Conformity with the principles of ethics. All individuals signed an informed consent to participate in the study. The study was approved by the local Ethics Committee of Siberian State Medical University (Minutes No. 8455/1 dated June 28, 2020).

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Особенности пролиферативной активности немелкоклеточного рака легкого при распространении опухоли по воздушным пространствам

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РЕЗЮМЕ

Цель исследования. Изучить пролиферативную активность немелкоклеточного рака легкого при распространении опухоли по воздушным пространствам.

Материалы и методы. В исследование включены 88 пациентов с немелкоклеточным раком легкого стадии IA–IIIA. Оценивался операционный материал. Гистологическое и иммуногистохимическое исследование выполнялись по стандартной методике. Применялись антитела к Ki67 (клон SP6, Cell Marque). В оцифрованных препаратах с применением автоматизированной системы Pannoramic MIDI с программным обеспечением Slide Viver V2.8. и многомодульной платформы Quant Center (3D HISTECH, Венгрия) подсчитывалась доля клеток (%) с позитивной ядерной экспрессией Ki67 в опухоли в целом и в зоне распространения по воздушным пространствам. Статистическая обработка проводилась с применением пакета программ Statistica 10.0. Статистически значимыми считали различия при $p < 0,05$.

Результаты. Экспрессия Ki67 (%) в ткани первичного опухолевого узла была выше в случаях с наличием распространения опухоли по воздушным пространствам, в том числе и при стадии IA аденокарциномы легкого. Экспрессия Ki67 (%) в зоне распространения опухоли по воздушным пространствам была выше в сравнении с экспрессией в опухоли в целом. Более высокая пролиферативная активность в зоне распространения опухоли по воздушным пространствам наблюдалась в случаях с обширным распространением в сравнении со случаями с ограниченным вариантом распространения по воздушным пространствам.

Заключение. Полученные результаты дополняют сведения о механизмах распространения опухоли по воздушным пространствам как формы инвазивного роста и одного из вариантов опухолевой прогрессии, характерного для рака легкого.

Ключевые слова: рак легкого, распространение по воздушным пространствам, пролиферативная активность, Ki67

Конфликт интересов. Авторы заявляют об отсутствии конфликта интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Авторы заявляют об отсутствии финансирования при проведении исследования.

Соответствие принципам этики. Все лица подписали информированное согласие на участие в исследовании. Исследование одобрено локальным этическим комитетом СибГМУ (протокол № 8455/1 от 28.06.2020).

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INTRODUCTION

Tumor spread through air spaces (STAS) is defined as the presence of tumor cells in the alveolar spaces beyond the margin of the primary tumor node. For a long time, this morphological picture was considered as an artifact resulting from mechanical spread of tumor cells during surgical interventions or tissue resection [1].

Currently, STAS is assessed as an invasion criterion associated with a high risk of recurrence in patients with early non-small cell lung cancer

after lung resection [2, 3]. However, most studies are devoted to lung adenocarcinoma. The presence of STAS in patients with lung adenocarcinoma was associated with decreased overall and recurrence-free survival after segmental resection [4].

Given that STAS has been consistently associated with a high risk of recurrence after segmental resection in patients with early-stage lung cancer, this prompted the need to study the biological characteristics of this phenomenon.

There is evidence of an association between STAS and the histological subtype of adenocarcinoma, size, degree of differentiation of the primary tumor, certain ALK mutations, and PD-L1 status of the primary tumor [5, 6].

A.Hashinokuchi et al. classified STAS as limited (with a maximum spread distance of ≤1000 μm) and extensive (with a maximum spread distance >1000 μm). In cases of extensive STAS, a more advanced disease stage, vascular invasion, and shorter overall and recurrence-free survival were more frequently observed [7].

There are data on the association of STAS with the proliferative activity of non-small cell lung cancer. At the same time, the association of this feature with prognosis remains ambiguous. According to several authors, in patients with lung adenocarcinoma with a poor outcome, a high percentage of Ki67 expression was noted, while in patients with squamous cell carcinoma, a low percentage of Ki67 expression was found. Low proliferative activity in squamous cell lung cancer is associated with epithelial – mesenchymal transition, immunosuppressive microenvironment, angiogenesis, and higher frequency of metastasis [8, 9].

Currently, more and more data are emerging indicating the need to classify STAS as a special variant of tumor progression. In this regard, studying the proliferative activity in the STAS zone is of interest.

The aim of the study was to investigate the proliferative activity of non-small cell lung cancer in tumor spread through air spaces.

MATERIALS AND METHODS

Surgical material from 88 patients with non-small cell lung cancer T1-3N0-1M0, stages IA-IIIA, who underwent pneumonectomy or lobectomy in the Thoracoabdominal Unit of the Cancer Research Institute of Tomsk National Research Medical Center was studied. The disease severity was determined according to the international TNM classification system (8th Edition, 2017). The histological type of the tumor was established based on the World Health Organization classification (5th Edition, 2021). The study included only cases with invasive non-mucinous adenocarcinoma or squamous cell carcinoma with central localization.

Surgical material was evaluated, including the primary tumor nodule with adjacent lung tissue and all removed lymph nodes. The material was fixed in a 10–12% solution of neutral buffered formalin. Material processing was carried out using a histological processing machine (Leica, Germany).

The characteristics of the main parameters of the study patient groups are presented in the table (Table 1).

Table 1

Characteristics of the Study Groups of Patients with Non-Small Cell Lung Cancer

Parameters	Adenocarcinoma, $n = 39$	Squamous Cell Carcinoma, $n = 49$	p
Age, *Me* [Q_1; Q_3]	60.0 [56.0; 64.0]	60.0 [53.0; 65.0]	0.821
Sex, *n* (%)			
Men	28 (72%)	46 (94%)	0.005
Women	11 (28%)	3 (6%)	
Characteristics of the primary tumor nodule, *n* (%)			
T1	20 (51%)	8 (16%)	0.002
T2	10 (26%)	24 (49%)	
T3	9 (23%)	17 (35%)	
Characteristics of lymphogenous metastases, *n* (%)			
N0	22 (56%)	27 (55%)	0.902
N1	17 (44%)	22 (45%)	
Stage, *n* (%)			
IA (T1cN0M0)	13 (33%)	6 (13%)	0.093
IB (T2aN0M0)	5 (13%)	9 (18%)	
IIB (T1cN1M0, T2aN1M0, T3N0M0)	16 (41%)	29 (59%)	
IIIA (T3N1M0)	5 (13%)	5 (%)	
Grade of malignancy, *n* (%)			
Grade 1	11 (28%)	2 (4%)	0.005
Grade 2	17 (44%)	33 (67%)	
Grade 3	11 (28%)	14 (29%)	
STAS, *n* (%)			
No	26 (67%)	31 (63%)	0.740
Yes	13 (33%)	18 (37%)	
Type of STAS, *n* (%)			
Limited	4 (31%)	12 (67%)	0.048
Extensive	9 (69%)	6 (33%)	

Histological preparations were made using the standard method with a rotary microtome HM300 (Thermo Fisher Scientific, USA) and a water bath for flattening sections Leica HI1210 (Leica, Germany).

The preparations were stained with hematoxylin and eosin using a staining machine (Shandon Varistain Gemini, USA). Histological examination was performed using a light microscope Axio Scope A1 (Carl Zeiss, Germany). The degree of tumor differentiation was assessed: grade 1 – high; grade 2 – moderate; grade 3 – low.

In the lung tissue adjacent to the main tumor nodule, the presence of tumor fragments and individual tumor cells was determined. In cases of their detection, the presence of STAS was confirmed. The type of spread was established: limited if spread was at a distance of less than or equal to 1,000 μm and extensive if spread was at a distance greater than 1,000 μm.

Immunohistochemical analysis was performed using antibodies Ki67 (clone SP6, Cell Marque) (Figures 1, 2). Ki67 expression was evaluated on slides digitized by whole-slide scanning using the automated Pannoramic MIDI system with SlideViewer V2.8 software and the multi-module QuantCenter platform (3D HISTECH, Hungary). The percentage of tumor cells with positive nuclear Ki67 expression was counted both in the overall tumor tissue and separately in the STAS zone (Fig. 1–4).

Statistical analysis was performed using the STATISTICA 10.0 software package. The Shapiro–Wilk test was used to assess the normality of distribution. Due to the non-normal distribution of the variables studied, the significance of differences between medians of two independent samples was tested using the nonparametric Mann–Whitney test. For three or more independent samples, the Kruskal–Wallis test was applied.

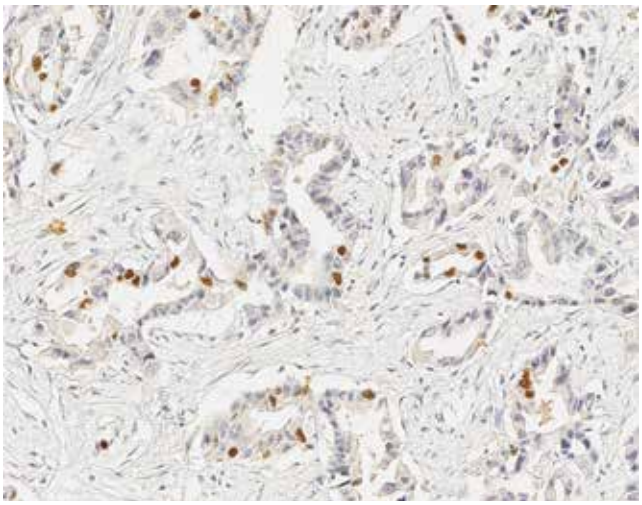

Fig. 1. Positive Ki67 expression in lung adenocarcinoma. Immunohistochemical analysis using antibodies Ki67 (clone SP6, Cell Marque). Magnification ×400.

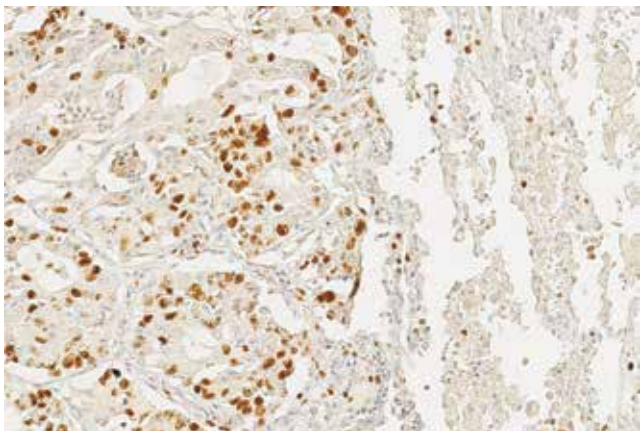

Fig. 2. Positive Ki67 expression in lung adenocarcinoma within the tumor spread through air spaces (STAS) area. Immunohistochemical analysis using antibodies Ki67 (clone SP6, Cell Marque). Magnification ×400.

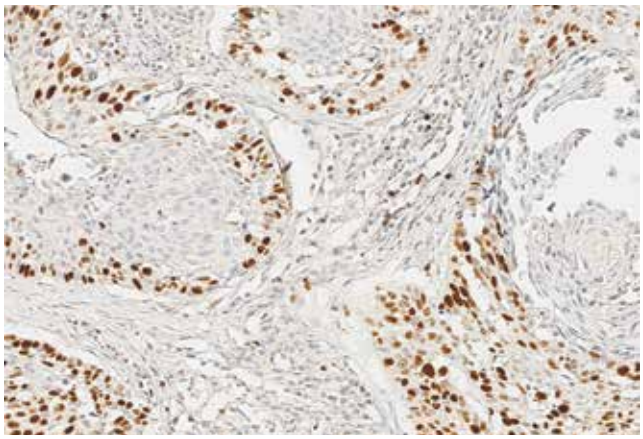

Fig. 3. Positive Ki67 expression in squamous cell lung carcinoma. Immunohistochemical analysis using antibodies Ki67 (clone SP6, Cell Marque). Magnification ×400

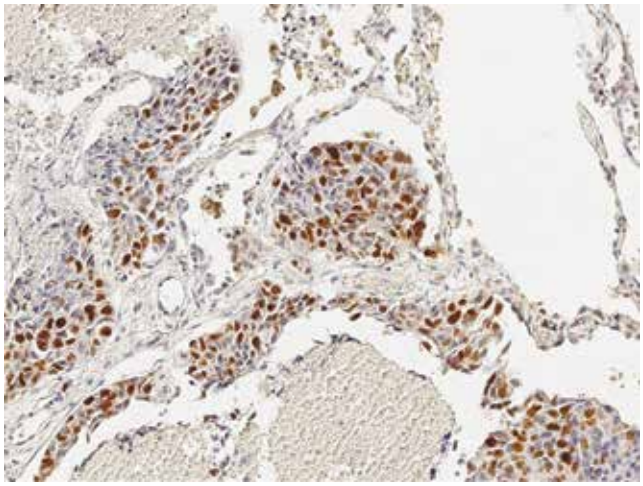

Fig. 4. Positive Ki67 expression in squamous cell lung carcinoma within the tumor spread through air spaces (STAS) area. Immunohistochemical analysis using antibodies Ki67 (clone SP6, Cell Marque). Magnification ×400.

The Pearson's chi-square (χ^2) test was used for comparison of qualitative data. Quantitative data were presented as median and interquartile range *Me* [Q_1; Q_3]; qualitative data were presented as absolute numbers and percentages (%). Differences were considered statistically significant at $p < 0.05$.

RESULTS

In the study of proliferative activity of non-small cell lung cancer, it was found that in both the group of patients with adenocarcinoma and those with squamous cell carcinoma, the percentage of Ki67 expression in the tumor was higher in cases with STAS compared to cases without STAS (Table 2).

Table 2

Proliferative Activity of the Primary Tumor in Groups of Patients with Adenocarcinoma and Squamous Cell Lung Carcinoma, Taking into Account STAS, *Me* [Q_1; Q_3], %

Parameter	Ki67		*p*
	Without STAS	With STAS	
Adenocarcinoma	20.5 [8.0; 30.3] (*n* = 26)	65.5 [54.5; 75.9] (*n* = 13)	0.001
Squamous Cell Carcinoma	37.6 [8.2; 51.5] (*n* = 31)	71.3 [62.6; 82.6] (*n* = 18)	0.001

The study of proliferative activity of the primary tumor considering STAS and the main clinical and pathological parameters in groups of patients with lung adenocarcinoma or squamous cell carcinoma was of interest. In the group of patients with adenocarcinoma without STAS, the percentage of Ki67 expression was higher in cases with T2 and T3 compared to T1 cases. However, in the presence of STAS, this pattern disappeared due to extremely high proliferative activity observed in T1 cases.

Both in N0 and N1 cases, proliferative activity was higher in tumor tissue with STAS. A similar pattern was observed regarding the degree of adenocarcinoma differentiation. Concerning the disease stage, in the absence of STAS, proliferative activity was higher at stage IIIA than at stages IA or IIB; however, this association was absent when STAS was present due to high proliferative activity in IA stage cases (Table 3).

In the group of patients with squamous cell lung carcinoma, the proliferative activity of the primary tumor was higher in cases with STAS compared to those without STAS, regardless of T stage, N stage, disease stage, and degree of squamous cell carcinoma differentiation (Table 4).

Table 3

Proliferative Activity of the Primary Tumor Considering STAS and Main Clinical and Pathological Parameters in the Group of Patients with Lung Adenocarcinoma, *Me* [Q_1; Q_3], %

Parameter	Ki67		*p*
	Without STAS (*n* = 26)	With STAS (*n* = 13)	
Characteristics of the primary tumor nodule, %			
T1	10.2 [6.3; 21.9]	73.5 [54.5; 75.9]	0.001
T2	26.7 [10.8; 32.7]	41.1 [17.8; 79.8]	0.254
T3	27.6 [19.1; 50.7]	60.8 [58.5; 77.2]	0.156
p	0.019	0.697	–
Characteristics of lymphogenous metastases, %			
N0	20.5 [10.8; 31.5]	53.9 [34.9; 73.5]	0.006
N1	16.6 [6.4; 26.7]	75.9 [58.5; 79.8]	0.005
p	0.693	0.074	–
Stage, %			
IA (T1cN0M0)	11.9 [8.0; 22.0]	73.5 [34.9; 74.8]	0.014
IB (T2aN0M0)	32.7 [30.3; 39.6]	32.4 [17.8]	1.000
IIB (T1cN1M0, T2aN1M0, T3N0M0)	11.6 [6.4; 26.3]	70.7 [60.8; 79.8]	0.001
IIIA (T3N1M0)	50.7 [22.4; 92.9]	67.9 [58.5; 77.2]	0.773
p	0.019	0.202	–
Grade of malignancy, %			
Grade 1	22.8 [12.4; 32.7]	76.6 [75.9; 77.2]	0.045
Grade 2	10.9 [6.6; 26.5]	73.5 [65.5; 74.8]	0.002
Grade 3	19.1 [12.4; 22.4]	56.5 [34.9; 60.8]	0.171
p	0.365	0.206	–

Table 4

Proliferative Activity of the Primary Tumor Considering STAS and Main Clinical and Pathological Parameters in the Group of Patients with Squamous Cell Lung Carcinoma, *Me* [Q_1; Q_3], %

Parameter	Ki67		*p*
	Without STAS, *n* = 31	With STAS, *n* = 18	
Characteristics of the primary tumor nodule, %			
T1	28.5 [24.8; 43.8]	66.4 [60.0; 72.8]	0.067
T2	26.9 [7.6; 54.8]	76.5 [63.0; 82.9]	0.001
T3	43.5 [14.6; 53.8]	62.6 [62.3; 73.5]	0.031
p	0.704	0.448	–
Characteristics of lymphogenous metastases, %			
N0	26.9 [7.6; 44.4]	62.5 [59.7; 69.7]	0.011
N1	41.9 [23.4; 56.0]	76.6 [65.6; 86.2]	0.001
p	0.254	0.028	–
Stage, %			
IA (T1cN0M0)	28.5 [24.8; 43.8]	–	–
IB (T2aN0M0)	11.3 [4.7; 26.9]	69.7 [33.8; 76.5]	1.000
IIB (T1cN1M0, T2aN1M0, T3N0M0)	42.8 [10.4; 61.9]	68.2 [62.6; 82.6]	0.004

End of table 4

Parameter	Ki67		*p*
	Without STAS, *n* = 31	With STAS, *n* = 18	
IIIA (T3N1M0)	43.8 [23.4; 56.0]	84.4 [73.5; 95.2]	0.149
p	0.338	0.319	–
Grade of malignancy, %			
Grade 1	3.1 [3.1; 3.1]	69.7 [69.7; 69.7]	1.000
Grade 2	41.3 [13.9; 51.5]	68.2 [62.3; 79.7]	0.007
Grade 3	20.5 [9.6; 47.5]	74.7 [62.6; 82.9]	0.028
p	0.182	0.822	–

It was of interest to evaluate the proliferative activity of non-small cell lung cancer directly in the STAS zone and in the tumor as a whole. In STAS, the percentage of Ki67 expression was higher compared to the proliferative activity of the tumor as a whole (Table 5).

Table 5

Proliferative Activity of Non-Small Cell Lung Cancer in Cases with Spread Through Air Spaces in the Tumor as a Whole and in the Spread through Air Spaces Zone, *Me* [Q_1; Q_3], %

Localization	Ki67
In the tumor as a whole, *n* = 31	69.7 [60.0; 79.7]
In the STAS zone, *n* = 31	79.6 [71.5; 87.5]
p	0.007

Comparison of tumor proliferative activity in the STAS zone with proliferative activity in the tumor tissue as a whole, considering the histotype of non-small cell lung cancer represented by adenocarcinoma or squamous cell carcinoma, demonstrated differences at the level of a pronounced trend (Table 6).

Table 6

Proliferative Activity of the Tumor in Cases with Spread through Air Spaces in the Tumor as a Whole and in the Spread through Air Spaces zone, Considering the Histotype of Non-Small Cell Lung Cancer, *Me* [Q_1; Q_3], %

Localization	Ki67	
	Adenocarcinoma, *n* = 13	Squamous cell carcinoma, *n* = 18
In the tumor as a whole	65.5 [54.5; 75.9]	71.3 [62.6; 82.6]
In the STAS zone	79.6 [65.2; 84.5]	79.9 [72.5; 88.5]
Level of significance	*p* = 0.059	*p* = 0.054

The study of proliferative activity in the STAS area and in the tumor overall among patients with lung adenocarcinoma, taking into account the main clinical and pathological parameters, revealed no statistically significant differences in the percentage of Ki67 expression (Table 7).

No statistically significant differences were found in the percentage of Ki67 expression in the STAS area and in the tumor overall, taking into account the main clinical and pathological parameters, in the group of patients with squamous cell lung carcinoma (Table 8).

Table 7

Proliferative Activity in the STAS Area and in the Tumor Overall, Considering the Main Clinical and Pathological Parameters in Patients with Lung Adenocarcinoma, *Me* [Q_1; Q_3], %

Parameter	Ki67		*p*
	In the STAS zone, *n* = 13	In the tumor as a whole, *n* = 13	
Characteristics of the primary tumor nodule, %			
T1	81.6 [73.2; 84.5]	73.5 [54.5; 75.9]	0.193
T2	65.2 [56.4; 87.5]	47.1 [17.8; 79.8]	0.347
T3	73.5 [62.3; 86.5]	60.8 [58.5; 77.2]	0.400
p	0.831	0.697	–
Characteristics of lymphogenous metastases, %			
N0	69.4 [56.4; 81.6]	53.9 [34.9; 73.5]	0.123
N1	84.5 [73.2; 87.5]	75.9 [58.5; 79.8]	0.177
p	0.100	0.074	–
Stage, %			
IA (T1cN0M0)	81.6 [54.8; 83.5]	73.5 [34.9; 74.8]	0.488
IB (T2aN0M0)	60.8 [56.4; 65.2]	32.5 [17.8; 47.1]	0.205
IIB (T1cN1M0, T2aN1M0, T3N0M0)	82.1 [73.5; 87.5]	70.7 [60.8; 79.8]	0.142
IIIA (T3N1M0)	74.4 [62.3; 86.5]	67.9 [58.5; 77.2]	0.710
p	0.303	0.202	–
Grade of malignancy, %			
Grade 1	85.5 [84.5; 86.5]	76.6 [75.9; 77.2]	0.017
Grade 2	81.6 [73.2; 83.5]	73.5 [65.5; 74.8]	0.188
Grade 3	67.9 [56.4; 79.6]	56.5 [34.9; 60.8]	0.190
p	0.243	0.206	–

Table 8

Proliferative Activity in the STAS Area and in the Tumor Overall, Considering the Main Clinical and Pathological Parameters in Patients with Squamous Cell Lung Carcinoma, *Me* [Q_1; Q_3], %

Parameter	Ki67		*p*
	In the STAS zone, *n* = 18	In the tumor as a whole, *n* = 18	
Characteristics of the primary tumor nodule, %			
T1	79.9 [78.5; 81.3]	66.4 [60.0; 72.8]	0.175
T2	87.5 [72.5; 91.3]	76.5 [63,0; 82.9]	0.201
T3	76.5 [71.5; 86.5]	62.6 [62.3; 73.5]	0.378
p	0.831	0.697	–
Characteristics of lymphogenous metastases, %			
N0	72.0 [62.5; 76.5]	62.5 [59.7; 69.7]	0.191
N1	87.0 [76.9; 91.4]	76.6 [65.6; 86.2]	0.081
p	0.100	0.074	–
Stage, %			
IA (T1cN0M0)	–	–	–
IB (T2aN0M0)	72.5 [56.5; 87.6]	69.7 [33.8; 76.5]	0.488
IIB (T1cN1M0, T2aN1M0, T3N0M0)	78.5 [72.5; 88.5]	68.2 [62.6; 82.6]	0.073

End of table 8

Parameter	Ki67		p
	In the STAS zone, n = 18	In the tumor as a whole, n = 18	
IIIA (T3N1M0)	92.5 [86.5; 98.5]	84.4 [73.5; 95.2]	0.578
p	0.303	0.202	–
Grade of malignancy, %			
Grade 1	72.5 [72.5; 72.5]	69.7 [69.7; 69.7]	–
Grade 2	76.5 [71.2; 87.5]	68.2 [62.3; 79.7]	0.208
Grade 3	84.5 [72.5; 91.5]	74.7 [62.6; 82.9]	0.167
p	0.243	0.089	–

The study of the percentage of Ki67 expression in non-small cell lung cancer tissue in patient groups with different types of STAS showed that while proliferative activity in the tumor overall did not differ, in the STAS area proliferative activity was higher in cases with extensive spread compared to cases with limited STAS (Table 9).

Table 9

Proliferative Activity of Non-Small Cell Lung Cancer in the Tumor Overall and in the STAS Area with Different Types of Spread, *Me* [Q_1; Q_3], %

Localization	Ki67		p
	Limited STAS, n = 16	Extensive STAS, n = 15	
In the tumor as a whole	62.8 [59.9; 71.6]	75.9 [62.7; 79.8]	0.123
In the STAS zone	73.4 [66.9; 82.5]	84.5 [79.6; 87.6]	0.049

No statistically significant differences were found in the level of proliferative activity in the tumor overall and in the STAS area with different types of spread in cases of adenocarcinoma and squamous cell carcinoma.

DISCUSSION

The conducted study showed that STAS as a form of invasive growth in lung carcinomas is associated with higher proliferative activity in the primary tumor in both adenocarcinoma and squamous cell lung cancer. Moreover, tumor cells in the presence of STAS are characterized by high proliferative activity in cases of stage IA disease, which is more pronounced in adenocarcinoma. In the absence of STAS, proliferative activity increases from stage IA to IIIA. This may be due to the temporal selection of more aggressive clones of tumor cells. Conversely, high proliferative activity of tumor cells already at stage IA may be caused by the emergence of such clones at the early stages of carcinogenesis.

It has been shown for the first time that the proliferative activity of non-small cell lung cancer in the STAS area is higher compared to the proliferative activity in the tumor overall, and moreover, higher proliferative activity in STAS is observed in cases with extensive spread compared to cases with limited spread. Higher proliferative activity both in the tumor and in the STAS area is characteristic already at the early stages of carcinoma development.

This phenomenon indicates that STAS is based on active tumor growth in air spaces. This is further confirmed by the fact that with a greater degree of STAS, proliferative activity in the tumor tissue is higher than with limited STAS. The obtained results are a strong argument in favor of STAS being a true form of invasive tumor growth, rather than an artifact. The data on the association of STAS with proliferative activity have fundamental significance.

CONCLUSION

The obtained results supplement the knowledge about the mechanisms of STAS as a form of invasive growth and one of the variants of tumor progression characteristic of lung cancer.

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Heterogeneity of VEGF Dynamics in the Acute Period of Ischemic Stroke: Association with Disease Severity and Short-term Outcomes

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ABSTRACT

Aim. To evaluate serum vascular endothelial growth factor (VEGF) levels in the dynamics of the acute period of ischemic stroke in patients during clinical and functional recovery.

Material and methods. The study included 114 patients with ischemic stroke. Patient groups were the following: Group 1 – mild stroke (n = 57 patients), Group 2 – moderate stroke (n = 25 patients), Group 3 – severe stroke (n = 32 patients). Observation period was 14 days. Observation points included: I – the first 48–72 hours from the onset of the disease; II – the 14th day. We used the following assessment scales: National Institute of Health Stroke Scale (NIHSS) and the modified Rankin Scale (mRS). VEGF was determined in blood serum on a multiplex analyzer. Statistical processing of the results was carried out using the Statistica 13.0 software package.

Results. Patients of groups 1 and 2 showed a statistically significant decrease in points on the NIHSS and mRs scales ($p < 0.001$) in the dynamics of observation, in patients of group 3, no significant changes were found ($p = 0.157$ and $p = 0.315$, respectively). VEGF in the comparison group did not show reliable differences relative to patients at points I ($p_{z-1} = 0.73$, $p_{z-2} = 0.738$, $p_{z-3} = 0.129$) and II of observation ($p_{z-1} = 0.66$, $p_{z-2} = 0.817$, $p_{z-3} = 0.276$). Analysis of the dynamics of the marker revealed an increase in VEGF between points I and II of observation in group 3 ($p = 0.021$), ΔVEGF positively correlated with a higher score on the NIHSS scale at point I ($r = 0.691$; $p = 0.027$). No relationships were found in group 1 ($p_{I–II} = 0.078$, $r_{\Delta VEGF\text{-}NIHSS\_I} = –0.294$; $p_{\Delta VEGF\text{-}NIHSS\_I} = 0.237$) and group 2 patients ($p_{I–II} = 0.285$, $r_{\Delta VEGF\text{-}NIHSS\_I} = –0.305$; $p_{\Delta VEGF\text{-}NIHSS\_I} = 0.392$).

Conclusion. Heterogeneity of ischemic stroke pathogenesis reduces the prognostic value of VEGF as an isolated biomarker. A comprehensive analysis of the temporal patterns of VEGF regulation and other angiogenic factors is needed to understand the dynamics of vascular remodeling and predict the outcomes of ischemic stroke.

Keywords: vascular endothelial growth factor, angiogenesis, biomarker, prognosis, clinical recovery

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Conformity with the principles of ethics. The clinical trial protocol was developed in accordance with the requirements of the Russian National Standard P52379-2005 “Good Clinical Practice” (2005) and approved by the Ethics Committee of Siberian State Medical University of the Ministry of Healthcare of the Russian Federation (Minutes No. 8565/1 dated January 21, 2021). Prior to inclusion in the study, all subjects or their immediate family members were informed of the nature, objectives, and potential risks of the study and provided a voluntary informed written consent to participation.

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Гетерогенность динамики VEGF в остром периоде ишемического инсульта: взаимосвязь с тяжестью заболевания и краткосрочными исходами

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РЕЗЮМЕ

Цель: оценка динамики сывороточного уровня фактора роста эндотелия сосудов (VEGF) у пациентов в остром периоде ишемического инсульта в контексте клинического и функционального восстановления, с акцентом на различия между патогенетическими подтипами и тяжестью заболевания.

Материалы и методы. Исследуемая выборка составила 114 пациентов с ишемическим инсультом головного мозга. Группы пациентов: 1-я группа – легкий инсульт (n = 57 пациентов), 2-я группа – средней степени тяжести (n = 25 пациентов), 3-я группа – тяжелый инсульт (n = 32 пациента). Период наблюдения: 14 сут. Точки наблюдения: I – первые 48–72 ч от начала заболевания; II – 14-е сут. Оценочные шкалы: шкала инсульта Национального института здоровья (NIHSS), модифицированная шкала Рэнкина (mRS). Уровень VEGF определяли в сыворотке крови на мультиплексном анализаторе. Статистическая обработка результатов проводилась с использованием пакета прикладных программ Statistica 13.0.

Результаты. У пациентов 1-й и 2-й групп обнаружено статистически значимое снижение количества баллов по шкалам NIHSS и mRs ($p < 0{,}001$) в динамике наблюдения, у пациентов 3-й группы значимых изменений не выявлено ($p = 0{,}157$ и $p = 0{,}315$ соответственно). Уровень VEGF в группе сравнения не показал достоверных различий относительно пациентов в I ($p_{z-1} = 0{,}73$; $p_{z-2} = 0{,}738$; $p_{z-3} = 0{,}129$) и во II точке наблюдения ($p_{z-1} = 0{,}66$; $p_{z-2} = 0{,}817$; $p_{z-3} = 0{,}276$). Анализ динамики маркера выявил увеличение уровня VEGF между I и II точками наблюдения у пациентов 3-й группы ($p = 0{,}021$), ΔVEGF положительно коррелировала с более высоким баллом по шкале NIHSS в I точке ($r = 0{,}691$; $p = 0{,}027$). Корреляционных взаимосвязей в 1-й группе ($p_{I-II} = 0{,}078$, $r_{\Delta VEGF\text{-}NIHSS\_I} = -0{,}294$; $p_{\Delta VEGF\text{-}NIHSS\_I} = 0{,}237$) и 2-й группе пациентов ($p_{I-II} = 0{,}285$; $r_{\Delta VEGF\text{-}NIHSS\_I} = -0{,}305$; $p_{\Delta VEGF\text{-}NIHSS\_I} = 0{,}392$) не выявлено.

Заключение. Гетерогенность патогенеза ишемического инсульта снижает прогностическую ценность VEGF как изолированного биомаркера. Необходим комплексный анализ временных закономерностей регуляции VEGF и других ангиогенных факторов для понимания динамики сосудистого ремоделирования и прогнозирования исходов ишемического инсульта головного мозга.

Ключевые слова: фактор роста эндотелия сосудов, ангиогенез, биомаркер, прогноз, клиническое восстановление

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Авторы заявляют об отсутствии финансирования при проведении исследования.

Соответствие принципам этики. До включения в исследование все субъекты или их ближайшие родственники были осведомлены о характере, целях, возможных рисках исследования и дали добровольное информированное письменное согласие на участие. Протокол клинического исследования разработан в соответствии с требованиями Национального стандарта РФ ГОСТ Р52379-2005 «Надлежащая клиническая практика» GCP (2005 г.) Good Clinical Practice и одобрен этическим комитетом СибГМУ (заключение № 8565/1 от 21.01.2021).

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INTRODUCTION

Strokes consistently represent a key medical and social problem worldwide, accounting for their high morbidity and mortality rates, leading to temporary disability and eventual permanent disability [1]. Scientific communities are continually developing and improving diagnostic algorithms and management strategies for patients with cerebral ischemia. However, issues of predicting the clinical and functional outcomes of the disease remain unsolved to this day. A comprehensive understanding of the neurobiological processes underlying ischemic stroke is crucial to develop early diagnostic, prognostic, and therapeutic approaches. Recently, particular attention has been paid to growth factors, which play a key role in neovascularization mechanisms and brain tissue recovery after acute ischemia [2, 3].

Vascular endothelial growth factor (VEGF) is one of the main growth factors, which is regulated by hypoxia-inducible factor (HIF) in response to acute cerebral ischemia. In cerebral stroke, VEGF is expressed on the surface of astrocytes, neurons, and endothelial cells in the infarct core and in the ischemic penumbra. Outside the central nervous system, VEGF is derived from a variety of cells, including macrophages and platelets [4]. Experimental studies on models of acute cerebral ischemia in rodents have shown that VEGF has a pleiotropic effect. On the one hand, its activation triggers angiogenesis and has a neuroprotective effect [5, 6]. On the other hand, VEGF promotes the disruption of the blood – brain barrier (BBB) and increased vascular permeability, leading to the progression of cerebral edema [7].

Despite the fact that VEGF has been studied as a prognostic marker for ischemic stroke since the 1970s, and a significant body of scientific data has been accumulated, a consensus on the role of this vascular factor in clinical and functional recovery has still not been reached. A 2013 study by R. Matsuo et al. demonstrated a sustained increase in plasma VEGF levels over a 90-day period following ischemic stroke in 171 patients, regardless of the stroke pathogenetic subtype, compared to a control group [8]. In contrast, a 2021 meta-analysis by A. Seidkhani-Nahal et al. showed that serum VEGF levels on day 1 and day 7 of acute cerebral ischemia were not statistically different between 769 patients and 621 controls [9].

The research team of A. Bhasin et al. in 2019 conducted a clinical and laboratory examination of 250 patients with cerebral ischemic stroke using the National Institutes of Health Stroke Scale (NIHSS) and the Modified Rankin Scale (mRS). In their work, the authors identified an association between VEGF levels and disease outcomes on day 7 and day 90 of cerebral ischemia [10].

Thus, the current clinical data on the role of VEGF as a potential marker for outcomes of ischemic stroke do not allow for definitive conclusions. The study of VEGF – a key factor in angiogenesis and vascular remodeling in the recovery processes of patients with ischemic stroke – is of significant scientific and practical interest. Such research is important both to understand the pathogenetic mechanisms of cerebral ischemia and to find new effective tools for forecasting rehabilitation potential, aiming for a future personalized treatment approach.

The aim of the study was to assess the changes in serum VEGF levels in patients during the acute phase of ischemic stroke in the context of clinical and functional recovery, with a focus on differences between pathogenetic subtypes of stroke and disease severity.

MATERIALS AND METHODS

This study was conducted at the Neurology and Neurosurgery Division of the Siberian State Medical University (SibSMU) in collaboration with the Laboratory of Molecular Genetics and Biochemistry at the Mental Health Research Institute, Tomsk National Research Medical Center. The study included 114 patients (51 women, 63 men) with ischemic stroke, hospitalized at the Regional Vascular Center of Tomsk Regional Hospital within 48–72 hours after the onset of focal neurological symptoms.

The median patient age was 65 (59; 70) years. The diagnosis of stroke was verified according to the WHO clinical criteria and confirmed by neuroimaging data. The nosological form of the disease was established in accordance with the International Classification of Diseases, 10th Revision (ICD-10). An informed consent was obtained from the patients or their immediate family members prior to inclusion in the study. Exclusion criteria were as follows: transient ischemic attack,

hemorrhagic stroke, history of stroke, nervous system damage of other etiology (traumatic, autoimmune, neurodegenerative, neoplastic, or epilepsy), and extracranial pathology (connective tissue diseases, musculoskeletal system diseases, hereditary disorders, or neoplasms).The patient population was divided into three groups based on the severity of neurological deficit assessed using the NIHSS scale (Goldstein et al., 2011): group 1 – mild stroke (NIHSS score 1–6, *n* = 57); group 2 – moderate stroke (NIHSS score 7–13, *n* = 25); group 3 – severe stroke (NIHSS score 14–42, *n* = 32) [11]. The clinical and demographic characteristics of the patient cohort are presented in Table 1. The comparison group consisted of 13 volunteers with a median age of 64 (58; 71) years, with no history of cerebrovascular or other organic lesions of the central nervous system, comparable to the study population in terms of sex, age, and cardiovascular risk factors.

Table 1

Clinical and Demographic Characteristics of the Study Population

Characteristic	Group 1, *n* = 57	Group 2, *n* = 25	Group 3, *n* = 32
Gender, *n* (%)			
– men	34 (59.6%)	12 (48%)	17 (53.1%)
– women	23 (40.4%)	13 (52%)	15 (46.9%)
Age, years *Me* [Q_1;Q_3]	65 [59; 69]	66 [59; 68]	69 [62; 74]
Body mass index, kg/m^2, *Me* [Q_1;Q_3]	28.26 [25.95; 30.85]	28.13 [25,25; 35.14]	27.99 [23.15; 31.22]
Arterial hypertension, *n* (%)	57 (100%)	25 (100%)	32 (100%)
Atherosclerosis of the aorta and heart valves, *n* (%)	45 (78.9%)	15 (60%)	20 (62.5%)
Diabetes mellitus, *n* (%)	12 (21.1 %)	6 (24%)	8 (25%)
Coronary heart disease, *n* (%)	15 (26.3%)	7 (28%)	14 (43.8%)
Myocardial infarction, *n* (%)	6 (10.5%)	3 (12%)	5 (15.6%)
Stenting and artificial heart valves, *n* (%)	2 (3.5%)	1 (4%)	2 (6.3%)
Atrial fibrillation, *n* (%)	13 (22.8%)	3 (12%)	13 (40.6%)
Dyslipidemia, *n* (%)	48 (84.2%)	19 (76%)	18 (56.25%)
Smoking, *n* (%)	15 (26.3%)	5 (20%)	2 (6.3%)
Affected cerebral hemisphere			
– right, *n* (%)	29 (50.9%)	15 (60%)	16 (50%)
– left, i (%)	28 (49.1%)	9 (40%)	16 (50%)
Stroke subtype according to TOAST criteria:			
– atherothrombotic, *n* (%)	8 (14%)	8 (32%)	10 (31.3%)
– cardioembolic, *n* (%)	13 (22.8%)	3 (12%)	13 (40.6%)
– lacunar, *n* (%)	3 (5.3%)	1 (4%)	0
– other established etiology, *n* (%)	0	0	0
– unknown etiology, *n* (%)	33 (57.9%)	13 (52%)	9 (28.1%)

The observation period was 14 days. Assessment time points were as follows: I – the acute stroke period (first 48–72 hours), II – the subacute period (day 14 of the disease). Neurological deficit was assessed using the NIHSS, and functional disability was evaluated with the mRS scale. Patient serum was used as the biological material for analysis. VEGF concentration was measured using the MAGPIX multiplex analyzer (Luminex, USA) and the HNDG3MAG-36K panel from MILLIPLEX MAP (Merck, Darmstadt, Germany). The results were expressed in pg/mL. Statistical analysis was performed using the Statistica 13.0 software package. The critical significance level for testing statistical hypotheses was set at 0.05 (p is the obtained significance level). Categorical variables were presented as absolute numbers and relative frequencies *n* (%). Quantitative and ordinal variables were presented as median and interquartile range, *Me* [Q_1; Q_3].

The Kruskal–Wallis test was used to compare multiple independent groups, with the Mann–Whitney U test and Bonferroni correction used for pairwise comparisons. Changes between the two time points were assessed using the Wilcoxon signed-rank test. Correlation analysis between variables was performed using non-parametric methods.

Table 2

Dynamics of Clinical and Laboratory Parameters in Acute Ischemic Stroke across Observation Groups

Criteria	Patient Groups			Comparison			
				Evaluation of dynamics, p_{I-II}			Intergroup comparison, $p_{1,2,3}$
	1 (n=57)	2 (n=25)	3 (n=32)	Groups			
				1	2	3	
mRs_I	3 [2; 3]	4 [4; 5]	5 [5; 5]	<0.001*	<0.001*	0.157	<0.001*
mRs_II	2 [1; 2]	4 [3; 4]	5 [5; 5]				<0.001*
NIHSS_I	4 [3; 5]	10 [2; 3]	18 [16; 21]	<0.001*	<0.001*	0.315	<0.001*
NIHSS_II	3 [2; 3]	7 [5; 8]	20 [12; 23]				<0.001*
VEGF_I pg/mL	83.0 [35.3; 113.6]	70.2 [47.6; 88.1]	53.5 [25.4; 90.6]	0.078	0.285	0.021*	0.377
VEGF_II pg/mL	90.3 [47.6; 150.2]	100.1 [64.8; 113.6]	110.9 [61.8; 228.2]				0.724
Δ VEGF pg/mL	18 [–5; 53]	13 [5; 17]	68 [38; 105]				0.065

* $p < 0.05$.

RESULTS

During the study, patients at point I demonstrated significant differences in the severity of neurological deficit according to the NIHSS and functional impairment according to the mRS across all groups.

In patients of groups 1 (mild stroke) and 2 (moderate stroke), a statistically significant decrease in NIHSS and mRS scores was found ($p_{I-II} < 0.001$), indicating a regression of neurological deficit and functional recovery by day 14 of the disease (Table 2). Moderate positive correlations between ΔNIHSS and ΔmRS further confirmed the clinical improvement and functional independence of patients in groups 1 and 2 at the second observation point ($r_1 = 0.645$, $p_1 < 0{,}001$ and r_2=0.507, p_2=0.001, respectively).

In group 3 patients with severe stroke, no significant quantitative changes in the studied scores were found at the observation points (Table 2). At the same time, ΔNIHSS significantly correlated with mRs_II, reflecting the degree of disability in patients with severe stroke in the absence of recorded clinical improvement on day 14 of the disease ($r_3 = 0.418$, $p_3 = 0.019$).

The serum VEGF concentration in the comparison group was 70.2 [47.6; 138.6] pg/mL and did not statistically significantly differ in the patient groups, either within the first 48–72 hours of ischemic stroke ($p_{z-1} = 0.73$, $p_{z-2} = 0.738$, $p_{z-3} = 0.129$) or on day 14 of stroke ($p_{z-1} = 0.66$, $p_{z-2} = 0.817$, $p_{z-3} = 0.276$). No significant differences in the marker levels in the peripheral blood of patients with varying severity of ischemic stroke were found either (Table 2).

A comparative analysis at observation point II revealed a significant increase in serum VEGF concentrations during the dynamics of the acute period in group 3 patients with severe stroke ($p_{I-II} = 0.021$). Here, ΔVEGF was 68 [38; 105] pg/mL and positively correlated with a higher NIHSS_I score ($r = 0.691$; $p = 0.027$).

Within the study, patients were also divided into stroke subtypes according to the TOAST (Trial of ORG 10172 in Acute Stroke Treatment) classification [12]. This resulted in the following groups: atherothrombotic stroke ($n = 26$), cardioembolic stroke ($n = 29$), and a combined group including patients with lacunar stroke and stroke of unspecified etiology ($n = 59$), due to the insufficient number of patients in the first category. No cases of stroke of other established etiologies were registered in the cohort.

The results of the comparative analysis showed that in the group of patients with the cardioembolic subtype of stroke, VEGF levels in the first 48–72 hours of the disease were significantly lower compared to the control group ($p = 0.039$).

Correlation analysis revealed significant relationships between clinical and laboratory parameters exclusively in the group of patients with atherothrombotic stroke, where ΔVEGF was 10 [–22; 63] pg/mL and positively correlated with the NIHSS score at both observation points I ($r = 0.754$; $p = 0.012$) and II ($r = 0.695$; $p = 0.026$). A similar correlation was found between ΔVEGF and mRs_II ($r = 0.695$; $p = 0.026$). The obtained results indicate the presence of a reliable relationship between the changes in VEGF

growth, the severity of neurological deficit, and the degree of functional impairment by the end of the 14-day observation period.

DISCUSSION

In acute ischemic brain injury, VEGF is one of the main regulators of angiogenesis and neuronal survival, determining the clinical outcome of stroke [13]. Immunohistochemical studies conducted on experimental models of middle cerebral artery occlusion in rats showed that VEGF expression in astrocytes in the ischemic core increased after 2 hours and then gradually decreased over 6 hours. Increased VEGF immunoreactivity in hypertrophied astrocytes and endothelial cells was also detected in the ischemic penumbra zone 24 hours after the onset of ischemia and persisted for 14 days [14].

The results of the present study showed that serum VEGF concentrations in patients during the first 48–72 hours of ischemic stroke remained at a level comparable to those in the comparison group. It is likely that the neuroprotein content in the peripheral blood does not reflect the level of expression in the ischemic focus. VEGF produced locally in brain tissue in response to ischemic injury does not reach the systemic circulation in equal concentrations during the first 48–72 hours, accumulating within the infarct zone. Furthermore, the neuroinflammatory process that develops during acute cerebral ischemia can modulate VEGF expression, counteracting its predicted increase [15]. Clinical studies presented in the international literature demonstrate a negative correlation between leukocyte levels and C-reactive protein compared to angiogenic growth factors, including VEGF, supporting the hypothesis of a negative impact of neuroinflammatory responses on angiogenesis [16].

Of particular interest are the differences in VEGF changes depending on the subtype of ischemic stroke. In cardioembolic stroke, hypoxia develops rapidly, which can lead to suppression of VEGF expression due to severe energy deficit in neurons and glial cells. In contrast to atherothrombotic stroke, in which occlusion develops gradually, the sudden cessation of blood flow in cardioembolism limits the activation time of HIF-1α, a key regulator of VEGF synthesis. As a result, in the first 48–72 hours of cardioembolic stroke, VEGF-dependent pathways likely remain functionally inactive [17]. An additional pathogenetic factor affecting VEGF expression in cardioembolic stroke is the concomitant systemic inflammatory response and hypercoagulability characteristic of atrial fibrillation and other cardiac sources of embolism. Elevated levels of proinflammatory cytokines (in particular, interleukin-6 and tumor necrosis factor-α) in combination with markers of coagulation cascade activation (such as D-dimer) create a microenvironment that suppresses angiogenic processes, which further inhibits VEGF production [18, 19]. In contrast, atherothrombotic stroke, which develops against the background of progressive stenosis of large cerebral arteries, is characterized by chronic hypoxia, which induces persistent compensatory VEGF expression. In lacunar stroke, caused by damage to small arteries, the ischemic response is minimal, which explains the absence of significant changes in VEGF levels [20, 21].

Of particular note is the observation that patients with mild to moderate stroke demonstrated better short-term outcomes of ischemic stroke on day 14 of disease without a significant increase in VEGF over time. This finding may indicate the activation of alternative signaling pathways regulating neuronal recovery and clinical outcomes that do not require vascular remodeling and a significant increase in VEGF expression. Recent research data support the hypothesis that activation of neuronal plasticity mechanisms, and in particular brain-derived neurotrophic factor (BDNF), plays a key role in the motor recovery of patients with ischemic stroke during the first 14 days [22].

In patients with severe ischemic stroke, a significant increase in VEGF in the absence of significant clinical and functional recovery was observed on the 14th day of disease. This increase is likely due to the need for continuous VEGF expression during the acute period to stimulate angiogenesis and neurogenesis [23]. However, angiogenesis may have limited efficacy in restoring lost functions in the short term. In severe stroke, accompanied by deeper damage to the conduction pathways located below the ischemic focus, the need for angiogenesis to restore impaired functions is presumably higher compared to mild or moderate strokes. Activation of angiogenic pathways in response to neuronal damage during acute brain

tissue ischemia likely requires a certain amount of time, which explains the absence of a significant increase in VEGF concentration in the peripheral blood, indicating a delayed activation of vascular remodeling reactions [24, 25]. Thus, VEGF may be a potential marker of functional outcomes in more remote periods of ischemic stroke (end of acute and early recovery).

The identified relationship between increased serum VEGF levels and worsening clinical and functional outcomes in atherothrombotic stroke can be explained by a complex of pathophysiological mechanisms characteristic of this subtype of stroke. The chronic nature of atherosclerotic vascular lesions creates conditions for prolonged hypoxia, which leads to sustained activation of HIF-1α and continuous expression of VEGF. This leads to the development of vascular preconditioning manifested by increased expression of VEGF-R2 receptors and changes in their sensitivity, which, in combination with the activation of matrix metalloproteinase-9 releasing matrix-bound VEGF creates the preconditions for increased vascular permeability [26, 27].

These changes contribute to the development of vasogenic edema due to destabilization of endothelial tight junctions and lead to extravasation of proinflammatory cytokines, which increases the risk of hemorrhagic transformation. It is important to note that atherothrombotic lesions are accompanied by pathological angiogenesis, producing functionally immature vessels, which exacerbates ischemic damage. Chronic hypoxia maintains constant activation of VEGF-dependent signaling pathways, which contributes to prolonged damage to the BBB and more pronounced neurological deficits [20, 28]. Increased VEGF expression in patients with the atherothrombotic subtype of ischemic stroke may serve as a marker of persistent pathophysiological processes, including a progressive increase in the volume of the ischemic lesion [29]. This explains the association we identified between increased VEGF and worse clinical and functional outcomes in this pathogenetic subtype of stroke.

CONCLUSION

The heterogeneity of the pathogenetic mechanisms of ischemic stroke limits the prognostic value of VEGF expression levels as a standalone biomarker during the acute phase of the disease. The prognostic value of VEGF is limited by the variability of the response depending on the severity and pathogenetic subtype of stroke. This study highlights the need to investigate the temporal changes in VEGF regulation and other angiogenic factors to understand the mechanisms of vascular remodeling and functional recovery after cerebral ischemia. Comprehensive analysis of VEGF in combination with other neuron-specific proteins may facilitate a more accurate assessment of compensatory processes in ischemic stroke and will become a valuable tool for predicting disease outcomes.

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Kucherova K.S. – conception and design, data interpretation. Koroleva E.S. – critical revision for important intellectual content. Alifirova V.M. – final approval of the manuscript for publication. Boiko A.S. – laboratory research and analysis of the results. Brazovskaya N.G. – statistical processing of the data. Ivanova S.A. – laboratory research, final approval of the manuscript for publication.

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Risk Assessment of Germline Transition of Adeno-associated Virus Vector of Chimeric Serotype PHP.eB into Mouse Oocytes

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ABSTRACT

Aim. To assess the risk of germline transmission of an adeno-associated virus vector of the chimeric serotype PHP.eB into the germ cells of female mice based on the development of an original method for obtaining mouse oocytes devoid of somatic cells and free vector particles.

Materials and methods. The vector under study was administered intravenously to female outbred CD-1 mice at a dose of 5×10^{10} vector genomes/mouse using an original technique which included hormonal superovulation of exposed animals, oocyte isolation, their purification from contaminating somatic cells, and direct quantitative PCR on lysed oocytes. The vector DNA content in the brain tissue, ovaries, and oocytes was assessed on days 1, 3, 7, 14, 30, and 90 after vector administration.

Results. Using the developed technique, we examined the ability of the adeno-associated virus vector of the chimeric serotype PHP.eB to undergo germline transmission into mouse oocytes. It was established that, despite persistence in brain and ovarian tissue for up to 3 months, vector DNA was not detected in oocytes at any time after vector administration.

Conclusion. The obtained data demonstrate the absence of germline transmission of the studied genetic construct into mouse oocytes. The detection of the adeno-associated vector in the ovaries, despite its absence in oocytes, confirms the effectiveness of the developed technique for obtaining mouse oocytes devoid of somatic cells.

Keywords: adeno-associated vector, chimeric serotype PHP.eB, germline transmission, oocytes, mice, direct PCR

Conflict of interest. The authors declare the absence of obvious or potential conflicts of interest related to the publication of this article.

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Оценка риска вертикального переноса аденоассоциированного вектора химерного серотипа PHP.eB в ооциты мышей

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РЕЗЮМЕ

Цель. Оценить риск вертикального переноса аденоассоциированного вирусного вектора химерного серотипа PHP.eB в половые клетки самок мышей на основе разработки оригинального способа получения ооцитов мышей, неконтаминированных соматическими клетками и свободными векторными частицами.

Материалы и методы. Исследуемый вектор вводили внутривенно самкам аутбредных мышей CD-1 в дозе 5×10^{10} векторных геномов на мышь. Использовали оригинальную методологию, включающую гормональную суперовуляцию экспонированных животных, выделение ооцитов, их очистку от контаминирующих соматических клеток и прямую количественную полимеразную цепную реакцию (ПЦР) на лизированных ооцитах. Содержание векторной ДНК в ткани головного мозга, яичников и в ооцитах оценивали на 1, 3, 7, 14, 30 и 90-е сут после введения вектора.

Результаты. С использованием разработанного подхода исследована способность к вертикальному переносу в ооциты мышей аденоассоциированного вирусного вектора химерного серотипа PHP.eB. Установлено, что несмотря на персистенцию в ткани головного мозга и яичников до 3 мес, векторная ДНК не выявляется в ооцитах ни на одном из сроков после введения вектора.

Заключение. Полученные данные демонстрируют отсутствие вертикального переноса в ооциты мышей исследуемой генной конструкции. Выявление аденоассоциированного вектора в яичниках при его необнаружении в ооцитах подтверждает эффективность разработанной методики получения ооцитов мышей, неконтаминированных соматическими клетками.

Ключевые слова: аденоассоциированный вектор, химерный серотип PHP.eB, вертикальный перенос, ооциты, мыши, прямая ПЦР

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

Gene therapy is a cutting-edge, innovative method for treating hereditary, oncological, cardiovascular, hematological, and other diseases by means of restoring the functions of defective genes or expressing new genes in cells using genetically engineered constructs based on recombinant nucleic acids [1, 2]. Like any drug therapy, gene therapy is associated with certain risks that are specific to the nature of the active agent. For DNA-based drugs designed for *in vivo* gene therapy, one potential and significant safety risk

is the germline (unintentional) transmission of vector DNA sequences into germ cells [3–5]. The discovery of the potential for germline transmission limits the applicability of gene therapy drugs for patients with reproductive potential [6, 7].

According to regulatory guidelines, if vector DNA is detected in the gonads at three consecutive time points (i.e., it persists) during a biodistribution study, additional studies are required to determine its transduction directly into germ cells [7–9]. The irreversibility of transduction and, consequently, a high risk of germline transmission are evidenced by the persistence of vector DNA in spermatozoa for more than 3 cycles of spermatogenesis [8, 9]. The identification of vector DNA in oocytes at any time point is considered as a high risk of germline transmission, since the entire non-renewable ovarian reserve is affected [8, 9].

Despite strict regulatory restrictions on conducting gene therapy when the risk of germline transmission is identified, there is currently no scientifically based, standardized methodology for its assessment. This problem is particularly relevant for female germ cells, since, unlike male germ cells, risk assessment for them can be based only on data of preclinical studies [9]. The EMA guidelines specifically note that the development and validation of animal models for these purposes are a pressing issue [9].

To identify target DNA sequences in germ cells, nucleic acid amplification methods (polymerase chain reaction (PCR) or more sensitive quantitative PCR) are recommended [9]. Alternatively, *in situ* hybridization or immunohistochemistry may be used, provided their sensitivity and reliability are properly validated [9]. However, these methods are costly, labor-intensive, and difficult to standardize. The main disadvantage of PCR-based methods is the high risk of false-positive results due to contamination of germ cells with free vector particles and/or somatic cells (spermatozoa by epididymal or vas deferens cells and oocytes by cumulus cells of the oocyte-cumulus complex) when standard isolation methods are used. In the case of oocytes, the analysis is further complicated by the extremely small amount of cellular material available for testing.

The aim of this study was to assess the risk of germline transmission of an adeno-associated viral vector of the chimeric serotype PHP.eB into the germ cells of female mice based on the development of an original method for obtaining mouse oocytes uncontaminated with somatic cells and free vector particles.

MATERIALS AND METHODS

Production of PHP.eB

In this study, we used the recombinant adeno-associated virus rAAV9-PHP.eB-CASI-Luc, which is an AAV of the chimeric PHP.eB serotype, constructed in the laboratory of Dr. Viviana Gradinaru [10] and carrying the firefly luciferase gene under the control of a modified chicken β-actin (CASI) promoter.

The rAAV9PHPeB-CASI-Luc virus was constructed using the pAAV-CASI-Luc, pAAV2/9-PHPeB, and pHelper Vector plasmids (Cell Biolabs, USA). The pAAV-CASI-Luc was constructed using the CASI promoter sequence together with the cloning sites and the WPRE sequence and the polyadenylation signal that were synthesized at Evrogen (Russia) with subsequent cloning of the synthesized sequence into the pAAV-CMV-Vector plasmid (Cell Biolabs, USA) between the left and right ITRs, replacing the existing expression cassette. Next, the codon-optimized sequence of the firefly luciferase gene used in our previous study [11] was cloned between the CASI promoter sequence and the WPRE element sequence. The pAAV2/9-PHPeB plasmid was derived from the pREPCAP2/9 plasmid using insertional mutagenesis by PCR with specific primers in order to introduce the appropriate amino acid substitutions into the capsid protein sequence of the serotype 9 AAV [10].

To produce the PHP.eB vector, the HEK293 cell line (from the cell culture collection of Gamaleya National Research Center for Epidemiology and Microbiology) was used. Cultivation and transfection were carried out under adherent conditions at 37°C and 5% CO_2, as described previously [12, 13]. The PHP.eB Viral preparations were purified using affinity chromatography (AC) on the POROS™ CaptureSelect™ AAVX Affinity Resin (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Additional purification and buffer exchange were performed using size-exclusion chromatography (SEC) on an XK 26/100 column packed with Superdex 200 sorbent (Cytiva, USA). Final vector formulation was performed on Amicon Ultra-15 100 kDa centrifugal concentrators (Merck, USA). The content of viral genomes in the preparation was assessed as described previously [11–13].

Work with Laboratory Animals

The study was conducted on mature female outbred CD-1 mice weighing 20–22 g and aged 8–9 weeks, obtained from Andreevka animal breeding

facility (Scientific Center of Biomedical Technologies of the Federal Medical and Biological Agency of Russia). The animals were kept in the vivarium of the Federal Research Center for Innovator and Emerging Biomedical and Pharmaceutical Technologies with a 12-hour light cycle, free access to water, and a standard complete diet. The conditions for animal housing and working with the animals complied with the requirements of Directive 2010/63/EU of the European Parliament and the EU Council on the protection of animals used for scientific purposes, as well as with Recommendation No. 33 of the Eurasian Economic Commission (November 14, 2023) "Guidelines for Working with Laboratory (Experimental) Animals in Preclinical (Non-Clinical) Studies".

Administration of Vector to Mice

A suspension of PHP.eB vector particles at a dose of 5×10^{10} vector genomes/mouse in a volume of 50 µl was injected into mice via the lateral tail vein at a flow rate of 70 µl/min using an MD-1001 BASi Bee Baby syringe drive (BASi Corporate Headquarters, USA).

Mice were divided into 7 groups of 5–6 animals each: I (control), II (1 day after injection), III (3 days after injection), IV (7 days after injection), V (14 days after injection), VI (1 month after injection), and VII (3 months after injection). Forty-eight hours prior to vector injection, mice of group II were intraperitoneally administered equine chorionic gonadotropin (eCG, Folligon, MSD Animal Health, Netherlands) at a dose of 5 IU/mouse. Seven hours after vector injection, mice were intraperitoneally administered human chorionic gonadotropin (hCG, PG600, MSD Animal Health, Netherlands) at a dose of 5 IU/mouse and euthanized 17 hours later. Mice of group III were administered eCG together with vector injection. After 55 hours, the mice were administered hCG and euthanized 17 hours later. Mice of groups I, IV, V, VI, and VII were administered PGFA and hCG 65 and 17 hours prior to euthanasia, respectively.

Isolation and Purification of Oocytes

The animals were euthanized by cervical dislocation. Ovaries with oviducts were transferred to drops of M2 medium (Sigma-Aldrich, USA) on a Petri dish, and the oviduct ampullae were dissected using a dissecting needle under a stereomicroscope (Stemi DV4, Carl Zeiss, Germany). For denudation, oocytes released in the oocyte-cumulus complex were transferred using an EZ-Grip microcapillary pipette (RI, UK) into a 100 µl drop of medium containing 150 IU/ml hyaluronidase Type II (Sigma, USA) and incubated for 20 min at room temperature. Oocytes were washed from the enzyme and cumulus cells by sequentially transferring them through 6–8 drops of fresh M2 medium. To remove the zona pellucida, oocytes were incubated in 0.025% collagenase Type I solution (Sigma-Aldrich, USA) for 20 min, followed by sequential washes in 6–8 drops of M2 medium to remove the enzyme.

For final purification from cumulus cells, the oocytes were transferred to the lower part of a drop of Percoll (Sigma-Aldrich, USA) at varying concentrations (10–70%) in a Petri dish and gently mixed with a circular motion of the microcapillary tip (Fig. 1). Within approximately 10 min, oocytes floated to the surface of the drop, after which they were collected and transferred to a fresh drop of Percoll, and the procedure was repeated. The oocytes were then washed in a drop of phosphate-buffered saline (pH 7.4), transferred to microtubes in a minimal volume of the buffer, frozen and stored at –20 °C until real-time PCR. To assess contamination with somatic cells, a part of the oocytes was fixed, stained, and examined under a microscope according to a previously described protocol [14].

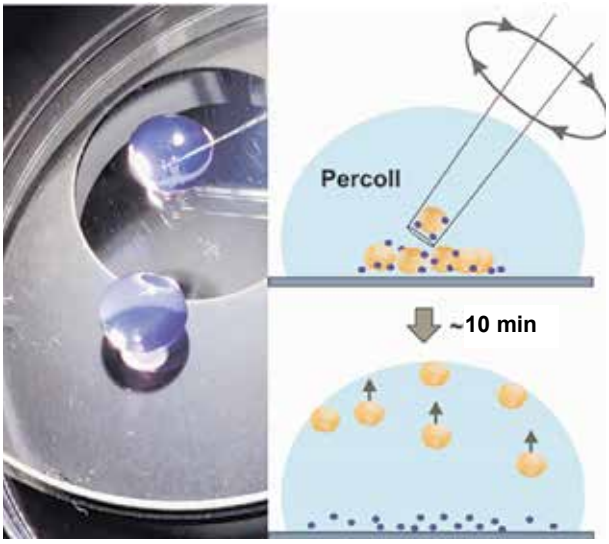

Fig. 1. Separation of oocytes and cumulus cells in a Percoll drop. A 100 µl drop of Percoll solution is placed on a polystyrene Petri dish, and the oocytes are transferred to the lower part of the drop. Using the tip of a microcapillary positioned at an angle of approximately 60°, the oocytes are gently distributed throughout the lower part of the drop by circular motions. After approximately 10 min, the oocytes that have floated to the surface of the drop are collected.\

After the release of oocyte-cumulus complexes, total DNA was extracted from ovaries with oviducts and brain tissue using the D-Tissues kit (Biolabmix,

Russia). The concentration of isolated DNA in the samples was measured using the dsDNA BR kit on a Qubit 4 minifluorometer (Thermo Fisher Scientific, USA). DNA was frozen and stored at –20°C until real-time PCR was performed.

Real-time PCR Procedure

To detect DNA sequences, direct PCR on lysed oocytes was performed [15]. Microtubes containing the oocyte suspension were thawed, an equal volume of lysis solution (500 μg/ml polyadenylic acid, 20 mM EDTA-Na_2, 500 mM dithiothreitol, and 1% N-lauryl sarcosine) was added, and the mixture was incubated at 37°C for 30 min. The lysate was then thoroughly resuspended and used for PCR. To assess the efficiency of direct PCR on lysed oocytes, PCR was performed for the sequences of both nuclear DNA gene (*Ptger2*) and mitochondrial DNA gene (*Cox-1*) of the mouse. The primer sequences are listed in Table 1.

Real-time PCR was performed on a QuantStudio 5 Real-Time PCR System (Applied Biosystems, USA). The reaction mixture included 1X qPCRmix-HS LowROX mixture (Eurogen, Russia), 2 μM SYBR Green I, 500 nM each of forward and reverse primers (DNA Synthesis, Russia), 5 μl of lysed oocyte suspension or 4 ng of DNA isolated from ovaries (PCR control).

Table 1

Primers Used in the Study for Real-time PCR

Gene Name	Primer Sequence		Amplicon size (bp)
Ptger2	Forward	5′-CCTGCTGCTTATCGTGGCTG-3′	186
	Reverse	5′-GCCAGGAGAATGAGGTGGTC-3′	
Cox-1	Forward	5′-ATTACAGCCGTACTGCTCCTAT-3′	150
	Reverse	5′-CCCAAAGAATCAGAACAGATGC-3′	
Luc	Forward	5′-GAGGCGAACTGTGTGTGAGA-3′	149
	Reverse	5′-GTGTTCGTCTTCGTCCCAGT-3′	

Thermal cycling conditions were as follows: 98°C for 1 min, then 35 cycles of 98 °C for 10 s, 6 1°C for 30 s, and 72 °C for 15 s. Melting curve was generated by increasing the temperature from 65 °C to 95 °C within 1 min (HRM mode: 20 data points per 1 °C). PCR efficiency (*E, %*) was calculated using the formula:

$$E = (10\text{^}(-1/a) - 1) \times 100$$

where a is the slope of the regression line describing the dependence of the threshold cycle Cq on the logarithm of the number of oocytes in the reaction (5, 10, 20, 40, 80, and 160 oocytes).

To detect vector DNA in the ovaries and oocytes, primers specific to the sequence of the luciferase gene cloned into the vector were used (Table 1). The reaction mixture included 1X BioMaster UDG HS-qPCR Lo-ROX SYBR (2X) mixture (Biolabmix, Russia), 600 nM each of forward and reverse primers (DNA Synthesis, Russia), 4 ng of DNA isolated from the ovaries or 5 μl of lysed oocyte suspension. Thermocycling conditions were as follows: 50°C for 2 min (anti-contamination treatment), 95°C for 5 min followed by 40 cycles of 95 °C for 15 s, 62 °C for 15 s, and 72 °C for 15 s. Melting curve was generated by increasing the temperature from 65 °C to 95 °C within 1 min (HRM mode: 20 data points per 1°C). The number of vector genome copies per microgram of total DNA (ovaries) or per haploid genome (oocytes) was determined based on a calibration curve constructed using control solutions with known vector concentrations. PCR efficiency was calculated using the formula provided above. The matrix effect and recovery were determined in accordance with the recommendations [16]. Amplification of the target DNA sequence was confirmed by the melting curve of the product.

RESULTS

In laboratory mice, the number of oocytes ovulating during one estrous cycle ranges from 8 to 12, depending on the strain and age [17]. To obtain a larger number of oocytes, hormonal superovulation is induced by sequential administration of eCG which stimulates follicle growth and hCG which resumes meiotic divisions of oocytes and causes ovulation [15]. In female CD-1 mice, the number of ovulated oocytes under conditions of superovulation was 20.1 ± 6.4 per mouse.

If the standard isolation procedure is used, after denudation the oocyte suspensions with an intact zona pellucida contain a large number of cumulus cells (Fig. 2, *a*). Removal of the zona pellucida followed by repeated washing significantly reduces contamination, but it does not allow to completely remove cumulus cells from the suspension (Fig. 2, *b*), since oocyte selection and transfer with

a microcapillary are performed in a small amount of medium/buffer. In a study by H. Okada et al. [18], a method of centrifugation in 22.5% Percoll was proposed for the purification of *in vitro* fertilized oocytes and two-cell mouse embryos from cumulus cells and spermatozoa. However, this approach was found to be unsuitable for unfertilized oocytes with removed zona pellucida. Centrifugation in Percoll resulted in the loss of over 90% of oocytes due to their mechanical destruction (own data).

In this study, we tested an approach based on the separation of oocytes and cumulus cells using differences in buoyancy in a Percoll drop (Fig. 1). In 10, 20, 30, and 40% Percoll, oocytes remained in the lower part of the drop, whereas in 50, 60, and 70% Percoll, they floated to the surface within approximately 10 min and remained on the surface of the drop. Cumulus cells remained in the lower part of the drop in all Percoll solutions. Collecting oocytes from the surface of the drop using a microcapillary proved to be most convenient in 70% Percoll, due to the decrease in surface tension of the Percoll solution with increasing density. To assess contamination, we conducted a series of four experiments with oocyte purification in 70% Percoll (a total of 119 oocytes). In none of the cases were cumulus cells detected on oocyte micropreparations after their purification (Fig. 2, *b*).

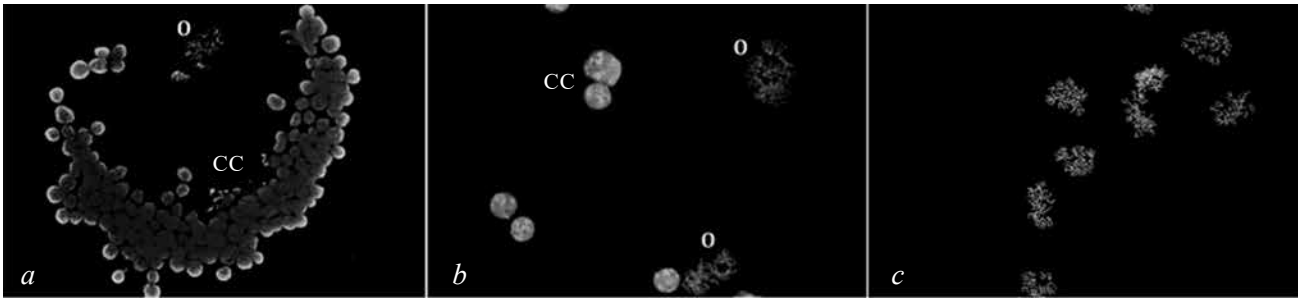

Fig. 2. Cytogenetic preparations of mouse oocytes. *a* – with intact zona pellucida, *b* – after removing the zona pellucida, *c* – after washing in 70% Percoll. O – oocyte (metaphase MII), CC – cumulus cells. Stained with Hoechst 33258; magnification ×400

Traditional DNA extraction methods (for PCR analysis) are not applicable to oocytes due to the limited availability of the material. To detect DNA (or RNA) sequences in oocytes, it is advisable to perform PCR directly on lysed cells. This method makes it possible to analyze the entire pool of isolated cells without loss, increasing the accuracy and sensitivity of detection. We used the "spanning" protocol developed by S.Tsuchita et al. [15], which involves cell lysis and DNA deproteinization using the detergent N-lauryl sarcosine at a concentration that does not inhibit PCR. The authors demonstrated high efficiency and a low false-positive rate when using this protocol to amplify DNA regions in single blastomeres and lymphocytes.

The amplification efficiency using oocyte lysate as a template for the mitochondrial DNA gene (*Cox-1*) and for the nuclear DNA gene (*Ptger2*) comprised 98.2% and 90.3%, respectively. The higher efficiency and earlier threshold cycles of *Cox-1* amplification are due to the large number of mitochondrial DNA copies (200,000–400,000) in mature oocytes [19]. PCR for the *Ptger2* gene showed linearity (R^2 = 0.975) with the number of oocytes in the reaction ranging from 5 to 160 (Fig. 3). With the

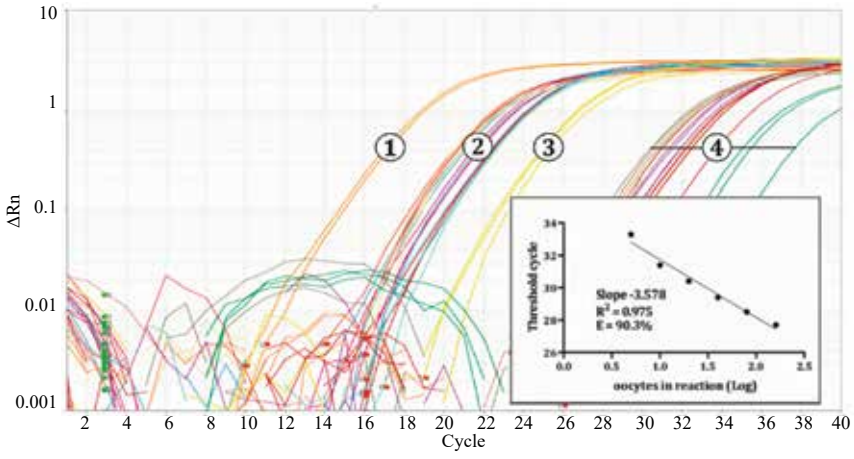

Fig. 3. Amplification curves for the *Ptger2* gene with ovarian DNA as a template (1), for the *Cox-1* gene with oocyte lysate (2), for the *Cox-1* gene with ovarian DNA (3), and for the *Ptger2* gene with oocyte lysate (4). The X-axis shows the amplification cycle, and the Y-axis shows the normalized change in the fluorescent signal at each amplification cycle (ΔRn). The inset shows the curve of dependence of the threshold cycle on the logarithm of the number of oocytes in the reaction for the *Ptger2* gene (slope of the regression line (slope), correlation coefficient (R^2), and reaction efficiency (E)).

number of oocytes less than 5, the linearity of the reaction significantly decreased, although the signal identified by the melting curve of the product was still detectable even with 3 oocytes in the reaction, which corresponds to the theoretically achievable sensitivity of the quantitative PCR method [16, 20].

The amount of vector DNA in mouse brain and ovarian tissues was similar at various time points after vector administration (Fig. 4). On days 1 and 3, vector genome copies were detected in all exposed animals, while on day 7 – in 5 out of 6 animals. Vector persistence was observed in both tissues for up to 3 months after administration. Vector DNA was not detected in the oocytes of any of the animals.

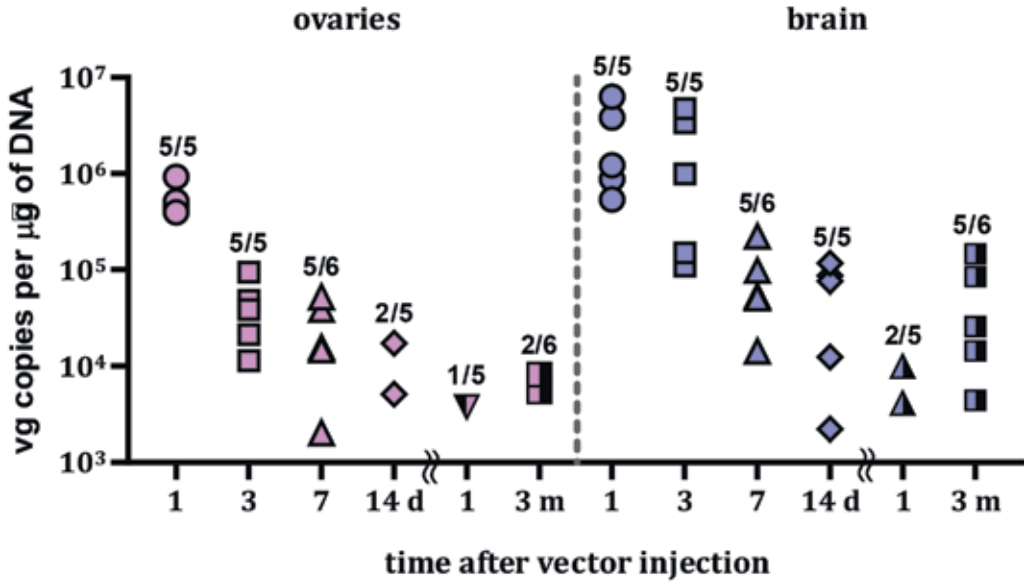

Fig. 4. The content of vector genomes (vg) in the ovaries and brain of mice. The numbers indicate the number of mice in which vector DNA was detected, relative to the total number of animals in the group

DISCUSSION

Immature oocytes in primordial ovarian follicles are protected from potentially dangerous agents by regulated transport through the basal membrane and the layer of granulosa cells, as well as by the absence of direct vascularization [21]. As oocytes mature, which is accompanied by structural and transport changes in the follicles, the blood–follicle barrier is progressively formed. The barrier includes the zona pellucida and cumulus cells surrounding the oocyte, as well as granulosa and theca cells of the antrum [21]. Theoretically, the "molecular sieve" of the blood–follicle barrier is impermeable to viral vectors due to their large size (total virion mass exceeds 5 MDa) [22]. However, AAVs have been shown to be capable of passing through the zona pellucida and transducing pre-implantation embryos [23, 24]. When injected into the ovarian stroma, AAVs are capable of penetrating the blood–follicle barrier via transcytosis and transducing the cumulus cells surrounding the oocyte [25]. In antral follicles, intercellular exchange between oocytes and somatic cells, in addition to transzonal projections, is carried out by circulating in the follicular fluid extracellular vesicles (EVs) that can transfer large molecules and viruses into the oocyte [21, 26]. For instance, the capacity for germline transmission into human oocytes and embryos has been demonstrated for the hepatitis B virus [27]. Taken together, this indicates a potential risk of germline transmission of viral and non-viral vectors into female germ cells and emphasizes the need to develop methodological approaches to risk assessment. For this purpose, we developed and validated an original methodology that includes hormonal superovulation of vector-exposed animals, oocyte isolation, their purification in a Percoll drop, and direct quantitative PCR on lysed oocytes.

Hormonal superovulation is a crucial step in the study. The estrous cycle in mice lasts for 4–5 days and culminates in the ovulation of 8–12 oocytes [15]. Hormonal stimulation synchronizes ovulation, allowing to collect an increased number of oocytes in a narrow time frame. For maximum effectiveness, it is recommended to design the experiment so that at the time of superovulation induction the animals are in the

diestrus phase, when the ovaries are most responsive to hormonal stimulation [28]. In addition to the increased number of oocytes, superovulation also results in more than a twofold increase in the size of the ovaries and oviducts, significantly simplifying their visual identification and retrieval during necropsy.

Mature oocytes contain a significant amount of lipids in the form of triglycerides in lipid droplets and membrane phospholipids which together constitute 10 to 30% of the oocyte's dry weight [29]. This property, along with the low cytoplasmic density and large size of oocytes, ensures their buoyancy in high-concentration Percoll solutions, in which somatic cells and free vector particles remain immobile. Purification in a Percoll drop completely removes cumulus cells that contaminate oocytes. As mentioned above, vectors are capable of transducing cumulus cells, which may lead to false-positive results. Performing direct quantitative PCR without a DNA extraction step allows to avoid material loss, which increases the accuracy and sensitivity of the analysis and reduces the likelihood of false-negative results [15, 16].

Using the developed methodology, we investigated an AAV of the chimeric serotype PHP.eB capable of effectively crossing the blood – brain barrier and transducing brain and spinal cord cells in mice. The choice of a synthetic AAV capsid was based on the fact that AAV engineering is currently actively developing and new variants of artificially created chimeric AAV serotypes with cell-type- and tissue-specific tropism appear every year [30]. In 2024, the drug BEQVEZ™ (fidanacogene elaparvovec-dzkt) based on the chimeric serotype rh74var was approved for the treatment of hemophilia B.

Thus, the study of the tropism and biodistribution of chimeric AAV serotypes is of particular scientific interest. Considerable data have accumulated on the efficacy of chimeric capsids of the PHP family in penetrating the blood – brain barrier in various animal species and even mouse strains [31]. However, there are no data on the ability of such chimeric AAVs to transduce oocytes or perform germline transmission. In the present study, the vector under investigation persisted for up to 3 months both in the targeted brain tissue and in the ovaries; however, vector DNA was not detected in oocytes at any time point after the exposure. These findings indicate a low risk of germline transmission of AAVs of the chimeric serotype PHP.eB into female germ cells.

AAVs have high tropism for ovarian tissue [32]. J. Zhao et al. studied the biodistribution of AAV of serotype 9 in mice using the high-precision RNAscope® *in situ* hybridization method [33]. Following intravenous administration, vector DNA was detected in the medulla and corpus luteum of the ovaries, as well as in the theca cells of the follicles, but not in oocytes. AAV of serotype 2 administered intravenously or into the spleen was detected in the ovaries and testes of mice but was not transmitted to their offspring [34]. AAV of serotype 8 persisted in the ovaries of mice for 150 days after intravenous administration [35]. Using *in situ* hybridization, vector DNA was identified in oocytes. However, a study of the offspring of vector-exposed females did not confirm the risk of germline transmission, raising questions about the accuracy and reliability of *in situ* hybridization for its assessment.

To date, there are only a few studies on the risk assessment of germline transmission of viral and non-viral vectors into female germ cells, which is obviously due to the methodological challenges in conducting such studies. For these purposes, the methodology we developed and described in this article can be used.

CONCLUSION

An original methodology for preclinical assessment of the risk of germline transmission of genetic constructs into the germ cells of female mice was developed and validated using an adeno-associated viral (AAV) vector of the chimeric serotype PHP.eB. The methodology is based on direct quantitative PCR; it is characterized by high sensitivity, accuracy, and relatively low labor intensity. It does not require specialized equipment or materials and can be integrated into biodistribution studies. With appropriate adaptation, it can be used in experiments on other mammalian species.

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Author Contribution

Malikova A.D. – conception and design, administration of the vector into the animals, selection of experimental conditions, data analysis and interpretation, and drafting of the initial manuscript. Zhanataev A.K.and Esmagambetov I.B. – conception, analysis of results and literature sources, drafting of the initial manuscript, and scientific editing. Anisina E.A. – work with laboratory animals, conducting PCR assays, processing of the obtained data, and revision of the manuscript.Pligina K.L. – work with laboratory animals, oocyte isolation, processing of the obtained data, and revision of the manuscript. Chaika Z.V. – DNA isolation, processing of the obtained data, and revision of the manuscript. Ryabova E.I., Dovgiy M.A., and Hossain R.M. – production and purification of vector particles and revision of the manuscript. Durnev A.D. – conception, analysis of results, revision of the manuscript, critical revision for important intellectual content. Dorofeev V.L. – critical revision for important intellectual content and final approval of the manuscript for publication.

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Th1/Th17 Cytokines of the Immune Response in Patients with Bronchial Asthma after COVID-19

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ABSTRACT

Aim. To study the content of interleukin 1β (IL-1β) in exhaled breath condensate (EBC) and interleukin 6 (IL-6) and 17A (IL-17A) in the blood serum of patients with bronchial asthma who experienced COVID-19 of varying severity.

Materials and methods. We examined 124 adult asthma patients of both sexes 6–12 months after COVID-19. The design included a general examination to determine the objective status of patients, asthma severity and control, assessment of the lung function, and measurement of IL-1β in EBC and IL-6, IL-17A in the serum of peripheral blood.

Results. The patients were divided into 2 groups. Group 1 consisted of 90 patients with mild persistent asthma. Group 2 included 34 patients with moderate asthma. The content of IL-6 and IL-17A in the blood serum of patients in group 1 was significantly lower than in group 2 ($p = 0.047$ and $p = 0.049$, respectively). The concentration of IL-1β in the EBC of patients in group 1 was significantly higher than in group 2 ($p = 0.019$). COVID-19-associated pneumonia was experienced by 40% of patients in group 1 and by 79% of patients in group 2. Post-COVID pulmonary fibrosis was registered in 19 and 62% of cases, respectively. In group 1, a relationship was revealed between the content of IL-17A and IL-6 in the blood ($Rs = 0.69$; $p < 0.001$). In group 2, a correlation was found between the content of IL-17A and IL-6 in the blood ($Rs = 0.32$; $p = 0.025$), as well as between the forced expiratory flow at 75% of forced vital capacity (FEF_{75}), reflecting the patency of small bronchi, and the levels of IL-6 ($Rs = –0.32$; $p = 0.023$) and IL-1β ($Rs = 0.49$; $p = 0.021$).

Conclusion. In patients who experienced COVID-19, a rise in the content of Th1/Th17 cytokines was observed as the severity of asthma increased. High concentrations of IL-17A and Th17-associated IL-1β and IL-6, which activate neutrophilic inflammation, may increase the risk of systemic inflammation and the development of pulmonary fibrosis.

Keywords: bronchial asthma, COVID-19, cytokines IL-1β, IL-6, and IL-17A, Th1/Th17-induced inflammation.

Conflict of interest. The authors declare the absence of obvious or potential conflict of interest related to the publication of this article.

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Цитокины Th1/Th17 иммунного ответа у больных бронхиальной астмой после перенесенной коронавирусной болезни 2019

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РЕЗЮМЕ

Цель. Исследовать содержание интерлейкина *(IL) 1β в конденсате выдыхаемого воздуха (КВВ) и IL-6, IL-17A в сыворотке крови* у пациентов с бронхиальной астмой (БА), переболевших коронавирусной болезнью 2019 (coronavirus disease 2019, COVID-19) разной степени тяжести.

Материалы и методы. Взрослые пациенты с БА (n = 124) обоего пола обследованы спустя 9–12 мес после перенесенной COVID-19. Дизайн предусматривал общий осмотр с определением объективного статуса больных, степени тяжести БА, уровня контроля над болезнью, оценку вентиляционной функции легких, измерение содержания IL-1β в КВВ и IL-6, IL-17A в сыворотке периферической крови.

Результаты. Больные распределены на две группы: 1-ю группу составили 90 пациентов с легкой персистирующей БА, 2-ю группу – 34 пациента со среднетяжелой БА. Содержание IL-6 и IL-17A в сыворотке крови пациентов 1-й группы было достоверно ниже, чем во 2-й (p = 0,047 и p = 0,049 соответственно). Концентрация IL-1β в КВВ у пациентов 1-й группы была существенно выше, чем во 2-й группе (p = 0,019). В 1-й группе 40% больных и 79% во 2-й перенесли COVID-19-ассоциированную пневмонию. Постковидный пневмофиброз зарегистрирован в 19 и 62% случаев соответственно. В 1-й группе прослеживалась взаимосвязь между содержанием IL-17A и IL-6 в крови (Rs = 0,69; p < 0,001), во 2-й группе – между содержанием IL-17A и IL-6 в крови (Rs = 0,32; p = 0,025), а также между максимальной объемной скоростью на уровне 75% форсированной жизненной емкости легких ($МОС_{75}$), отражающей проходимость мелких бронхов, и уровнем IL-6 (Rs = –0,32; p = 0,023) и IL-1β (Rs = 0,49; p = 0,021).

Заключение. У пациентов, перенесших COVID-19, по мере нарастания степени тяжести БА наблюдалось увеличение содержания цитокинов Th1/Th17. Высокие концентрации IL-17A и Th17-связанных IL-1β и IL-6, активирующих нейтрофильное воспаление, могут повышать риск системного воспаления и развития пневмофиброза.

Ключевые слова: бронхиальная астма, COVID-19, цитокины *IL-1β, IL-6 и IL-17A,* Th1/Th17-индуцированное воспаление

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Авторы заявляют об отсутствии финансирования при проведении исследования.

Соответствие принципам этики. Все пациенты подписали информированное согласие на участие в исследовании. Исследование одобрено локальным этическим комитетом ДНЦ ФПД (протокол № 137 от 24.05.2022).

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INTRODUCTION

Studies investigating the cooccurrence of bronchial asthma (BA) and COVID-19 have shown that older age, a large number of comorbidities, as well as eosinopenia and lymphopenia significantly increase susceptibility to SARS-CoV-2 infection [1]. It is hypothesized that diabetes mellitus and hypertension may upregulate the expression of angiotensin-converting enzyme 2 (ACE2), whereas the use of inhaled corticosteroids contributes to its downregulation, thereby hindering SARS-CoV-2 entry into the epithelium [2]. Significant factors for mortality among patients with COVID-19 and BA include a history of asthma exacerbation

within a year prior to COVID-19 and an increase in asthma severity [3].

Of particular note is the increased susceptibility to SARS-CoV-2 in patients with non-allergic BA. This phenotype is frequently associated with severe COVID-19, necessitating intensive care, mechanical ventilation, and / or leading to a fatal outcome [4, 5]. This observation can be explained by higher ACE2 expression levels in these patients compared to those with the allergic asthma phenotype [6, 7].

The critical course of COVID-19, driven by the cytopathic effect of SARS-CoV-2 on target cells expressing ACE2 receptors and coreceptors – transmembrane serine protease TMPRSS2 and cathepsin L – leads to the release of damage-associated molecular patterns (DAMPs) and is accompanied by the induction of cellular pyroptosis. The generation of numerous inflammatory mediators, neutrophil activation with the formation of neutrophil extracellular traps (NETs) that contribute to lung epithelial cell death, the development of macrophage activation syndrome (MAS), hyperinflammation, and cytokine storm are all associated with the overproduction of IL-1β, IL-6, and IL-17 among other proinflammatory cytokines [8–10]. In patients with non-allergic BA, IL-1β, IL-6, and IL-17 act as central regulators of Th2/Th17 or Th1/Th17 inflammatory patterns with predominant bronchial neutrophilic infiltration [11]. Severe uncontrolled non-atopic asthma is dominated by a Th1/Th17 immune response and increased production of proinflammatory cytokines in the airways, which modify the respiratory tract structure, potentiate remodeling and bronchial obstruction, and cause a decrease in forced expiratory volume in 1 second (FEV_1) [12, 13].

Since IL-1β, IL-6, and IL-17 are key players in systemic inflammation and complications of COVID-19, as well as in the Th1/Th17 immune response in BA, profiling these cytokines in patients with both diseases holds significant prognostic value.

The aim of the study was to investigate the levels of IL-1β in exhaled breath condensate and the levels of IL-6 and IL-17A in serum in patients with BA of varying severity who recovered from COVID-19.

MATERIALS AND METHODS

A total of 124 adult patients with BA were enrolled in a single-center, observational, cross-sectional, cohort study 9–12 months after a confirmed COVID-19 infection. The diagnosis of BA was based on the ICD-10 codes and the GINA criteria [14].

A prior COVID-19 infection was confirmed using medical records, which documented the verification of SARS-CoV-2 RNA in oropharyngeal and / or nasopharyngeal swab specimens by nucleic acid amplification tests or the detection of SARS-CoV-2 antigen by the immunochromatographic assay. The COVID-19 diagnosis was established according to the version of the temporary methodological guidelines of the Russian Ministry of Health "Prevention, Diagnosis, and Treatment of Novel Coronavirus Infection (COVID-19)" that was in effect at the time of the patient's examination.

Clinical material was collected in 2022–2023 during patient visits at the Far Eastern Scientific Center of Physiology and Pathology of Respiration. The study was approved by the local Ethics Committee at this Scientific Center (Minutes No.137 dated May 24, 2022).

Inclusion criteria were the following: adult individuals of both sexes; diagnosis of BA corresponding to the ICD-10 codes J45.1, J45.8, J45.9, persistent mild and moderate BA; a history of COVID-19 of varying severity, confirmed by laboratory methods; presentation for the study 9–12 months after the completion of COVID-19 therapy; ability to technically correctly perform maneuvers during instrumental testing; a written informed consent to the examination.

Exclusion criteria: BA corresponding to the ICD-10 code J45.0, severe BA; presence of comorbid pathology and drug therapy that could lead to distortion of the results of the collected biological material analysis; lack of interest or failure to provide a written informed consent.

Study design: patient selection at the stage of presentation to the Far Eastern Scientific Center of Physiology and Pathology of Respiration; general examination with assessment of the objective status, asthma severity, and level of disease control; evaluation of lung function; collection of biological fluids – peripheral blood and exhaled breath condensate (EBC).

Following the completion of sample collection, the patients were divided into groups based on disease severity: group 1 included 90 individuals with mild persistent BA, and group 2 comprised 34 patients with moderate disease severity. The main clinical characteristics of the examined patients are presented in Table 1.

BA symptoms were objectively assessed using the validated questionnaires Asthma Control Test (ACT) and Asthma Control Questionnaire (ACQ-5).

Table 1

Main Clinical Parameters of Patients with Bronchial Asthma

Parameter	Group 1	Group 2	*p*
Age, years, *Me* [Q_1; Q_3]	42[31;53]	50[49;65]	<0.001
BMI, kg/m², *Me* [Q_1; Q_3]	26.6[23.2;30.5]	29.3[26.1;32.7]	0.007
Sex (male/female), %	49/51	44/56	>0.05
Proportion of smokers, %	22	38	<0.05; $\chi^2 = 4.6$
Smoking, pack – years, *Me* [Q_1; Q_3]	12[5;20]	17[3;30]	>0.05
ACT score, *Me* [Q_1; Q_3]	18[15;21]	12[10;13]	0.002
ACQ-5 score, *Me* [Q_1; Q_3]	2.0[1.0;3.0]	2.8[2.4;3.2]	0.057
SaO_2, %, *Me* [Q_1; Q_3]	97[96;98]	96[94;97]	<0.001
IgE, IU/ml, *Me* [Q_1; Q_3]	32[13;74]	160[48;266]	<0.001

Note. BMI – body mass index; ACT – Asthma Control Test; ACQ-5 – Asthma Control Questionnaire-5; SaO_2 – oxygen saturation; IgE – immunoglobulin E, *p* – the significance level for differences between group 1 and group 2 (here and further).

Lung function was evaluated by spirometry using the Easy on-PC electronic spirometer (ndd Medizintechnik AG, Switzerland) equipped with an ultrasonic flow sensor based on ndd True Flow™ technology. The measured parameters included forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), maximum forced expiratory flow at 50% and 75% of FVC (FEF_{50} and FEF_{75}, respectively), and mid-expiratory flow between 25% and 75% of FVC (MEF_{25-75}). The measurement and analysis of the recorded parameters followed the methodological guidelines for conducting studies and interpreting results and quality standards recommended by the Russian Respiratory Society, which are in line with the standards of the American Thoracic Society (ATS) and the European Respiratory Society (ERS) [15, 16]. The patient's actual values were expressed as a percentage of predicted values based on the European Community for Steel and Coal (ECSC) reference values for individuals over 18 years. Reversibility of obstructive abnormalities was assessed via a bronchodilation test using a short-acting β_2-agonist (salbutamol 400 mcg) [15].

Additionally, patients in group 1 with FEV_1 greater than 75% underwent a bronchoprovocation test with 3-minute isocapnic cold air hyperventilation (–20 °C) to verify cold air hyperresponsiveness [17].

EBC samples were collected using the ECoScreen II device (VIASUS Healthcare GmbH, Germany). Collection was performed once before noon or sequentially before and after the cold air hyperventilation challenge test. Prior to the procedure, the patients rinsed their oral cavity twice with distilled water. Subsequently, while breathing calmly through a mouthpiece for 20 minutes, they ventilated air through the device, with nasal breathing occluded by a nose clip. Upon completion, the container with the biological material was removed from the device. Following thawing, the liquid condensate was aliquoted in 1000-μl volumes into sterile 1.5 ml Eppendorf-type plastic tubes using a Light DPOP-1-100-1000 single-channel pipette dispenser (Thermo Scientific). The tubes were sealed with airtight caps and immediately placed in a freezer at approximately –80 °C, where they were stored for no more than two weeks until biochemical analysis. The concentration of IL-1β (in pg/ml) in the EBC was determined using commercial LEGENDplex™ Human T Helper Cytokine Panel Version 2 kits on a FACS Canto II flow cytometer (Becton Dickinson, USA) with the FACS Diva 6.0 software (Becton Dickinson, USA).

Peripheral blood was collected once from the median cubital vein in the morning (before 9:00 AM). A 2 ml-volume of venous blood was drawn into vacuum tubes containing a coagulation activator, incubated for 30 minutes at room temperature, and subsequently centrifuged at 3,000g for 10 minutes at 4 °C. The obtained serum was stored at –20 °C until analysis. Cytokine concentrations of IL-6 and IL-17A (pg/ml) were measured using commercial LEGENDplex™ Human T Helper Cytokine Panel Version 2 kits on a FACS Canto II flow cytometer (Becton Dickinson, USA) with the FACS Diva 6.0 software (Becton Dickinson, USA).

Statistical analysis was performed using the Automated Medical Examination System software (Russia) [18]. The normality of distribution was assessed using the Kolmogorov – Smirnov test, the Pearson – von Mises test, and measures of skewness and kurtosis. For comparing two independent samples, the Student's *t*-test was applied when data followed a normal distribution and group variances were homogeneous according to the Fisher's test; otherwise, the Mann – Whitney *U*-test or Kolmogorov – Smirnov test was used. For comparing two dependent samples, the Wilcoxon signed-rank test was employed. Quantitative parameters were presented as either $M \pm SD$ (where *M* is the arithmetic mean and *SD* is

the standard deviation) or as $Me[Q_1;Q_3]$ (where *Me* is the median and Q_1–Q_3 is the interquartile range). Frequencies of categorical variables were analyzed using the χ^2 (Pearson›s chi-squared) test. Correlation between two random variables was determined using the Spearman's nonparametric correlation analysis (*Rs*). The differences were considered to be statistically significant at $p < 0.05$.

RESULTS

Analysis of the blood cytokine profile involved in the Th1/Th17 immune response in BA patients revealed significantly higher levels of IL-6 and IL-17A in group 2 compared to group 1 (Table 2). In the meantime, group 2 showed significantly lower levels of IL-1β in EBC than group 1 (Figure). Notably, in patients who underwent the cold air challenge test, IL-1β levels increased after the test, suggesting the active role of the cytokines in mediating the acute response to cold air bronchoprovocation (Table 2).

This finding is consistent with our previous research, which demonstrated an association between IL-1β and the non-atopic asthma phenotype, cold air hyperresponsiveness, and the probable development of a Th1/Th17 immune response, regulated by this cytokine [19].

Table 2

Levels of IL-6 and IL-17A in Peripheral Blood of Asthma Patients, pg/ml, *Me* [Q_1; Q_3]

Parameter	Group 1	Group 2	*p*
IL-6	6.70 [5.10;11.92]	10.20 [5.40;17.60]	0.047
IL-17A	0.14 [0.04;0.36]	0.28 [0.18;0.46]	0.049

The assessment of clinical and functional data revealed that patients in group 2 had poorer disease control compared to those in group 1 (Table 1). Both groups demonstrated low median ACT scores. In group 1, asthma was newly diagnosed in 60% of patients and required therapeutic intervention. Poor disease control was observed in 16% of cases, while only 24% of individuals exhibited partially controlled disease. Bronchospasm in response to cold air inhalation during the isocapnic hyperventilation challenge was detected in 36% of group 1 patients. In group 2, asthma exacerbation was present in 50% of cases, with uncontrolled disease, in 30% of cases and with partially controlled disease, in only 20%. Notably, COVID-19-associated pneumonia was reported in 40% of group 1 and 79% of group 2 patients. According to multispiral computed tomography results, bilateral post-COVID pulmonary fibrosis with polysegmental distribution

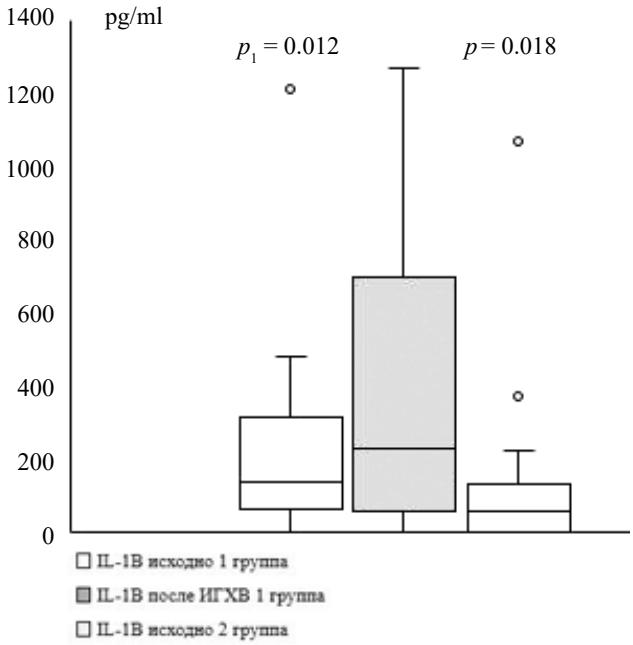

Fig. Level of IL-1β in exhaled breath condensate, pg/ml: *p* – significance level for the differences (Mann – Whitney *U*-test) between group 1 and group 2; p_1 – significance level for the differences (Wilcoxon signed-rank test) between group 1 patients before and after the isocapnic cold air hyperventilation challenge.

was documented in 62% of group 2 patients. Foci of pulmonary fibrosis were also observed in 19% of group 1 patients.

Comparison of key flow – volume curve parameters (FEV_1, FEV_1/FVC) and distal airway patency (FEF_{50}, FEF_{75}, MEF_{25-75}) indicated significantly worse lung function in group 2 patients compared to group 1 (Table 3).

Table 3

Flow – volume Curve Parameters of Forced Expiration and Changes in FEV_1 (ΔFEV_1bd) after Short-acting β_2-agonist Inhalation, *M* ± *SD*

Parameter	Group 1	Group 2	*p*
FEV_1, % predicted	94.3 ± 11.4	71.1 ± 22.2	<0.001
FEV_1/FVC, %	74.7 ± 7.6	64.9 ± 9.9	<0.001
FEF_{50}, % predicted	62.0 ± 33.2	42.8 ± 20.4	<0.001
FEF_{75}, % predicted	51.0 ± 30.3	35.7 ± 17.5	<0.001
MEF_{25-75}, % predicted	58.0 ± 27.5	41.2 ± 18.7	0.007
ΔFEV_1bd, %	7[3;12]	17[3;23]	0.004

Despite mean group values for FEV_1 and FEV_1/FVC in mild BA patients falling within the normal range, individual analysis revealed that 18% of patients had FEV_1 below 80% of the predicted value and the FEV_1/FVC ratio below 0.7. Furthermore,

isolated small airway obstruction was observed in 17% of patients. High bronchial lability (ΔFEV_1bd), exceeding 12% in the salbutamol test, was identified in 27% of group 1 and 57% of Group 2 patients. Two patients exhibited a paradoxical response to the short-acting bronchodilator, with FEV_1 decreasing by 11 and 30%, respectively.

The correlation analysis revealed significant associations between cytokine levels in EBC and serum and impaired lung function. In group 1, significant correlations were observed between serum IL-17A levels and bronchial response (ΔFEV_1) to the cold air hyperventilation challenge ($Rs = –0.40$, $p = 0.047$), serum IL-17A and IL-6 levels ($Rs = 0.69$, $p < 0.001$). In group 2, in addition to the positive correlation between serum IL-17A and IL-6 levels ($Rs = 0.32$, $p = 0.025$) that was also present in group 1, significant correlations were found between small airway patency (FEF_{75}) and IL-6 ($Rs = –0.32$, $p = 0.023$) and IL-1β levels ($Rs = 0.49$, $p = 0.021$).

DISCUSSION

Our findings suggest that IL-1β-induced synthesis of type 17 cytokines (IL-17A and IL-17F), whose primary function is neutrophil recruitment and activation, promotes the mobilization of the neutrophilic component in non-Th2-mediated asthma inflammation [20, 21]. As several authors contend, IL-1β plays a leading role in polarizing CD4+ T cells into the CD4+ T-helper 17 (Th17) subset, with IL-6 serving to amplify this process [22]. Furthermore, IL-1β-dependent IL-17 production is associated with the stimulation of innate immune cells belonging to specific minor subpopulations –namely, γδ T cells and group 3 innate lymphoid cells (ILC3s). These cells emerge during the immune response to pathogen invasion and possess the ability to produce IL-17 to maintain immune homeostasis, particularly in mucosal tissues [23].

According to another perspective, IL-1β further amplifies the function of IL-6, which is crucial for Th17 differentiation [24]. By inducing the expression of the key Th17 transcription factor RORγt and the related RORα in naive CD4+ T (T_0) cells, the content of which is associated with STAT3 activity, IL-6, acting via tyrosine residues of the signal transducer (subunit of the IL-6 receptor) gp130, activates STAT3 [25]. Activation of the IL-6 – gp130/STAT3 signaling pathway is considered as an IL-6/STAT3-dependent mechanism of pulmonary neutrophilic inflammation, making its components promising therapeutic targets in BA [26].

Neutrophilic inflammation is most frequently associated with elevated levels of IL-17A, the primary effector chemoattractant for neutrophils produced by the Th17 lineage. IL-17A is expressed by Th17 cells, and ILC3s are considered as a risk factor for the development of severe asthma [21, 27]. Key proinflammatory IL-17A-related cytokines and chemokines associated with activation of the transcription factor NF-κB — which is critical for the development of chronic airway inflammation — include IL-6, IL-1β, IL-8, and GM-CSF [23]. GM-CSF enhances neutrophil survival, adhesion, migration, and phagocytosis, promotes NET formation, stimulates the secretion of IL-6 and IL-23 by monocytes / macrophages, and participates in the expression of RORγt (necessary for Th17 cell differentiation) and CCL17/TARC, a key chemokine for recruiting these cells to the airways [23, 28, 29].

Our study revealed a strong association between BA severity and impaired lung function with elevated levels of serum IL-17A and its functionally related cytokine IL-6, both of which were significantly higher in the group of patients with moderate BA. It is reasonable to suggest the involvement of IL-17A and IL-6 in this disease phenotype, potentially mediated through neutrophil recruitment to the airway inflammatory infiltrate driven by increased production of these cytokines. This finding is supported by multiple publications demonstrating correlations between increased neutrophilic infiltration in the bronchi and elevated IL-17A levels in sputum, bronchoalveolar lavage fluid, and bronchial biopsy specimens (including epithelial cells, the subepithelial mucosal layer, and leiomyocytes) from patients with moderate-to-severe non-atopic steroid-resistant BA [13, 21, 27].

Previous studies have demonstrated a direct correlation between the number of Th17 cells in peripheral blood, sputum, and bronchoalveolar lavage fluid and the severity of airway remodeling in BA patients [24]. The elevated levels of IL-17A and IL-6 observed in group 2 patients, accompanied by probable escalation of bronchial inflammation through potential neutrophil mobilization and synthesis of proinflammatory cytokines, may adversely affect bronchial barrier function and stimulate airway remodeling, thereby worsening BA severity. BA severity is a factor that can exacerbate the infectious process following SARS-CoV-2 infection. Moderate-to-severe asthma is considered as a predictor of poor COVID-19 prognosis, with evidence indicating

a significantly higher mortality rate among these patients compared to those with mild disease (13.8% vs. 5.5%, $p = 0.006$) [3].

Based on multispiral computed tomography findings, we identified a substantial number of patients with post-COVID pulmonary fibrosis, particularly among those with moderate BA. These fibrotic changes in lung tissue should be considered in the context of the development and outcome of polymorphonuclear inflammation permeated by disintegrating neutrophils, resulting from the organization of exudative pneumonia foci caused by SARS-CoV-2. It is plausible that pre-existing neutrophilic airway inflammation in asthma patients — mediated by activation of Th1/Th17 immune response cytokines prior to SARS-CoV-2 infection — could have contributed to more severe lung damage during COVID-19.

The significance of neutrophilic infiltration as a key structural component of exudative pneumonia is supported by autopsy data from COVID-19 fatalities [30]. Microscopic examination of lung tissue in most cases revealed pronounced infiltration of interalveolar septa by neutrophils and mononuclear cells. Alveolar lumens, lined by hyaline membranes, contained abundant fibrinopurulent exudate rich in macrophages. Reactive hyperplasia and desquamation of alveolar epithelium were observed, along with thrombosis and hyalinosis of blood vessels in the pulmonary interstitium, fibroblast proliferation, and features of acute suppurative bronchiolitis with destruction and metaplasia of the bronchiolar epithelium.

Neutrophilia, along with eosinopenia, lymphopenia, and elevated levels of C-reactive protein in peripheral blood, has been identified among potential predictors of pulmonary fibrosis and long-term deterioration of lung function in COVID-19 patients. Other indicators associated with the risk of fibrotic remodeling and abnormal residual lung function include: patient age, severity of SARS-CoV-2 infection combined with chronic internal organ diseases, duration of intensive care unit stay for hospitalized patients, mechanical ventilation, and markers of hyperinflammation [31].

Assuming that BA patients had elevated concentrations of IL-1β, IL-6, and IL-17A prior to COVID-19, it is highly plausible that these cytokines contributed to the SARS-CoV-2-initiated lung injury, pneumonia, and hyperinflammation. In severe COVID-19, the cytopathic effect of SARS-CoV-2 triggers the release of damage-associated molecular patterns (DAMPs) from target cells. Released surface glycoproteins, ATP, and nucleic acids are recognized by neighboring epithelial cells, endothelial cells, and macrophages, stimulating pyroptosis — a highly inflammatory form of programmed cell death accompanied by IL-1β overproduction. Defects in apoptosis, caused by reduced cytolytic activity of NK cells and CD8+ T lymphocytes, may prolong the survival of virus-infected cells, leading to the accumulation of hyperactivated immune cells in the lungs and prolonged interaction between innate and adaptive immune cells. This cascade results in the generation of proinflammatory cytokines, cytokine storm, and the development of macrophage activation syndrome (MAS) [8, 10]. IL-1β and IL-6 serve as key inducers of hyperinflammation and MAS, which manifests as activation, uncontrolled expansion, and persistence of macrophages; massive cytokine secretion; induction of their synthesis by myeloid cells; hemophagocytosis; fibrinolytic coagulopathy; and multiorgan failure [10, 32]. Pathogenetic mechanisms predisposing to MAS include neutrophil hyperactivation, driven by the recruitment of granulocytes to the inflammation site via attractants, such as IL-7, IL-8, IFNγ, IP-10, as well as IL-1β, IL-6, and IL-17 from the Th1/Th17 subset. Neutrophil degranulation and the formation of abundant NETs promote inflammation escalation, damage to the vascular endothelium of the lungs and internal organs, and the development of microthromboses. NETs induce macrophage expression of IL-1β — a key mediator of MAS — which, in turn, activates pulmonary neutrophilic infiltration, neutrophilia, and NET formation [9, 10].

Thus, Th1/Th17-dependent activation of the neutrophilic component in the chronic airway inflammatory infiltrate of BA patients most likely contributes to the exacerbation of acute lung injury during COVID-19, laying the foundation for subsequent fibrotic replacement of respiratory tissue.

CONCLUSION

In asthma patients who have recovered from COVID-19, disease severity is associated with increased levels of Th1/Th17 cytokines in the blood. In mild BA, elevated IL-1β levels in exhaled breath condensate may be explained by its involvement in regulating the Th1/Th17 immune response and its contribution to cold air hyperresponsiveness. In contrast, patients with moderate BA demonstrated increased production of IL-6 and IL-17A, which was associated with impaired small airway patency, poor disease control, and likely activation of neutrophilic airway inflammation. Elevated concentrations of IL-

17A and related Th17 cytokines (IL-1β and IL-6) may increase the risk of systemic inflammation and pulmonary fibrosis development.

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Author Contribution

Prikhodko A.G. – conception and design, analysis and interpretation of the data, drafting of the manuscript. Pirogov A.B. – analysis of the data, manuscript rationale, critical revision of the manuscript for important intellectual content, drafting of the manuscript. Gassan D.A. – carrying out of biochemical assays. Perelman J.M. – critical revision of the manuscript for important intellectual content, final approval of the manuscript for publication, accountability for the integrity of all article sections.

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Risk Factors for Cognitive Decline in Patients in Long-term Periodof Coronary Artery Bypass Grafting

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ABSTRACT

Aim. To identify risk factors leading to a cognitive decline 5–7 years after CABG, and to develop a model for predicting the development of POCD in patients in the long-term period of CABG.

Materials and methods. The observational prospective study included 146 patients; average follow-up period was 6.4 years. The patients underwent general clinical, neurological, and instrumental examinations 3–5 days before and 5–7 years after surgery. Neuropsychological testing included assessment of psychomotor and executive functions, attention, and short-term memory. The method of binary logistic regression was used to build a predictive model.

Results. Cognitive decline was detected in 67 patients (45.9%) at 5–7 years after CABG. The presence of carotid artery (CA) stenosis (p = 0.01), smoking (p = 0.005), reduced left ventricular ejection fraction (p = 0.039), and high triglyceride levels (p = 0.011) were associated with a cognitive decline. The model`s sensitivity was 0.61 and specificity was 0.82, indicating a good quality. Results indicate that the model can accurately predict the presence or absence of cognitive decline with a high level of accuracy.

Conclusion. Five to seven years following CABG, 46% of patients experienced a decrease in cognitive functions, manifested in the form of neurodynamic dysfunction, as well as deterioration of short-term memory. The factors included in the prognostic model were CA stenosis, reduced left ventricular ejection fraction, and high triglyceride levels, as well as smoking. The findings indicate the need to improve approaches to postoperative follow-up of patients who have undergone cardiac surgery in order to minimize adverse neurological consequences.

Keywords: cognitive functions, CA stenosis, coronary artery bypass grafting, long-term postoperative period

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Факторы риска когнитивного снижения у пациентов в отдаленном периоде коронарного шунтирования

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РЕЗЮМЕ

Цель. Выявление факторов риска снижения когнитивных функций через 5–7 лет после КШ, а также разработка прогностической модели, способной предсказать вероятность развития ПОКД у пациентов в отдаленном периоде КШ.

Материалы и методы. В наблюдательное проспективное исследование включены 146 пациентов, средний период наблюдения составил 6,4 года. Пациенты прошли общее клиническое, неврологическое и инструментальные обследования за 3–5 дней до и через 5–7 лет после операции. Нейропсихологическое тестирование включало оценку психомоторных и исполнительных функций, внимания и кратковременной памяти. Для построения прогностической модели использовался метод бинарной логистической регрессии.

Результаты. Когнитивное снижение через 5–7 лет после операции выявлено у 67 (45,9%) пациентов. Установлено, что наличие стенозов сонных артерий (СА) (p = 0,01), факт курения (p = 0,005), низкий уровень фракции выброса левого желудочка (p = 0,039) и высокий уровень триглицеридов (p = 0,011) были ассоциированы с развитием когнитивного снижения через 5–7 лет после проведения КШ. Уровень чувствительности составил 0,61; специфичности – 0,82, обеспечивая успешное определение наличия или отсутствия снижения когнитивных функций, что говорит о хорошем качестве прогностической модели.

Заключение. Через 5–7 лет после проведения операции КШ у 46% пациентов наблюдается снижение когнитивных функций, проявляющееся в виде нейродинамических нарушений, а также ухудшения кратковременной памяти. Факторами, вошедшими в прогностическую модель, являлись стенозы СА, низкий уровень фракции выброса левого желудочка и высокий уровень триглицеридов, а также курение пациентов. Это свидетельствует о необходимости совершенствования подходов к послеоперационному наблюдению за пациентами, перенесшими кардиохирургические операции, с целью минимизации неблагоприятных неврологических последствий.

Ключевые слова: когнитивные функции, стенозы СА, коронарное шунтирование, отдаленный послеоперационный период

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

Coronary artery bypass grafting (CABG) is a cardiac surgical procedure with a high risk of complications like postoperative cognitive dysfunction (POCD) [1]. It can be either short-term or long-term [2]. POCD is associated with an increase in medical care costs, longer hospital stay, and an increase in one-year mortality rate [3]. A significant part of patients referred for myocardial revascularization have chronic cerebral ischemia and are more vulnerable to cognitive decline after surgery [4]. Chronic cerebral circulatory insufficiency and impaired cardiac pumping function are cited as the causes of cognitive impairment in the cohort of patients who are considered as candidates for elective CABG [5].

Identification of modifiable risk factors and mechanisms contributing to the development of POCD can help prevent this dangerous condition and, as a result, improve the effectiveness of patient treatment. There is a large number of studies on the risk factors for cognitive decline in patients in the early postoperative period of cardiac surgery [6–9]. It has been shown that age, cardiopulmonary bypass (CPB) time, and arterial hypertension can be significant predictors of the development of POCD [10]. The role of intraoperative hypoperfusion in the development of POCD is particularly emphasized [7]. The brain and kidneys are the organs most sensitive to changes in blood pressure during CPB, however, optimal cerebral perfusion pressure to maintain stable cerebral blood flow is still a subject of discussion. [11].

The development and aggravation of postoperative cognitive impairment in the long-term period largely negates the success of cardiac surgery, leading to disability, lower quality of life, and higher mortality [12, 13]. The data obtained emphasize the importance of timely identification of patients at high risk of POCD both in the early and long-term period.

However, one should note that studies on the risk factors for the development and aggravation of POCD in the remote postoperative period of CABG are scarce [14, 15]. It has been shown that the patient`s age, smoking history, hypertension, diabetes mellitus, heart failure, and preoperative cognitive impairment are predictors of persistent POCD one year after CABG [15]. However, it remains unclear whether these factors will have the same significance in the long term, for example, 5–7 years after surgery. Recurrent strokes and progression of coronary stenosis, development of atrial fibrillation, as well as the level of adherence to treatment can also be considered as possible predictors of the development of cognitive decline in the remote postoperative period. In this regard, the aim of this study was to identify risk factors for cognitive decline 5–7 years after CABG, as well as to develop a model for predicting the likelihood of developing POCD in patients in the long-term period of CABG.

MATERIALS AND METHODS

The selective observational prospective study was performed to assess the neurological and cognitive status of patients with coronary artery disease (CAD) over a 5–7-year period after CABG, with an average follow-up of 6.4 years. The study included 152 patients with stable CAD who were admitted to an inpatient unit to undergo a surgery. The study was conducted in strict accordance with international standards of Good Clinical Practice and the Declaration of Helsinki (2008). The protocol of the study was approved by the Institutional Review Board of the Research Institute for Complex Issues of Cardiovascular Diseases (Minutes No. 20 dated January 25, 2011). Prior to inclusion in the study, all participants received all the necessary information and provided their consent to participate.

The inclusion criteria were as follows: age 45–69 years, male gender, normal or adjusted to normal vision and hearing, elective primary CABG with CPB. The exclusion criteria were: prior acute cerebrovascular accident (CVA), traumatic brain injury, depression (more than 8 points on the Beck`s Depression Inventory), dementia (less than 24 points according to the Mini-Mental State Examination (MMSE), carotid artery stenosis (CA) greater than 50%, severe respiratory, renal and hepatic insufficiency, and oncological diseases. The clinical and history data of the patients are presented in Table 1.

Table 1

Preoperative Clinical Characteristics of Patients

Characteristics	Patients, $n = 152$
Age, years, *Me* [Q_{25}; Q_{75}]	57 [53; 61]
Arterial hypertension, *n* (%)	129 (85)
Carotid artery stenosis, *n* (%)	56 (37)
Duration of carotid artery stenosis, years, *Me* [Q_{25}; Q_{75}]	4 [2; 8]
Coronary artery disease duration, years, *Me* [Q_{25}; Q_{75}]	5 [3; 8]
Myocardial infarction in history, *n* (%)	114 (75)
SYNTAX score, *Me* [Q_{25}; Q_{75}]	23 [16; 28]
Number of affected arteries (coronary angiography), *Me* [Q_{25}; Q_{75}]	2 [2; 3]
Left ventricular ejection fraction, %, *Me* [Q_{25}; Q_{75}]	60 [51; 63]

Comprehensive examinations of patients, including clinical, neurological, and instrumental diagnostics, were conducted both before surgery and 5–7 years after it. MMSE and the Beck`s Depression Inventory were used as screening methods [16–18]. Psychophysiological Complex software was used for complex neuropsychological testing with the determination of indicators of psychomotor and executive functions, attention, and short-term memory [19]. Changes in cognitive functions were calculated using 13 parameters from a common set of tests. Cognitive decline was determined by the "20–20" criterion: postoperative indicators of cognitive functions should be lower by 20% or more compared to preoperative values in 20% of all the indicators used in the study.

The patients were treated in accordance with the general principles of therapy for patients with CAD, chronic heart failure (CHF), and arterial hypertension. The planned procedure was performed with CPB, with normal body temperature and intravenous anesthesia with propofol. The duration of CPB averaged 100.2 ± 2 8.2 minutes, and the time of aortic cross clamping was 62.8 ± 16.86 minutes. The average number of the grafts was 2.6 ± 0.71. During the surgical intervention, patients underwent invasive and continuous hemodynamic monitoring and cerebral oximetry (INVOS-3100, SOMANETICS, USA). Outpatient follow-up at the place of residence was conducted for all patients after discharge from the hospital.

Statistical analysis was performed using IBM SPSS Statistics 21 software. When describing the analysis results, continuous variables were expressed as median and interquartile range Me [Q_1; Q_3] and categorical variables were expressed in the form of values and percentages of n (%), the Pearson's and Wilcoxon tests $\chi 2$ were used to establish statistical differences.

To build a predictive model, we used the binary logistic regression method and regression coefficients. The regression equation was as follows: $y = a + b_1 \times X_1 + b_2 \times X_2 + ...b_i \times X_i$, where y – variable with two values: 0 means no event; 1 stands for an event occurred; a is constant; b_i is regression coefficients; X_i stands for variables. The probability of cognitive decline was determined according to a formula: $P = 1 / (1 + e^{\wedge}–y)$, where P is predicted probability, e is exponent, the approximate value of which is 2.718. A method based on the percent correctly reclassified and the Somers`D measures were used to assess the validity. The Hosmer–Lemeshow goodness-of-fit test was used to verify the overall consistency of the model with real data. The differences were considered statistically significant at $p < 0.05$.

RESULTS

Analysis of the results of clinical examinations revealed that 5–7 years after CABG, 8 patients (5.3%) had myocardial infarction, and 7 (4.6%) had a stroke. The majority of the study participants had CHF with functional class (FC) no higher than II (Table 2).

Table 2

Clinical Characteristics of Patients, *n* (%)

Characteristics	Patients, n = 152		p
	Before the procedure	After 5 years	
Class I-II angina pectoris	86 (57)	30 (20)	0.0004
Class III angina pectoris	33 (22)	5 (3)	< 0.0001
Class I-II CHF	116 (77)	146 (96)	0.001
Class III CHF	36 (24)	6 (4)	0.0007
Atrial fibrillation	5 (3)	12 (8)	0.1
Type 2 diabetes mellitus	21 (14)	39 (26)	0.002
CA stenosis	56 (37)	86 (57)	0.0001

A total of 146 patients out of 152 participated in a comprehensive neuropsychological testing using the STATUS PF computer program 5–7 years after CABG. Before the operation, three people dropped out due to not scoring enough points on the MMSE and Beck`s Depression Inventory scales; three more patients were unable to undergo comprehensive neuropsychological testing due to cognitive impairments after stroke and advanced dementia.

Cognitive decline, defined as a ≥ 20% decrease in postoperative cognitive indicators compared to the baseline level in ≥ 20% of tests from the entire test battery, was detected 5–7 years after surgery in 67 (45.9%) patients. Patients were most likely to

exhibit deterioration in postoperative parameters when performing neurodynamic (psychomotor speed and executive functions) and short-term memory tests. In the neurodynamic domain, 60.9% of patients had an increasing number of missed signals. In tests examining short-term memory (memorization of numbers, syllables, words), deterioration occurred in more than 20% of patients (Table 3).

Table 3

Prevalence of More than 20% Cognitive Decline in the Long-term Postoperative Period, *n* (%)

Cognitive domain	Parameters	Patients, *n* = 146
Neurodynamics	Mean reaction time	24 (16.4)
	Errors	57 (39.0)
	Missed signals	89 (60.9)
Attention	The Bourdon test, processed symbols:	
	– on the 1st minute	12 (8.2)
	– on the 4th minute	11 (7.5)
Memory	10 numbers memory test	31 (21.2)
	10 syllables memory test	30 (20.6)
	10 words memory test	36 (24.7)

The next stage of the study aimed at identifying the most significant risk factors for cognitive decline 5–7 years after CABG. For this purpose, we selected patients with cognitive decline 5–7 years after surgery (n = 67) and without cognitive decline (n = 79). The following predictors that could potentially influence the development of cognitive decline were included in the analysis: stroke, old age, smoking, arterial hypertension in history, heart failure with a left ventricular ejection fraction (LVEF) of less than 50%, CA stenosis, diabetes mellitus, impaired carbohydrate tolerance, atrial fibrillation, compliance with a four-component treatment regimen for CAD, achievement of target blood pressure, indicators of lipid metabolism and glucose in blood serum, and achievement of their target values.

Possible predictors identified both before surgery and during examination 5–7 years after CABG were considered both in their original form and in the form of binomial variables. Using the step-by-step inclusion method, we build the regression model. As a result, the following factors were the most significant in terms of cognitive decline: stroke, CA stenosis present 5–7 years after CABG, preoperative LVEF, smoking before surgery, cholesterol, HDL and triglyceride levels detected during examination 5–7 years after CABG (Table 4).

Analysis of the data presented in Table 4 allow us to conclude that CVA, smoking, CA stenosis, reduced LVEF, and high triglyceride and HDL cholesterol levels detected during examination 5–7 years after CABG increase the likelihood of cognitive decline in patients in the long-term postoperative period.

The developed model correctly predicts the absence of cognitive decline in 82.3% of cases and its presence in 61.2% of cases (Table 5). With the cut-off threshold equal to 0.5, we selected the most effective ratio of sensitivity (0.61) and specificity (0.82), ensuring the successful prediction of both the presence and absence of cognitive decline and good quality of the model.

Table 4

The Main Results of Binary Logistic Regression Predicting the Development of Cognitive Decline in Patients in the Long-term Period after Coronary Artery Bypass Grafting

Step 6	B coefficient	RMSE	Wald	*p*	Exp (B)
CVA	21.634	16644.068	0.000	0.999	2485603116.578
CA stenosis (5 years)	1.018	0.396	6.625	0.010	2.769
LVEF (before CABG)	-0.925	0.448	4.273	0.039	0.396
Smoking (before CABG)	1.067	0.381	7.841	0.005	2.906
HDL cholesterol (5 years)	1.171	0.617	3.605	0.058	3.226
Triglycerides (5 years)	0.533	0.209	6.527	0.011	1.704
Constant	-2.576	1.208	4.546	0.033	0.076

Table 5

Classification Matrix of the Predictive Model of Cognitive Decline in the Long-term Postoperative Period

Actual cognitive decline	Predicted cognitive decline		Percent correct classification, %
	Absent	Present	
Absent	65	14	82.3
Present	26	41	61.2
Total percent of correct classification, %			72.6

Note. The cut–off threshold value is 0.5.

DISCUSSION

The results of the study demonstrated that cognitive decline 5–7 years after coronary bypass grafting occurred in 46% of patients and in most cases manifested in the form of neurodynamic dysfunction, and disorders of verbal and symbolic short-term memory.

As previously shown, the pathogenesis of postoperative cognitive impairment is multifactorial [20]. During cardiac surgery, a systemic inflammatory reaction can lead to multiple organ failure, including brain tissue injury. It has been established that some CABG patients suffer blood – brain barrier disruption, accompanied by an increase in systemic inflammation detected in blood plasma, and the development of subacute neuroinflammation [21]. The progression of atherosclerosis is accompanied by changes in the microcirculatory system, resulting in reduced vascular elasticity [22]. In combination with decreased myocardial contractility, patients may experience a deterioration in blood supply to the brain during surgery, which is reflected in the predictive model developed in this study.

Other factors included in the predictive model were smoking and elevated triglyceride levels 5–7 years after CABG, which may indicate low patient adherence to prescribed treatment. There are several known factors that contribute to low patient adherence to prescribed treatment: lack of awareness, tendency to self-medicate, non-compliance with the medication regimen, and, most importantly, the relationship between cognitive impairment and low adherence [14, 23].

CA stenosis turned out to be a significant factor determining the development of cognitive decline 5–7 years after CABG. The progression of carotid stenosis increases the risk of developing cerebral circulatory disorders, and is also an independent factor predicting the likelihood of sudden cardiac death [24]. It has been established that during CABG, the presence of CA stenosis greater than 70% in a patient can lead to ischemic stroke [25, 26]. Studies indicate that patients suffering from cerebral atherosclerosis are at increased risk of decreased blood supply to the brain, atrophy of its tissues and cognitive decline [27]. There is a probability according to which patients with asymptomatic CA stenosis may experience decreased blood flow and microembolization of the brain during surgery, which in turn causes impaired adaptive mechanisms and deterioration of cognitive functions [28]. Among the factors contributing to the development of CA stenosis, surgical intervention on the coronary arteries can cause an exacerbation of the systemic inflammatory process and endothelial dysfunction. These changes create favorable conditions for the progression of the atherosclerotic process [29].

CONCLUSION

The present study demonstrated that 5–7 years after CABG, 46% of patients experience cognitive decline which manifested in the form of neurodynamic dysfunction and deterioration of verbal and symbolic short-term memory. The factors included in the predictive model are CA stenosis, reduced left ventricular ejection fraction, high triglyceride levels, and smoking in patients. The findings highlight the need to improve approaches to postoperative follow-up of patients who have undergone cardiac surgery in order to minimize adverse neurological consequences.

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Author Contribution

Syrova I.D. – drafting of the the manuscript, acquisition and interpretation of data, compilation of database, statistical processing of data, and final approval of the manuscript for publication. Tarasova I.V. – conception and design, drafting of the manuscript, editing of the manuscript, and final approval of the manuscript for publication.Trubnikova O.A. – conception and design, editing of the manuscript, and

final approval of the manuscript for publication. Sosnina A.S. – acquisition and interpretation of the data, compilation of database, and final approval of the manuscript for publication. Ivanov V.I. – statistical processing of data and final approval of the manuscript for publication. Barbarash O.L. – conception and design and final approval of the manuscript for publication.

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In vitro and *in vivo* Osteogenic Cell Response to High Temperature Exposure

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ABSTRACT

Aim. To evaluate the response of mesenchymal stromal/stem cells (MSCs) *in vitro* and the status of bone cells (osteoblasts, osteocytes) during regeneration of the femoral bone after local thermoablation at 55–60 °C.

Materials and methods. Morphology and viability (MTT assay) of human adipose tissue-derived MSCs were analyzed after incubation at 37 °C or 56 °C for 0–60 minutes. *In vivo*, a heating cuff was applied to the femur of anesthetized rabbits, and intraoperative thermoablation was performed for 30 minutes (bone marrow canal temperature: 55–60 °C). Bone tissue was histologically examined (hematoxylin – eosin and Einarson staining) immediately and 14 days post-treatment. Quantitative morphometry was implemented in ImageJ, with subsequent statistical analysis performed in R.

Results. *In vitro*, signs of massive MSC death were observed after 15 minutes of heating; at 30 minutes, viable fibroblast-like cells were nearly absent. *In vivo*, local thermoablation caused direct death of osteoblasts and osteocytes, evidenced by morphological signs of apoptosis and necrosis, as well as impaired DNA/RNA synthesis. Morphological and molecular markers of cellular damage significantly increased by day 14 post-treatment.

Conclusion. Moderate thermoablation (55–60 °C) exerts significant direct and delayed damaging effects on osteogenic cells, from stem to mature forms. These findings are relevant for combined treatment of tumor and metastatic bone lesions.

Keywords: mesenchymal stromal/stem cells, cell culture, rabbit femurs, osteoblasts, osteocytes, thermalablation, signs of cell death

Conflict of interest. The authors declare the absence of obvious or potential conflict of interest related to the publication of this article.

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In vitro и *in vivo* реакция остеогенных клеток на высокотемпературное воздействие

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РЕЗЮМЕ

Цель исследования: оценить реакцию мезенхимных стромальных/стволовых клеток (МСК) *in vitro* и состояние костных клеток (остеобластов, остеоцитов) в процессе восстановления бедренной кости после локальной термоабляции в диапазоне 55–60 °С.

Материалы и методы. Морфологию и жизнеспособность (МТТ-тест) культуры МСК, выделенной из жировой ткани человека, изучали при культивировании в термостатах при 37 °С или 56 °С в диапазоне 0–60 мин. На бедренную кость наркотизированных кроликов накладывали нагревательную манжету и проводили интраоперационную термоабляцию в течение 30 мин (температура в костно-мозговом канале 55–60 °С). Состояние костной ткани анализировали гистологически (окраска гематоксилином и эозином и по Эйнарсону) сразу и через 14 сут после воздействия. Проводили компьютерную морфометрию с использованием ImageJ и статистический анализ в R.

Результаты. Морфологические признаки массовой гибели МСК в культуре *in vitro* наблюдались через 15 мин нагревания; через 30 мин живые фибробластоподобные клетки практически отсутствовали. Прижизненная локальная термоабляция бедренной кости кроликов вызывала прямую гибель остеобластов и остеоцитов, зафиксированную по морфологическим признакам апоптоза и некроза, а также нарушению синтеза ДНК и РНК. Морфологические и молекулярные маркеры клеточного повреждения статистически значимо увеличивались к 14-м сут после нагревания.

Заключение. Умеренные режимы термоабляции (55–60 °С) обладают значительным прямым и отсроченным повреждающим эффектом на остеогенные клетки от стволовых до зрелых форм. Полученные результаты важны для практики комбинированного лечения опухолевых и метастатических поражений костной ткани.

Ключевые слова: мезенхимные стромальные стволовые клетки, клеточная культура, бедренные кости кролика, остеобласты, остеоциты, термоабляция, признаки клеточной смерти

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

Optimal remodeling of skeletal tissues in case of diseases and injuries, including those around implanted materials and products, remains an unsolved problem in traumatology, orthopedics, and surgical oncology. The central role in the sequential, reparative, and pathological regeneration of bone tissue is played by mesenchymal stromal/stem cells (MSCs) [1, 2].

Currently, various pharmacological and physicochemical methods for controlling reparative processes have been developed and tested in experiments and clinical settings. One of the areas that is being actively developed (primarily in oncology) is the use of high temperatures. These temperatures can be divided into two categories: hyperthermia (above 41 °C) and thermal ablation (above 55 °C) [3, 4]. Hyperthermia in the range of 43–46 °C enhances *in vivo* osteogenesis [5] and bone tissue mineralization [6] two weeks after heat stress [7]. We have previously shown the *ex vivo* or *in vivo* effect of thermal ablation at a local temperature of above 55 °C on the mechanical strength of bones [8, 9], as well as the morphology of bone tissue in experimental animals in the early periods (up to 7 days) after high-temperature exposure [10].

MSCs and osteoblasts are considered to be thermosensitive cells [11, 12] and a part of the tumor-associated microenvironment [12]. The response of these cells to heating determines the outcome of therapeutic manipulations and reparative processes under clinical thermal ablation conditions.

It should be noted that most studies on cells were performed under hyperthermic conditions (up to 45 °C). At the same time, thermal ablation is mainly used in the combined treatment of benign and malignant tumors and metastatic lesions of bone tissue [13, 14].

It is well known that direct thermal ablation has a direct effect on cells, leading to the development of coagulation necrosis [15]. This is accompanied by protein denaturation and disruption of the integrity and permeability of cell membranes [15]. Some researchers, such as [4], suggested that indirect thermal effects are associated with the reaction of the immune system. This, in line with the concept of osteoimmunology, has a significant impact on metabolic and reparative processes in bone tissue both under normal and pathological conditions or extreme influences [16].

However, the specific consequences of the immediate and delayed effects of high-temperature exposure on bone tissue are still poorly understood. This can be seen from our searches for the keywords “MSC thermoablation”, “osteoblast thermoablation” or “osteocyte thermoablation”, which did not return any results in the PubMed database (https://pubmed.ncbi.nlm.nih.gov/).

In light of this, the aim of the study was to investigate the response of MSCs *in vitro* and the state of bone cells (osteoblasts and osteocytes) *in vivo* after local thermal ablation at temperatures between 55 °C and 60 °C.

MATERIALS AND METHODS

In vitro Study of the Effect of Thermal Ablation on the MSC Culture

MSCs were obtained after four-passage stromal vascular fraction (SVF) expansion, isolated from adipose tissue lipoaspirate from an apparently healthy donor, as described previously by our group [17]. The presence of MSCs in the SVF was confirmed according to the minimal morphological criteria established by the International Society for Cellular Therapy (ISCT) [18]. In our study, the following culture parameters were used: 1) fibroblast-like morphology; 2) adhesion to a surface of culture plastic; 3) viability of 93% in the test with 0.4% trypan blue solution; 4) positive expression of CD73, CD90, and CD105 (95%); 5) low expression (1.3%) of hematopoietic cell markers CD45, CD14, CD20, and CD34 according to flow cytometry.

The isolated cells were cultured in a concentration of 5×10^4 living cells per 1 ml of a nutrient medium with the following composition: 90% Dulbecco’s modified Eagle’s medium (DMEM) (Servicebio, China), 10% fetal bovine serum (FBS) (Technozero, Russia), antibiotics (100 units / ml penicillin and 100 mg / ml streptomycin (Capricon scientific GmbH, Germany) and 2 mM L-glutamine (PanEco Company, Russia). The cultures were maintained at 5% CO_2 and 100% humidity in 24-well plates (Corning, USA). Exothermic exposure of MSC cultures was performed in two dry-air thermostats (Smolensk SKTB SPU, Russia) set to 37 °C or 56 °C (thermal ablation) for different time intervals. The experimental groups for *in vitro* thermal ablation were divided into the following categories based on external heating times: 1) 37 °C for 60 min (control group); 2) 37 °C + 56 °C in different combinations: 45 + 15, 30 + 30, 15 + 45 or 0 + 60 min, respectively. Five wells were used for each time group. The cells were then incubated in a CO_2 incubator (MCO-170I, Alphavita Bio-Scientific,

China) for up to 20 h at 37 °C, 5% CO_2, and 100% humidity.

After cultivation, the viability of the cell cultures was determined using the MTT test recommended by GOST ISO 10993-5-2023, as described previously [19]. Four hours before the end of 2-hour culture, the cells were removed using a 0.25% trypsin-EDTA solution (PanEco, Moscow, Russia), centrifuged at 1,500 rpm for 10 min and washed twice with phosphate-buffered-saline. A 0.5% solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (NeoFroxx, Germany) in a complete growth medium was then added to each well, and the samples were incubated for 4 hours at 37 °C. After incubation, the supernatant was carefully removed and a solubilizing solution (10% dodecyl sulfate (SDS) in 0.01 M HCl) was added to dissolve formazan crystals. The index of cytotoxicity (IC) was determined by spectrophotometric analysis of the samples at 540 nm using a Picon spectrophotometer (Picon, Uniplan, Russia) with a reference wavelength of 630 nm.

Cell morphology was analyzed using an ADF I350 phase-contrast inverted microscope (ADF Optics Co Ltd, China) with a built-in digital camera and corresponding software.

In vivo Study of the Effect of Thermal Ablation on the Bone

The design of the experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of the Central Research Laboratory of Siberian State Medical University (SibSMU) (Minutes No. 1 dated April 3, 2023). The study was conducted in the vivarium at the Central Research Laboratory (CRL) of SibSMU on 6 healthy mongrel rabbits aged 15 weeks and weighing 3–4 kg after a 7-day quarantine period in ambient conditions. The rabbits were randomly divided into two experimental groups. The criteria for exclusion from the experiment were signs of illness of an animal, which were detected by a veterinarian through a physical examination. These signs included lethargy, lack of appetite, hair loss, itching, redness of the skin, mucosal redness, and the presence of ear mites. Rabbits that underwent thermal ablation were also excluded from further observation if they had pathological fractures of their femurs.

Thermal Effects on Laboratory Animals

The procedure of local controlled intraoperative thermal ablation of the right femur in all rabbits was performed for 30 min, as described in detail previously [10], using the Phoenix-2 local hyperthermia complex (PromEl LLC, Tomsk), developed at Tomsk State University of Control Systems and Radioelectronics [20].

The animals were euthanized using carbon dioxide asphyxiation. Group 1 (Int 0 days 55–60 °C – contralateral limb; Temp 0 days 55–60 °C – limb exposed to thermal effects) was euthanized immediately after thermal ablation (n = 3). Group 2 (Int 14 days 55–60 °C; Temp 14 days 55–60 °C) was euthanized 14 days after thermal ablation (n = 3).

Histological Study

A histological study of rabbit femurs was conducted according to the method described in detail in [10] at the Division of Morphology and General Pathology of Siberian State Medical University (Tomsk). Whole femurs were extracted from each animal (heated and intact), fixed in 10% buffered formalin solution (pH 7.4, Biovitrum, Russia), and decalcified using the modified Gripp method [21]. The decalcified bone fragments were then placed in a 10% sodium sulfate solution (LenReaktiv JSC, Russia) for 24 hours, dehydrated using 6 changes of an IsoPrep isopropanol-based solution (Biovitrum, Russia), and embedded in HISTOMIX paraffin mixture (Biovitrum, Russia). Histological slides of bones (5–7-μm thick) were prepared using a semiautomatic microtome MZP 01 (Tekhnom, Russia) and mounted on glass slides. The preparations of the contralateral femur and the femur subjected to thermal ablation were stained with Gill's hematoxylin (Biovitrum, Russia) and eosin (Biovitrum, Russia) and according to the Einarson method [22]. The Einarson technique is highly specific for staining nucleic acids, especially under conditions of increased acidity in the environment [23].

Histological slides were examined using an Axioscope 40 light microscope (Zeiss, Germany). Digital photographs were taken under fixed lighting conditions using a Canon PowerShot A2200 camera (14.1 MP resolution; Canon Inc., China) and AxioVision 4.8 software (Zeiss, Germany).

The area, circularity index (CI), and conventional units of optical density (c.u.o.p.) of the stained zones of interest (nucleus, cytoplasm) were estimated according to the method of computer morphometry of digital images [24] using the tools of the ImageJ program (version 1.38, National Institutes of Health, Bethesda, USA; https://www.rsb.info.nih.gov/ij). To estimate CI, the following formula was used: , where S is the area and P is the perimeter of the selected area.

The number π was rounded to 7 decimal places (π ≈ 3.1415926). A CI value of 1 corresponds to a regular circle, while a value close to zero corresponds to a highly elongated polygon. The method of measuring optical density allows for obtaining photometric quantitative characteristics of an opaque object. For this purpose, the background brightness (*SF*) and the brightness of the studied area of the cell or tissue (*ST*) were determined in the image in grayscale mode. The optical density of the object (*D*) can be calculated using the formula: .

Statistical Data Analysis

Statistical analysis of the data was conducted using the R programming language (version 4.4.0) and the RStudio environment (version 2023.12.0+369). The MVN [25], stats [26], and brunnermunzel [27] packages were used. The normality of distribution of quantitative characteristics was tested using the Shapiro – Wilk test with the Royston correction [28]. The data were presented as $M \pm SD$ for normally distributed data and as Me (Q_1; Q_3) for non-normally distributed data.

To compare the means of two samples, the Smith – Welch – Satterthwaite test [29–31] was used as a more powerful criterion than the Student's *t*-test possessing good control over type I errors [32–34]. For rank and non-normally distributed quantitative data, the Brunner – Munzel test was used [35, 36], which does not require equal variances or distributions like the Mann – Whitney *U*-test and is reliable when these assumptions are met [37, 38]. Comparison of categorical features was performed using the Fisher's exact test with the Holm – Sidak correction for multiple comparisons.

Since the study groups included non-inbred rabbits whose bone conditions varied significantly, a personalized statistical approach was used. In this approach, values were standardized and compared to those of the intact contralateral femur, which did not undergo local thermal ablation.

RESULTS

In vitro Study of the Effect of Thermal Ablation on the MSC Culture

Cells in the control group (37 °C for 60 min) had an elongated, spindle-like or polygonal shape, as well as a large, regular, round or oval nucleus (Fig. 1, *a*), which corresponds to the typical fibroblast-like morphology of MSCs adhering to plastic. When assessing the morphofunctional state of MSCs after thermal ablation (56 °C, 15 min), the cell culture was represented by single cells adhered to the bottom of the well (Fig. 1, *b*), with cell morphology similar to that in the control group. Round cells of varying sizes with non-uniform, foamy/bubbly cytoplasm were also observed (Fig. 1, *c*), with a moderate amount of cell debris between the cells. A decrease in cell size, changes in the state of the cytoplasm, loss of pseudopodia and rounding suggest cell death by anoikis, a special case of apoptosis [39]. This state of the culture indicates the damaging effect of thermal ablation at 56 °C even after a 15-minute exposure. Indeed, the MTT test revealed that the average IC value was 62 ± 3%.

A further increase in the duration of temperature exposure up to 30 minutes or more (45–60 minutes) led to complete disappearance of cells with fibroblast-like morphology. The cells sharply decreased in size, the cytoplasm became less homogeneous, pseudopodia were absent, and a large number of cell debris were detected (Fig. 1, *d, e, f*). The described morphological changes indicate massive cell death after 30 minutes of thermal ablation at 56 °C. The MTT test confirmed these morphological changes, with IC for MSCs reaching 96 ± 2%.

Thus, the experiment conducted with external *in vitro* thermal ablation of the MSC culture at 56 °C demonstrated its high sensitivity to extreme effects.

In vivo Study of the Effect of Thermal Ablation on the Bone

The State of Bone Tissue Immediately after Local Thermal Ablation

The contralateral (relatively intact) left femurs of the rabbits immediately after thermal ablation of the right femurs showed no morphological signs of necrosis or inflammation when stained with hematoxylin and eosin (Fig. 2, *a, c*). The diaphysis was represented by lamellar bone tissue, with the medullary canal filled with red bone marrow. The bone was covered by periosteum, to which muscle fibers were adjacent. Numerous osteoblasts were visible in the endosteal zone (Fig. 2, *c*, arrow).

In the area of direct heating of the diaphysis of the right femurs (55–60 °C in the medullary canal and 60–65 °C on the periosteum side), signs of venous hyperemia in large bone marrow vessels were observed (Fig. 2, *b*). The lamellar bone tissue of the endosteum (Fig. 2, *d*) showed no obvious damage.

The microscopic examination of osteocytes in the group immediately removed from the experiment

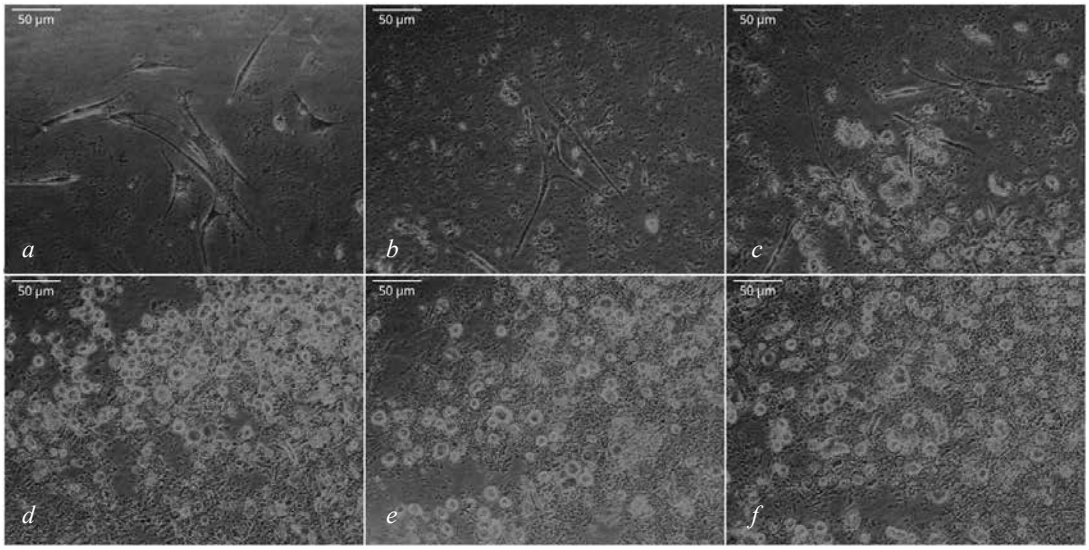

Fig. 1. Morphological state of a 20-h culture of mesenchymal stromal/stem cells after heating at 56 °C; *a* – control at 37 °C; thermal ablation*: b, c* – 15 min; *d* – 30 min; *e* – 45 min; *f* – 60 min followed by cultivation at 37 °C. Dark-field inverted microscopy, × 200.

(group 1) revealed numerous empty osteocyte lacunae in the area of thermal ablation within the compact bone substance. The number of these lacunae in the periosteal zone significantly increased by 20% compared to the contralateral, intact limb (Fisher's exact test; p = 0.0042, Fig. 3, *a*). However, the morphometric analysis of the compact bone close to the endosteum showed that lacuna closure after thermal ablation had increased but did not reach statistical significance (Fisher's exact test; p = 0.10; Fig. 3, *a*).

The emptying of the lacunae probably indicates osteocyte denucleation and cytolysis, which are the most significant morphological signs of cell death through necrosis or apoptosis. Consequently, 30-minute thermal ablation at 55–60 °C (from the endosteum side) and 60–65 °C under the heater cuff from the periosteum side increased the direct death of osteocytes in the diaphysis by 12–20% during the procedure. A higher periosteum heating temperature (~ 5 °C) also caused higher numbers (8%) of cell death.

When staining nucleic acids by the Einarson method, the morphology of osteocyte nuclei (Fig. 2, *g–j*) was visually different in the two groups. In the control group, the osteocyte nuclei were large, round or spindle-shaped, and intensely stained (Fig. 2, *g, i*; arrows); after thermal ablation, the nuclei were less intensely stained and appeared spindle-shaped or flattened (Fig. 2, *h, j*; arrows). However, the morphometric analysis of the morphofunctional parameters (area, CI, and optical density) of the osteocyte nuclei in the periosteum (Fig. 2, *i, j*) and endosteum (Fig. 2, *g, h*) showed no significant differences (Fig. 4, *a, d, g, m, p*) with the corresponding zones of interest in the intact diaphysis.

Some increase in the median area of the osteocyte nucleus near the endosteum (by 1.31 μm²; Brunner – Munzel test; p = 0.04; Fig. 4, *j*) may be associated with their swelling after heating, as the optical density of nucleic acid staining did not significantly decrease (Fig. 4, *m*). However, it is not possible to assess the state of the osteocyte cytoplasm using optical microscopy, as it is practically unstained using the Einarson method, and the outer cytoplasmic membranes of the cells are in close contact with the walls of the lacunae in many cases.

On the other hand, visual analysis (Fig. 2, *e, f*) revealed pronounced differences in the state of osteoblasts immediately after thermal ablation. In intact bones, there were numerous osteoblasts with signs of high synthetic activity, such as large basophilic cytoplasm and apical location of the nucleus (Fig. 2, *c, e*; arrows). In contrast, in the thermal ablation zone, osteoblasts were much fewer and showed low synthetic activity (Fig. 2, *f*; arrow). These observations were supported by the morphometric analysis. Cell shrinkage (Welch's *t*-test; $p < 0.001$; Fig. 5, *a*) and a decrease in cytoplasmic optical density (Brunner – Munzel test; p = 0.0036; Fig. 5, *d*) were observed. The area of nuclei also decreased (to 79%) from the intact value (Brunner – Munzel test; $p < 0.001$; Fig. 5, *g*), and their optical density increased to 171% of the intact level (Welch's *t*-test; $p < 0.001$; Fig. 5, *j*). These changes can be interpreted as signs of karyopyknosis.

From the perspective of the modern interpretation of the data obtained, it is possible to suggest that

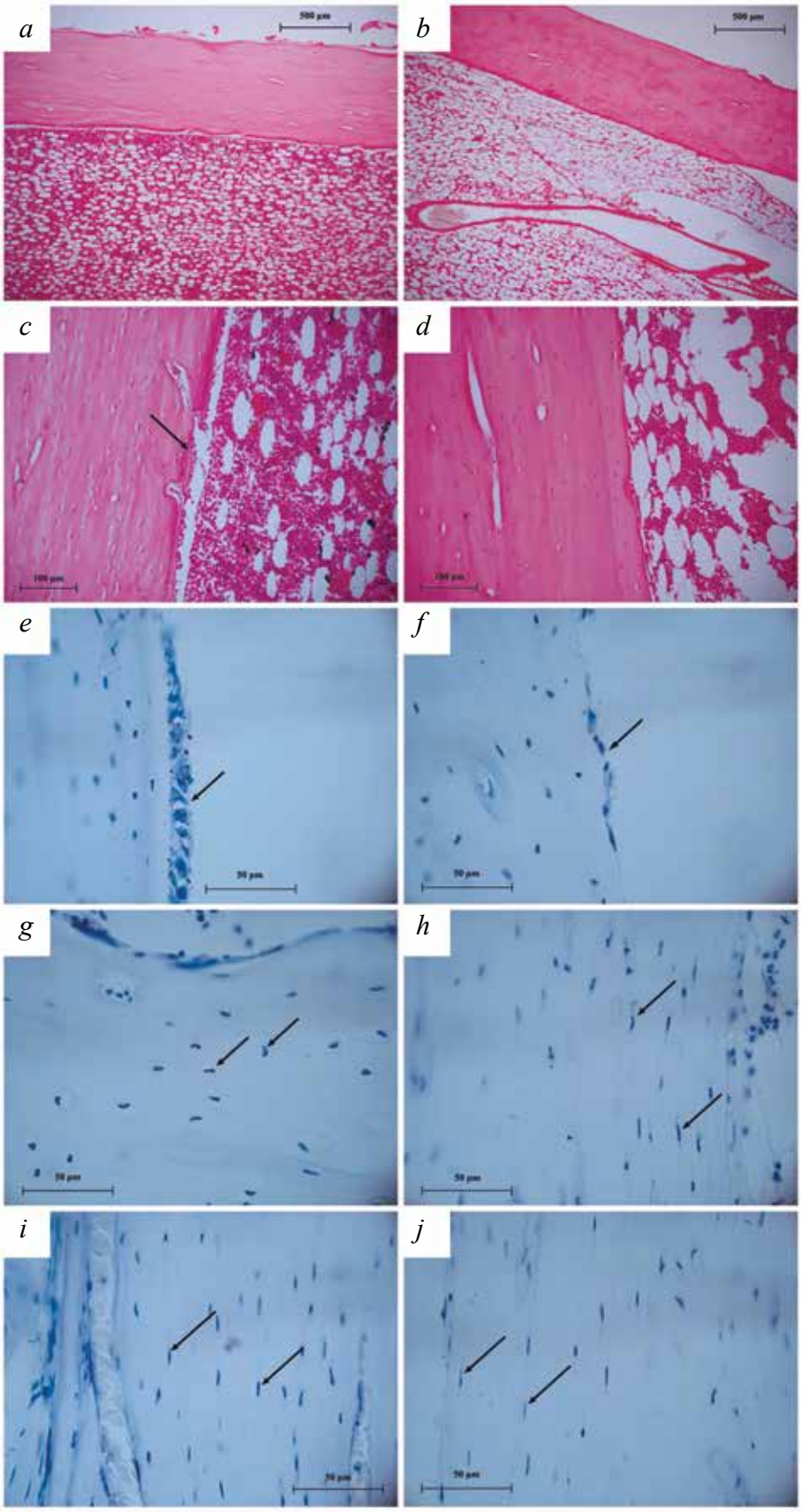

Fig. 2. Morphology of intact femurs (*a, c, e, g, i*) and bones immediately after thermal ablation (0 days) at 55–60 °C (*b, d, f, h, j*). In the diaphysis of intact specimens (*a, c, e, g, i*): lamellar bone tissue, red bone marrow (*a*), numerous active osteoblasts in the endosteum (*c, e*; arrows), osteocytes with intensely stained rounded (*g*; arrows) and fusiform (*I*; arrows) nuclei. After thermal ablation (*b, d, f, h, j*): hyperemia of bone marrow vessels (*b*), bone tissue in the endosteum zone without pronounced changes (*d*), single inactive osteoblasts (*f;* arrow); osteocytes with less intensely stained fusiform (*h*; arrows) and flattened (*j*; arrows) nuclei. Hematoxylin and eosin staining (*a–d*), Einarson staining (*e–j*). × 50 (*a, b*), × 200 (*c, d*), × 630 (*e–j*).

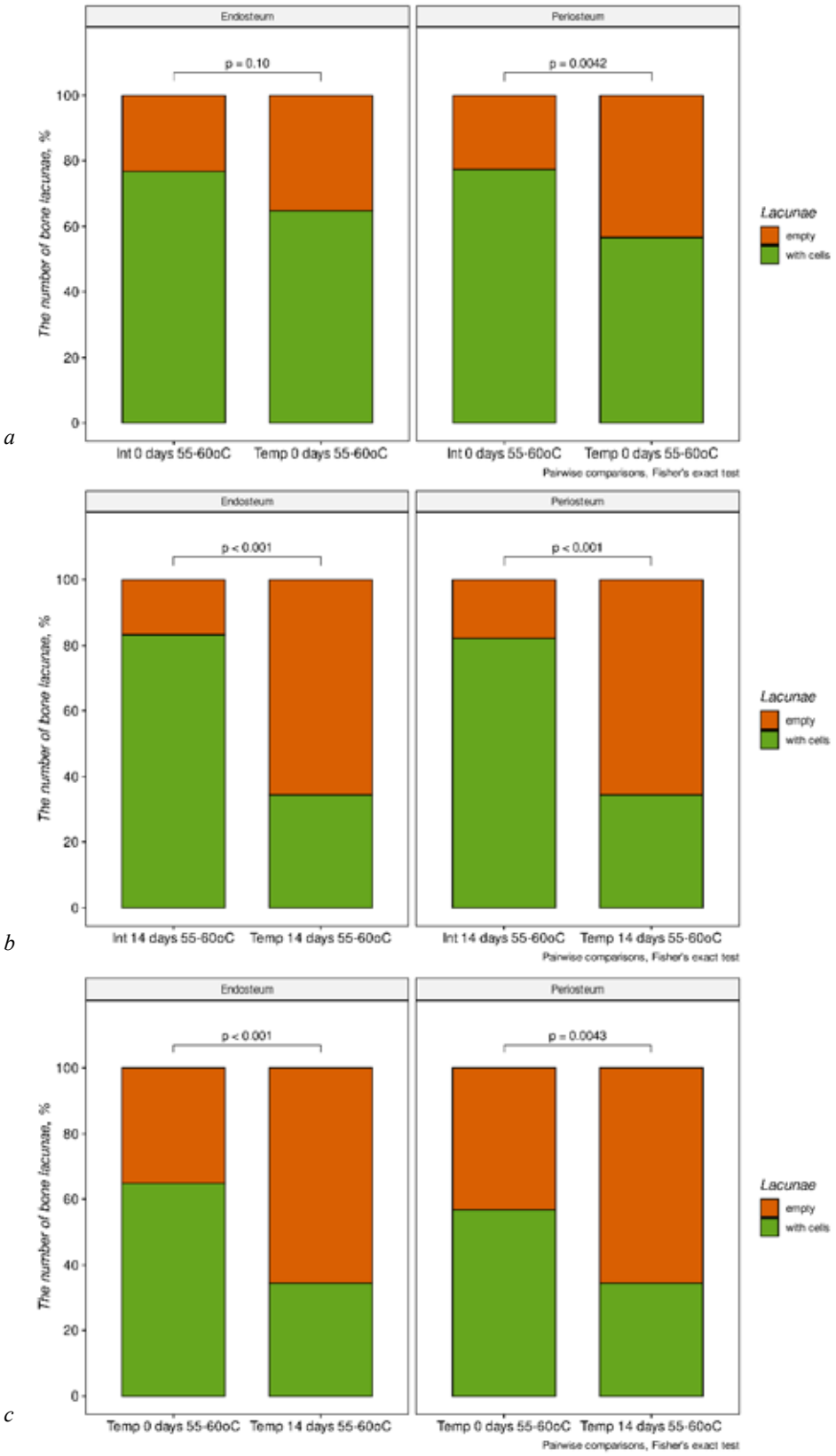

Fig. 3. The proportion of bone lacunae with and without osteocytes in sections of femoral diaphysis over time. Comparison of the index in intact bone and bone after heating on day 0 (*a*) and day 14 (*b*), as well as on days 0 and 14 after thermal ablation (*c*). Temperature range 55–60 °C. Data are presented as percentages. Comparison criterion – Fisher's exact test (*a–c*)

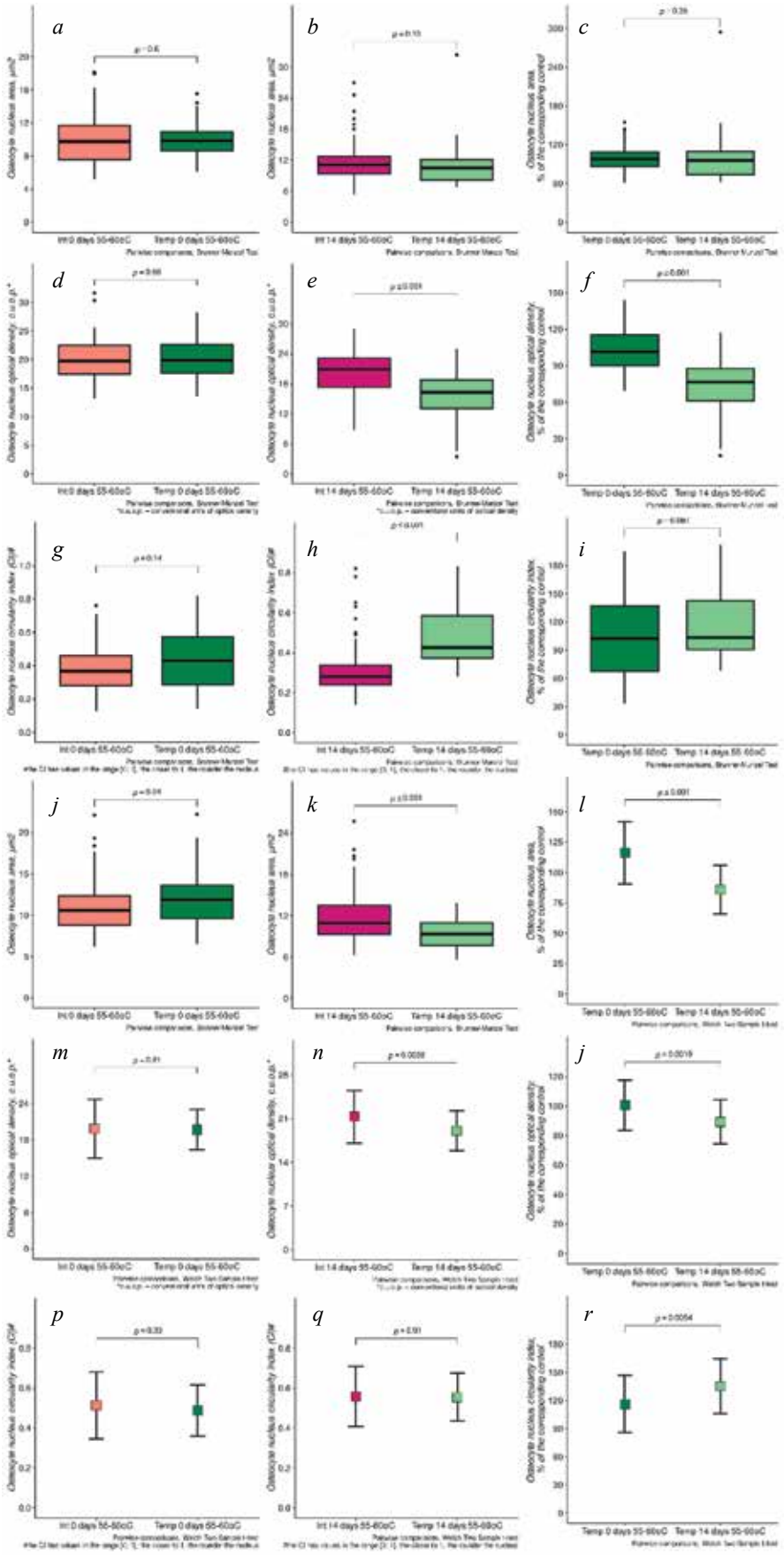

Fig. 4. Morphometric parameters of osteocytes in the periosteal (*a–i*) and endosteal zone (*j–r*) of the femoral diaphysis. Area of osteocyte nuclei (*a–c*, *j–l*) in the intact bone and bone after hyperthermia on day 0 (*a, j*) and day 14 (*b, k*), as well as in the bone after hyperthermia on days 0 and 14 (*c, l*). Optical density of osteocyte nuclei (*d–f, m–o*) in the intact bone and bone after hyperthermia on day 0 (*d, m*), day 14 (*e, n*), as well as in the bone after hyperthermia on days 0 and 14 (*f, o*). Comparison of CI in osteocyte nuclei (*g–i, p–r*) in the intact bone and bone after hyperthermia on day 0 (*g, p*), day 14 (*h, q*), and in the bone after hyperthermia on days 0 and 14 (*i, r*). Data are presented as *Me (Q1; Q3)* (*a–k*) and *X* ± *SD* (*l–r*). Comparison criteria– Brunner – Munzel test (*a–k*), Welch's *t*-test (*l–r*), c.u.o.p. – conventional units of optical density. CI has values in the range [0; 1], the closer to 1, the rounder the nucleus

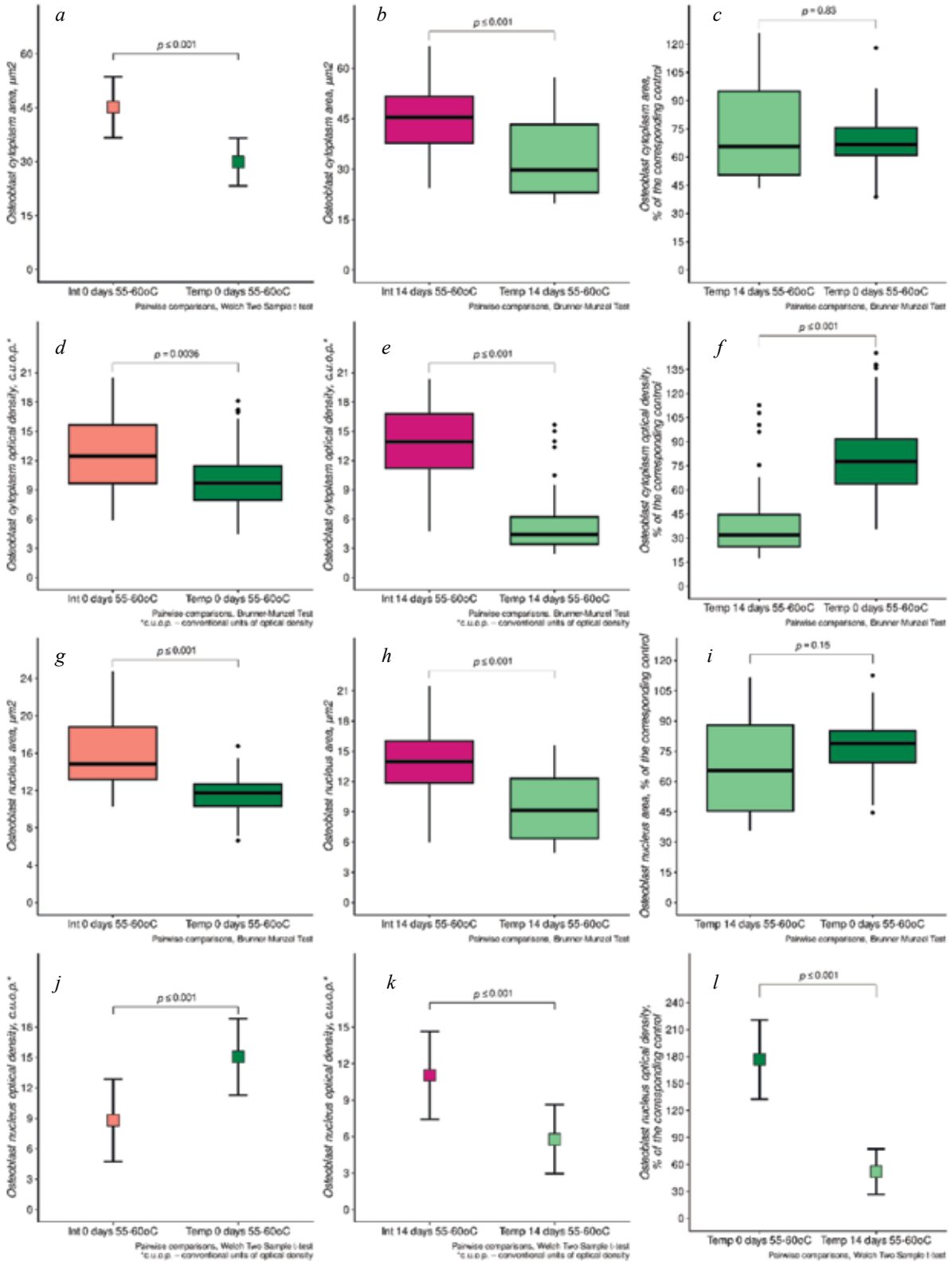

Fig. 5. Morphometric parameters of the cytoplasm (*a–f*) and nuclei (*g–l*) of the osteoblasts of the femoral diaphysis. Area (*a–c*) and optical density (*d–f*) of the osteoblast cytoplasm in the intact bone and bone after hyperthermia on day 0 (*a, d*), day 14 (*b, e*), and in the bone after hyperthermia on days 0 and 14 (*c, f*). Area (*g–i*) and optical density (*j–l*) of osteoblast nuclei in the intact bone and bone after hyperthermia on day 0 (*g, j*), day 14 (*h, k*), and in the bone after hyperthermia on days 0 and 14 (*i, l*). Data are presented as *Me (Q1; Q3)* (*b–i)* and $X \pm SD$ (*a, j–l*). The comparison criteria – the Brunner – Munzel test (*b–i*), the Welch's *t*-test (*a, j–l*), c.u.o.p. – conventional units of optical density

osteoblast death occurs by apoptosis, which is characterized by a decrease in cell size due to the compaction and fragmentation of the nucleus, as well as a loss of cellular integrity through the formation of apoptotic bodies [39]. A decrease in the optical density of the cytoplasm, when stained by the Einarson method, indicates reduction in the content of nucleic acids, primarily RNA molecules. As a consequence, it can be assumed that the synthetic activity of osteoblasts decreases immediately after the direct thermal damage at temperatures ranging from 55 to 60 °C.

The State of Bone Tissue 14 Days after Local Thermal Ablation

Standard histological analysis with hematoxylin and eosin staining of the femur sections of the contralateral bones (Fig. 6, *a, c*) revealed that the diaphysis retained a generally normal structure 14 days after thermal ablation. However, cavities containing tissue detritus and granulation tissue were observed in the endosteal zone (Fig. 6, *a*; arrows). These cavities were lined with osteoblasts (Fig. 6, *c*; arrows), which had a high content of nucleic acids when stained by the Einarson method (Fig. 6, *e*; arrow), suggesting the presence of bone tissue remodeling. These findings suggest that there may be a distant damaging effect of local heating mediated through biological environments and integral systems of the body.

The visual assessment of the femoral diaphysis under the heater cuff revealed extensive hemorrhages (hematoma type) in the medullary canal and areas adjacent to the endosteum (Fig. 6, *b*; arrows). At the same time, the lamellar bone tissue of the endosteum did not show any obvious signs of structural damage or active regeneration when stained with hematoxylin and eosin. Only single osteoblasts were visualized, which were weakly stained according to the Einarson technique (Fig. 6, *d, f*; arrows). The deterioration of the morphofunctional state of osteoblasts in the heating zone was quantitatively confirmed by computer morphometry data.

Thus, a significant drop by 1.5–3 times was noted in all measured parameters, including area and

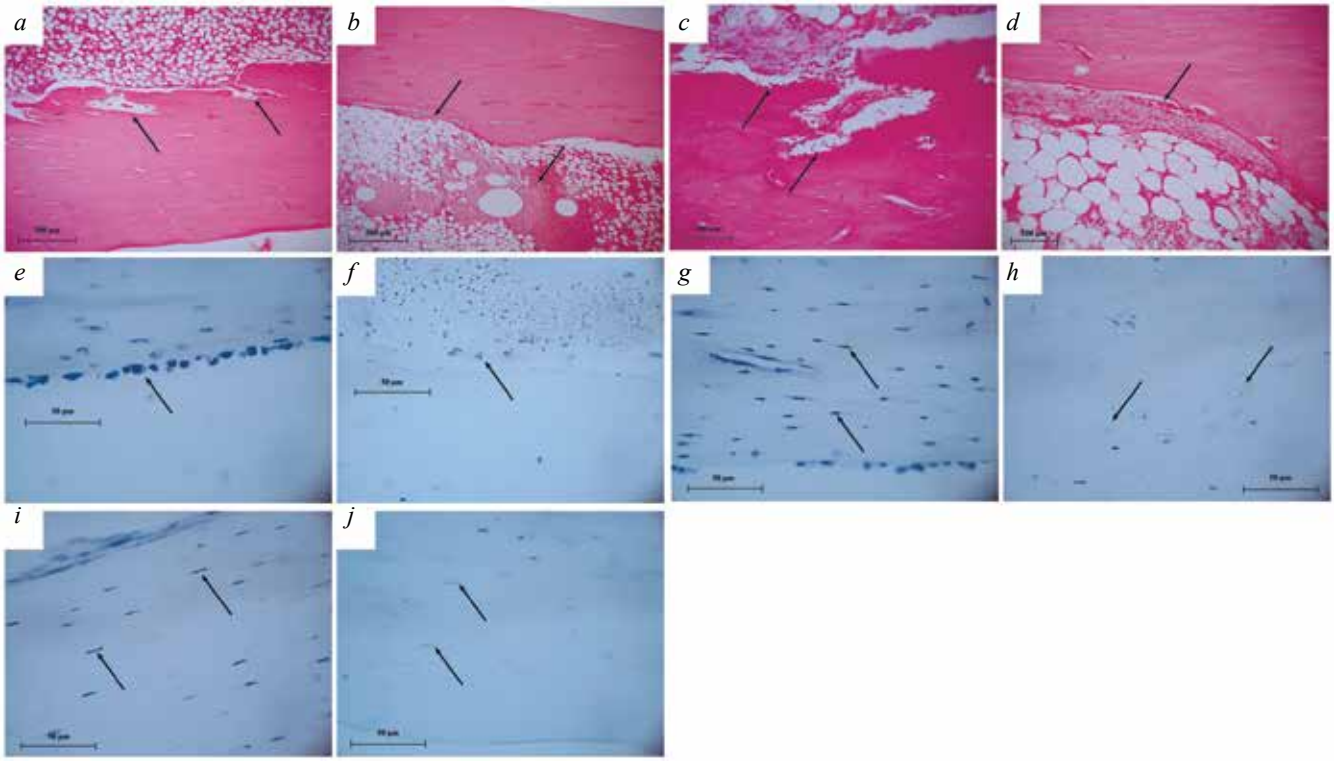

Fig. 6. Morphology of intact femurs (*a, c, e, g, i*) and bones 14 days after thermal ablation at 55–60 °C (*b, d, f, h, j*). In the diaphysis of the contralateral femurs (*a, c, e, g, i*): cavities with granulation tissue in the endosteal region (*a*; arrow); numerous active osteoblasts in the endosteum and cavities (*c, e;* arrows); osteocytes with large, intensely stained nuclei in the endosteal (*g*; arrows) and periosteal (*I*; arrows) zones. After thermal ablation (*b, d, f, h, j*): hemorrhages in the medullary canal and at the endosteum (*b*; arrows), single inactive osteoblasts (*d, f*; arrows), numerous empty osteocyte lacunae in the endosteal (*h;* arrows) and periosteal (*j*; arrows) zones. Hematoxylin and eosin staining (*a–d*), Einarson staining (*e–j*). × 50 (*a, b*), × 200 (*c, d*), × 630 (*e–j*)

optical density of cytoplasm and nuclei (Fig. 5, *b, e, h, k*). These morphofunctional changes in osteoblasts can be interpreted as signs of apoptosis, a form of programmed cell death in modern biology [39].

Therefore, delayed death of bone cells responsible for bone tissue regeneration occurred within two weeks after extreme exposure.

The morphometric analysis of the state of osteocyte lacunae within the compact bone tissue 14 days post-exposure found that their desolation under the periosteum (Fig. 6*, j*; arrows) increased by 43% compared to that in the contralateral femur (Fisher's exact test; $p < 0.001$; Fig. 3*, b*). Close to the endosteum, the desolation of lacunae following thermal ablation (Fig. 6, *h;* arrows) showed similar values (a decrease by 44% from the intact control, Fisher's exact test; $p < 0.001$; Fig. 3*, b*).

Fourteen days after the heating of the right femurs, osteocytes were relatively evenly distributed in the diaphysis of the contralateral bones. They had large, round or spindle-shaped nuclei that stained intensely for nucleic acids (Fig. 6, *g, i*; arrows). In contrast, in the thermal ablation zone, there were numerous empty osteocyte lacunae (Fig. 6, *h, j;* arrows). The few surviving osteocytes in this area showed signs of cell death, such as a decrease in size and nuclear staining (Fig. 6, *h, j*).

The quantitative assessment of the morphofunctional state of morphologically identifiable osteocytes in the local heating zone of the diaphysis revealed a decrease in the optical density of nuclear DNA staining both under the periosteum and under the endosteum. Under the periosteum, optical density decreased to 77% (Brunner – Munzel test; $p < 0.001$; Fig. 4, *e*) and under the endosteum, it decreased to 85% of the corresponding control (Welch's *t*-test; $p = 0.0038$; Fig. 4, *n*). In addition, under the endosteum, the area of nuclei in mature bone cells decreased to 86% of the control (Brunner – Munzel test; $p < 0.001$; Fig. 4, *k*).

Comparative Analysis of the State of Bone Tissue Immediately (0 days) and 14 days after Local Thermal Ablation

Since non-inbred rabbits were tested in the study groups and their initial bone conditions were significantly different, the dynamics of osteocyte changes in the femurs were compared immediately (0 days) and 14 days after thermal ablation in relative units (% of the corresponding value of the intact contralateral femur). This allowed to a certain extent to neutralize the influence of different initial (before exposure) conditions, as well as indirect cell death (for example, due to intoxication with tissue detritus products) in bone tissue in groups of non-inbred animals.

The results showed (Fig. 3, *c*) that the proportion of empty lacunae with dead osteocytes significantly increased by day 14 from 32 to 59% under the endosteum (Fisher's exact test; $p < 0.001$) and from 39 to 59% under the periosteum (Fisher's exact test; $p = 0.0043$). In other words, there was delayed (indirect) damage to the tissue structures of the compact bone that were not destroyed during high-temperature heating. Moreover, the morphofunctional signs of osteocyte death increased after the cessation of hyperthermic exposure, particularly near the endosteum, even though the intramedullary temperature of thermal ablation was approximately 5 °C lower than in the periosteum area (under the heater cuff). Thus, under the endosteum by day 14 of observation, in comparison with day 0, there was a decrease in both the area (Welch's *t*-test; $p < 0.001$; Fig. 4, *l*) and the intensity of staining for nucleic acids (Welch's *t*-test; $p = 0.0019$; Fig. 4, *o*) of osteocyte nuclei, as well as an increase in their roundness (Welch's *t*-test; $p = 0.0054$; Fig. 4, *r*). At the same time, there was a decrease in the staining (optical density) of nuclei for nucleic acids under the periosteum (Brunner –Munzel test; $p < 0.001$; Fig. 4, *f*).

Thus, after thermal ablation of the femoral diaphyses of the rabbits at 55–60 °C, increasing death of osteocytes during the first 14 days after extreme exposure was noted. In turn, when the histological sections of the diaphysis were stained by the Einarson method, the osteoblasts showed a pronounced decrease in optical density both in the cytoplasm and in the nucleus on day 14 compared to day 0. The decrease was by 2.5 times for the cytoplasm (Brunner – Munzel test; $p < 0.001$; Fig. 5, *f*) and by 3.4 times for the nucleus (Welch's *t*-test; $p < 0.001$; Fig. 5, *l*). Apparently, functional disorders of the synthetic processes in the dividing cells of bone tissue also increased after the end of the thermal effect.

DISCUSSION

According to [40], the sensitivity of normal and tumor cells to hyperthermia is approximately the same. At the same time, other authors suggest that tumor tissues may have lower tolerance to high-temperature exposure. This is due not only to direct temperature damage, but also to a combination of secondary effects, such as stress-modulating effects on the

immune system [41–43] and increased permeability of a well-developed vascular network. Consequently, both direct and indirect effects of heating make tumors vulnerable to drugs and immune cells [44–48].

To date, there is limited information available about the effects of high temperature on the structural and functional state of healthy stem cells. Specifically, culturing MSCs with intervals of hyperthermia at low temperatures (up to 43 °C) has been shown to have a positive effect on cell viability. In such cultures, MSCs are characterized by increased proliferation and resistance to stress factors, as well as a slower rate of cellular aging [49]. Additionally, these cells are able to actively differentiate into osteoblasts and adipocytes [49].

The effect of higher temperatures has been described in the literature fragmentarily. With short-term (45 seconds) exposure to a temperature of 58 °C, irreversible changes occur in the cell membrane, leading to the massive death of MSCs. However, the paradox exists in that a longer (150 seconds) exposure to 48 °C no longer has a significant effect on MSC metabolism and viability [50]. In clinical practice, osteosarcoma thermal ablation is performed by heating for longer period of time (up to 2.5 hours). Given this and ambiguous literature data, we conducted an experimental study of the direct effect of an increase in ambient temperature (up to 60 minutes exposure) on MSC survival *in vitro*.

The results of the study showed (Fig. 1) that exposure to exogenous thermal ablation at 56 °C for even 15 minutes caused morphological changes in MSCs, including loss of pseudopodia and cell rounding, which are signs of apoptosis and / or necrosis. The MTT assay also revealed intracellular metabolic changes associated with decreased cell viability. Further increases in the thermal ablation time led to complete death of cells in the culture *in vitro* (Fig. 1). These findings suggest that MSCs may be targeted in the process of bone tissue heating and their loss has significant implications for post-traumatic regenerative dynamics.

The damaging effects on bone tissue occur not only during therapeutic thermal ablation of primary nodes and metastases of malignant tumors. During osteosynthesis and endoprosthetic replacement of large joints, the bone is exposed to high temperatures caused by friction from surgical instruments, such as automatic drills and saws [51, 52].

It is believed that bone tissue can maintain its morphofunctional and biomechanical properties up to 60 °C [8, 9]. A subsequent increase in temperature leads to denaturation of proteins involved in the formation of the intercellular bone matrix [53–55], which may result in disruption of bone integrity. However, damage to bone cells has also been reported at lower temperatures, such as 48 °C [56], where culturing osteoblasts leads to irreversible changes in the cytoskeleton with subsequent activation of apoptosis through p53 and JNK signaling pathways. Lower temperatures, such as 42–45°C, not only cause reversible changes in the structure of actin filaments, but also a significant increase in heat shock protein 70 (Hsp70) levels, which is responsible for cell protection [56].

On the contrary, it has been established *in vivo* that hyperthermia in the range of 43–46 °C promotes osteogenesis [5] and mineralization of bone tissue [6]; The maximum effect occurs 2 weeks after exposure to heat stress [7]. At the same time, to date there is practically no data available on the state of bone cells after local thermal exposure at temperatures above 55 °C.

In this study, the object of *in vivo* research was rabbits, which are often used to investigate the state of bone tissue and joints during injuries and therapeutic manipulations [57]. It should be emphasized that in the work [60], significant variations in the morphometric indicators of the femurs of rabbits that did not undergo direct thermal ablation were revealed. In the study [58], significant variability of bone tissue markers was also revealed in humans. Therefore, when conducting statistical analysis and comparing the outcomes of local thermal ablation, we adhered to the principles of personalized biomedicine. For each animal, morphometric indices were normalized relative to the corresponding values in the contralateral femur that did not experience direct local thermal ablation, as we previously described [10].

Osteocytes are a conservative cell population that does not have the ability to self-renew (mitotic division) [59]. They are formed from osteoblast precursor cells [59]. Despite their low level of synthetic activity, they play a significant role in bone metabolism by actively participating in the regulation of bone matrix renewal through various mechanosensory mechanisms [60]. Osteoblasts are another type of cell with high synthetic activity. They are descendants of MSCs and are located mainly in the endosteum and periosteum [61]. Osteoblasts play a crucial role in the synthesis of the extracellular bone matrix [61].

The obtained results revealed morphofunctional changes (Fig. 3–5) in the zone of local thermal ablation

of the femoral diaphysis immediately after heating (55–60 °C in the medullary canal, 60–65 °C under the cuff on the periosteum side), with standard staining of sections with hematoxylin and eosin or according to the Einarson method. These changes were more pronounced in osteoblasts (Fig. 5), which can be interpreted as cell death by apoptosis, accompanied by a decrease in cell size due to nuclear compaction and fragmentation of nuclei, loss of cellular integrity through separation of apoptotic bodies [39], and a decrease in nucleic acid synthesis.

At the same time, during the period of bone tissue restoration (14 days after thermal exposure), the synthetic processes in bone cells continued to decrease. This is evident from a further decrease in the optical density of the nuclei and cytoplasm of cells compared to the values immediately after heating (Fig. 3, *c*, 4, *f, l, o, r*, 5, *f, l*). From the point of view of morphological changes in bone cells, identifying various forms of cell death (apoptosis, autophagy, and types of necrosis) can be challenging and is constantly evolving [62]. Nevertheless, the above-mentioned signs of morphofunctional changes in cells can be classified as apoptosis to a greater extent. In turn, the increasing desolation of bone lacunae during the 14-day observation period (Fig. 3, *c*) as a sign of osteocyte cytolysis is consistent with a necrotic phenotype [39].

Thus, thermal ablation of the femoral diaphysis of rabbits at an intramedullary temperature of 55–60 °C, in addition to the direct damaging effect "under the beam", has a long-term trace effect, manifested by morphological and functional signs of death of osteoblasts and osteocytes (primarily in the endosteum). Consequently, the expected post-stress recovery of bone tissue from the endosteum, despite a lower heating temperature, should hypothetically be slower compared to the periosteum. In fact, in the previous study [10], we observed early regeneration of bone tissue primarily in the periosteum, starting from the area of the proximal metaphysis of the femur that was not subjected to thermal ablation.

During thermal ablation of rabbit femoral diaphysis at temperatures between 55 and 60 °C, we did not observe any histological signs of femoral fracture in the early postoperative period. However, progressive 14-day bone cell death was observed, which may have implications for the long-term success of surgical osteosynthesis and endoprosthetic procedures [63].

Thus, *in vitro* experiments have shown that MSCs have high thermal sensitivity to a temperature of 56 °C already after the first 15 minutes of direct heating. Under *in vivo* conditions, bone tissue appears to shield the direct damaging effects of 30-minute thermal ablation (55–60 °C in the medullary canal, 60–65 °C under the cuff on the periosteum side) on osteocytes and osteoblasts. This protective effect may be important at short periods of bone heating, such as when using electric drills and/or saws during orthopedic and traumatological manipulations [64]. Nevertheless, 30 minutes of thermal ablation is sufficient to initiate the mechanisms of delayed cell death.

In line with the concept of osteoimmunology, delayed processes of bone cell death can be mediated by proinflammatory macrophages, as was described for chondrocytes [65]. In particular, pyroptosis is characterized by the release of a large number of intracellular peptide fragments into the extracellular environment, which can interfere with cellular adhesion [66, 67], which in turn leads to apoptosis of intact osteoblasts [68].

Under conditions of thermal damage to MSCs, a "vicious circle" of death and impaired bone regeneration occurs, which can lead to a prolonged period of restoration of bone cell populations in the diaphysis of femurs subjected to local thermal ablation at an intramedullary temperature of 55–60 °C. As a consequence, the processes of bone remodeling will be largely determined by the intensity of osteoconductivity from undamaged areas, such as the metaphysis, as we have previously noted [10].

CONCLUSION

With exothermic exposure, after only 15 minutes, a decrease in cell size, a change in the state of the cytoplasm, loss of pseudopodia and rounding were observed in the culture of human MSCs, suggesting that cell death by anoikis, a special type of apoptosis, had occurred. After 30 minutes of heating, fibroblast-like cells were practically not found.

In vivo, immediately after a single 30-minute local heating of the femoral diaphysis in rabbits, a significant increase in the number of empty osteocyte lacunae was observed in the compact bone under the periosteum compared to the contralateral femur. However, no significant change was seen in the endosteum. A higher heating temperature of the periosteum (~ 5 °C) resulted in a higher number of cell deaths (by 8%) compared to the endosteum. Additionally, there was a decrease in osteoblast area and concentration of nucleic acids (synthetic activity) in the cytoplasm, along with a decrease in nuclear area and an increase in

nuclear optical density, all of which can be interpreted as morphological signs of apoptosis.

Interestingly, 14 days after local thermal ablation of the femoral diaphysis of rabbits, signs of delayed damage to bone lacunae increased. Morphometric indices of bone cell death (osteoblasts and osteocytes) also increased significantly. Osteocytes died primarily in the endosteum and not the periosteum, as observed immediately after direct hyperthermic exposure.

Thus, even low levels of thermal ablation in the temperature range of 55–65 °C have a significant damaging potential for bone cells, from stem cells to mature cells, which increases over the course of 14 days of observation. These results should be considered when planning the treatment of benign and malignant tumors and metastases in bone tissue, as the individual response of healthy and pathologically altered cells to heat largely determines the success of therapeutic interventions and repair processes from the perspective of personalized medicine.

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The Role of a Medical Psychologist in Maintaining the Rehabilitation Potential of Patients after a Stroke at the Second Stage of Medical Rehabilitation

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ABSTRACT

Stroke can lead to motor, cognitive, and speech disorders that require long-term correction. Rehabilitation potential (RP) indicates the level of maximum possible recovery of a patient in the given time period. Maintaining RP at the second stage of medical rehabilitation (MR) is one of the important tasks of a medical psychologist. However, current Russian scientific literature has not sufficiently disclosed the scope and theoretical foundation of such work. The authors of this lecture analyzed the concept of RP from the theoretical and methodological perspectives of Russian medical (clinical) psychology. In addition, they investigated current studies on the relationship between psychological factors and neuroplasticity mechanisms. Scientific data allow to consider RP as a complex and dynamically changing system formed due to various effects, including psychological ones. The theoretical and methodological tradition of Russian medical (clinical) psychology gives grounds to regard illness perception (IP) as a psychological component of the RP, which is 1) social in origin, 2) mediated in structure, 3) subject to voluntary control. Within the second stage of MR of patients after stroke, formation and improvement of IP to maintain the RP require joint efforts of a neuropsychologist and a pathopsychologist. Modern studies on the relationship between psychological factors and neuroplasticity mechanisms provide scientific grounds for the methods of work of a medical psychologist in medical rehabilitation.

Keywords: medical rehabilitation; rehabilitation potential; stroke; medical psychology; illness perception; activity theory; consciousness; higher mental functions

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Роль медицинского психолога в поддержании реабилитационного потенциала пациентов с острым нарушением мозгового кровообращения на втором этапе медицинской реабилитации

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РЕЗЮМЕ

Острое нарушение мозгового кровообращения (ОНМК) может приводить к двигательным, когнитивным и речевым нарушениям, которые требуют продолжительной коррекционной работы. Реабилитационный потенциал (РП) является показателем уровня максимально возможного восстановления пациента в намеченный отрезок времени. Поддержание РП на втором этапе медицинской реабилитации (МР) – одна из важных задач работы медицинского психолога. Однако ее содержание и теоретический фундамент недостаточно раскрыты в современной отечественной научной литературе.

Авторы настоящей лекции проанализировали концепцию реабилитационного потенциала с теоретико-методологических позиций отечественной медицинской (клинической) психологии. Были изучены актуальные исследования связи психологических факторов с механизмами нейропластичности. Научные сведения позволяют заключить, что РП является сложной и динамически изменяющейся системой, которая формируется в результате действия различных влияний, в том числе психологических. Теоретико-методологическая традиция отечественной медицинской (клинической) психологии дает основания рассматривать внутреннюю картину болезни (ВКБ) как психологический компонент РП, который 1) социален по происхождению, 2) опосредован по строению, 3) доступен произвольному контролю.

В рамках второго этапа МР пациентов с ОНМК работа над формированием и повышением качества ВКБ для поддержания РП требует совместных усилий нейропсихолога и патопсихолога. Современные исследования связи психологических факторов с механизмами нейропластичности предлагают естественно-научные основания методам работы медицинского психолога в рамках медицинской реабилитации.

Ключевые слова: медицинская реабилитация, реабилитационный потенциал, острое нарушение мозгового кровообращения, медицинская психология, внутренняя картина болезни, теория деятельности, сознание, высшие психические функции

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Авторы заявляют об отсутствии финансирования при проведении исследования.

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INTRODUCTION

Progress in the state system of medical rehabilitation (MR) actualizes the concept of rehabilitation potential (RP) [1]. RP is the maximum possible level of recovery[1] of a patient within the given time period (Order of the Ministry of Health are of the Russian Federation No. 788n dated July 31, 2020). One of the primary targets of RP research is personalization of medical services [2]. Explicit RP encourages a multi-professional rehabilitation team (MPRT), a patient, and patient's social environment to expect a definitive rehabilitation outcome. RP prospect reflects the expectations of the MR participants and affects their mindset and motivation. All these make it possible to consider the significance of the psychological aspect of the RP. It is of special importance for the second stage of MR of patients who experienced stroke [3]. Being the key reason for the working population disability, stroke results in movement disturbances (with about 80% in the acute phase and around 40% in the first 6 months), speech (28–40%), cognitive (up to 72%), and other disorders [4–6]. Therefore, special efforts are required to overcome these difficulties, which often leads to a shift in the patient's way of living [7]. In this respect, analyzing the ways by which a medical psychologist can support the RP of patients after stroke at the second stage of MR is relevant.

The aim of this lecture was to study the concept of RP from the theoretical and methodological standpoints of Russian medical (clinical) psychology as well as to review relevant studies on the association between psychological factors and mechanisms of neuroplasticity.

REHABILITATION POTENTIAL: COMPLEXITY, MULTI-FACTOR AND SYSTEMATIC NATURE OF THE CONCEPT

The term rehabilitation potential was coined in the work of Herbert S. Whiting "Classification of Rehabilitation Potential" (1950) [8]. Developing ideas of Howard Rusk, the founder of American rehabilitation medicine, H.S.Whiting proposes a RP classification consisting of three components: 1) physical rehabilitation potential, 2) potential at home, and 3) professional potential. For the first component, the author introduces a graduated approach including five hierarchical levels [8].

With further MR development, the understanding of the RP has changed [9]. Two main driving factors for this transformation can be outlined from the analysis of the current literature. Firstly, the progress of neuroscience contributes to RP concept rethinking [10]. As the understanding of the mechanisms of recovery from dysfunction deepens, it increases the efficiency of the related methods of rehabilitation [11]. The morphological and functional basis of mental processes is being elucidated as well as their influence on somatic changes [12, 13]. This contributes to gradual abandonment of hyper-specific skill training in MR. At the same time, approaches based on patient systemic involvement in the recovery process (sensory feedback (SFB) and biological feedback (BFB), virtual and augmented reality (VR, AR), etc.) become more important [14]. Due to that we need to consider a constantly growing number of factors when determining the RP. We are starting to appreciate the complex structure and dynamic nature of the RP, as well as its dependence on environmental factors and patient's personal characteristics [9].

Secondly, broader application of personalized medicine [15] drives transformation of the goal behind determining the RP. While in the mid-20th century, its main task was to understand whether a patient should be sent to a rehabilitation facility, today such an approach is subject to criticism. [2]. A client-centered approach requires assessing the RP to determine which of the available rehabilitation services will provide the greatest benefit to the patient in their life circumstances.

Following the analysis of the literature on the RP from foreign scientific literature databases (Medline, CINAHL and Embase), Priscilla Lam Wai Shun and her colleagues (2022) outlined a scheme of the RP for acquired brain injury (Fig. 1) [9]. [На рисунке: в овале изменить Interpretating g на Interpreting; в самом нижнем правом прямоугольнике заменить Socio-economical на Socio-economic!!]

This model suggests that the RP is not just a property of a particular patient. On the contrary, it is rather a reflection of the environment and peculiarities of rehabilitation specialists. The experience, competences as well as personal traits of clinicians influence the way they perceive a patient and the forecast they give in every particular case. The state of the health care system, social stereotypes and mindset related to certain diagnoses, cultural peculiarities of

[1] return to previous professional or other labor activities, maintaining the ability to carry out daily activities, and restoring the ability to self-care.

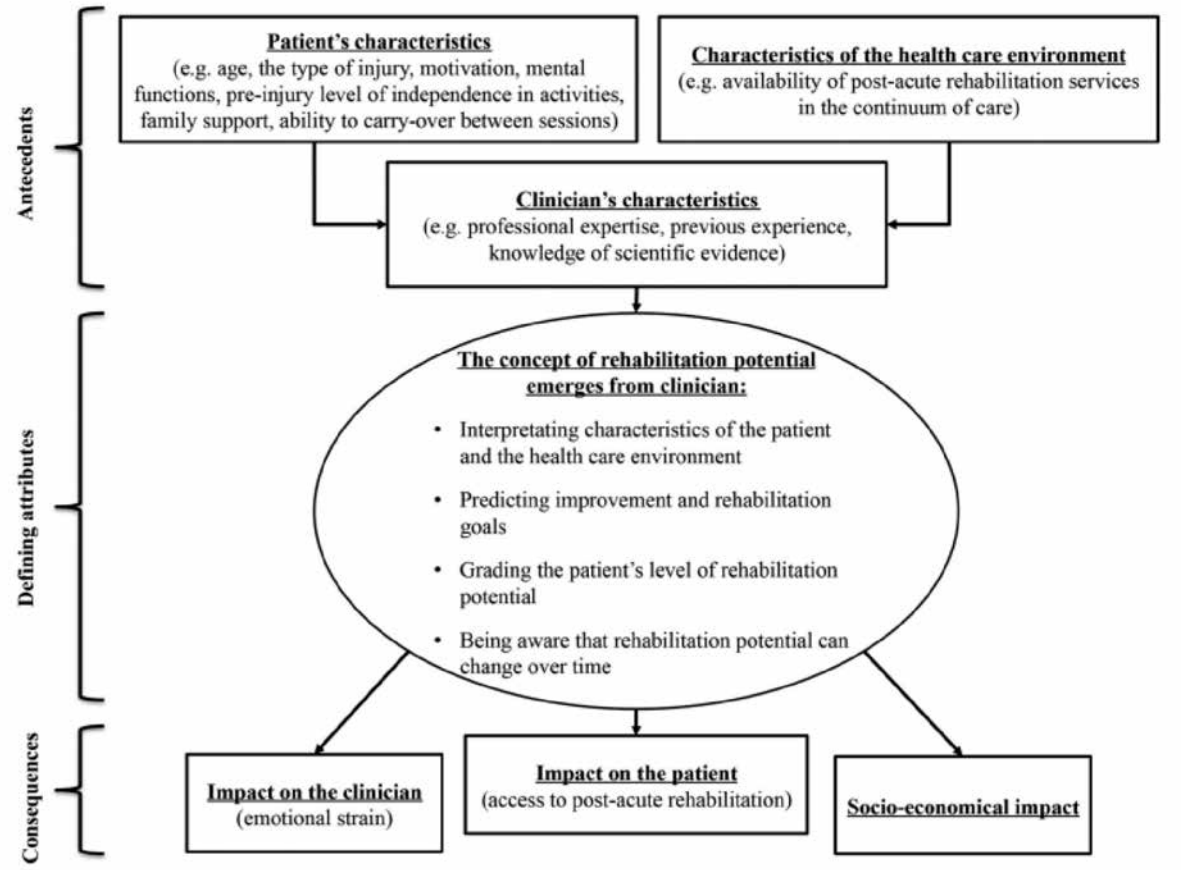

Fig. 1. Study of the RP, according to P. Lam Wai Shun et al. (2022) [9]

society, and other environmental factors determine rehabilitation options that in turn can also dramatically increase or decrease the patient's RP.

Thus, studies of RP allow us to consider this concept in terms of complex dynamic systems. This is in excellent agreement with the view of modern sciences on the subject in contemporary Russian methodology [16]. In particular, it is in line with ideas of complex self-developing systems as the cognitive object of postnonclassical type of rationality [17]. Unlike the usual object of medical and natural sciences, a patient's RP is inaccessible for visual objective examination. It cannot be studied with the use of classical empiric methods. The dictionary definition of the term "*potential*" attributes it to the category of the "*possible*" in contrast to the category of the "present" [9]. The RP is a complex cognitive and verbal construction created by MPRT members based on the incoming information. Similarly to the objects studied by synergetics and quantum physics, it is characterized by the stochastic nature and a spectrum of possible ways of further development.

All such properties allow to link the studies of RP to the methodology of Russian clinical psychology for which aforementioned conceptual grounds are inherent [18]. Memory, thinking, imagination, representation, etc. – the subjects matter of psychological examination – always include the components of subjective reality inaccessible for classical objective research [19]. In order to make them empirically visible, psychology developed the principles of research design that include the combination of qualitative and quantitative methods [20, 21]. From the point of view of this conceptual approach, the goal of rehabilitation is to "create psychologically justified conditions for the potentiation of active self-sustained personal development under newly emerged illness-restricted conditions" [19, page 8]. Using the term "*potentiation"* is illustrative in this context. It places special emphasis on the fact that a systemic psychological approach suggests not a static definition of the RP but rather the process of its continuous search in joint activity [7], creating conditions for its revealing and maintenance. A medical psychologist as a specialist aimed at the study and description of subjective phenomena monitors a complex system of psychic processes capable of restricting available physical abilities and uncritically ignoring present

issues. By establishing a contact and communication with a patient, a medical psychologist seeks to provide the conditions for the fullest possible understanding of: a) the emergence and flow of thoughts, motives, and emotions at any given moment in time; b) the value of personal feelings and individual experience; c) genuine existential needs; d) objective opportunities for growth in the present situation. Therefore, thanks to realization of the reflection potentials [22], communication with a medical psychologist is aimed at the formation, maintenance, and strengthening of the patient's RP. Modern studies of brain and psychic describe the mechanisms providing the material basis for these processes [23].

ILLNESS PERCEPTION AS A PSYCHOLOGICAL COMPONENT OF THE REHABILITATION POTENTIAL

One of the first foreign researchers who drew attention to the psychological component of the RP was S.Z. Nagi [24], the author of the social disability model. In his publication "A study in the evaluation of disability and rehabilitation potential: concepts, methods, and procedures" (1964), he described the psychological study of the RP along with medical, ergotherapeutic, and professional studies. According to S.Z. Nagi, the psychologcal evaluation of the RP must be carried out with the use of a clinical interview and psychometric testing. Together they elucidate the psychologcal traits of a patient and his/her view of the nature and repercussions of the disease [24]. Thus, the key psychological component of the RP was something often referred to as illness perception (IP) in modern psychological studies (Fig.2).

Discussing the approaches utilized by a medical psychologist, S.Z. Nagi places emphasis on the use of standard tests (Wechsler Adult Intelligence Scale (WAIS), Minnesota Multiphasic Personality Inventory (MMPI), etc.). Such a view of psychological diagnostics is characteristic of the Western tradition focused on the quantitative approach [25]. However, unlike foreign researchers, since its early stages of formation, Russian psychology has been inclined to the so-called activity paradigm [26]. Its main feature consists in assigning greater value to the qualitative observation of execution of various tasks rather than quantitative evaluation of the results. In this paradigm, the psyche is perceived as a complex dynamic system that exists and develops in close connection with the instrumental activity of a person in the environment [27] (Fig. 3).

Study of a particular person's psyche requires a qualitative description of their activity unfolding in a real-world situation. This allows a medical psychologist (Fig. 4) to determine: a) to which representations a subject is referring in problem-solving, b) how they are interconnected, and c) what drives and guides these connections.

According to the activity approach and cultural – historical concept of higher mental functions (HMF), IP is treated by the Russian psychological school as the result of the process of *nosognosia*, i.e. lifetime, culturally determined development of the person's understanding of the disease during their lifetime [29]. The description of the semiotic model of IP is based on such components of consciousness as sensible tissue, meaning, and personal sense (Fig. 5).

Thus, the Russian model allows to analyze the emergence and development of IP in patients after stroke during the second stage of MR more precisely.

1. *Sensible tissue.* Depending on the volume and localization of a lesion, stroke can lead to disturbance in multidimensional sensual composition of particular representations of reality [30]. Furthermore, the initial stunning impact of stroke provokes the feeling of extreme insecurity [31]. Patients feel loss of control over their body that gives rise to intense frustration. Thus, neuropsychological work on activation of sensory representations and regaining control in the flow of the perceived information of various modalities is one of the components aimed at the improvement of IP. However, for better support of the RP during the second stage of MR, psychological interventions aimed at emotion and affect regulation are just as important [32, 33].

2. *Meaning.* The vocabulary of available means for sensory experience provides its "initial designation" [29]. It guides the flow of associations, gradually building up a narrative that allows one to interpret one's experience in this way or another. Patients suffering from various forms of aphasia due to stroke experience particular difficulties in this respect. In order to create the IP that maintains the RP within MR, they first need neuropsychological and speech therapy so as to restore disordered syntagmatic and paradigmatic associations of verbal processes [34]. However, with the speech function retained, the meaning system can also require professional attention. At the second stage of MR of patients after stroke, a medical psychologist can deal with a mixed differentiation of the vocabulary describing mental processes ("Am I not that crazy yet?", "I have

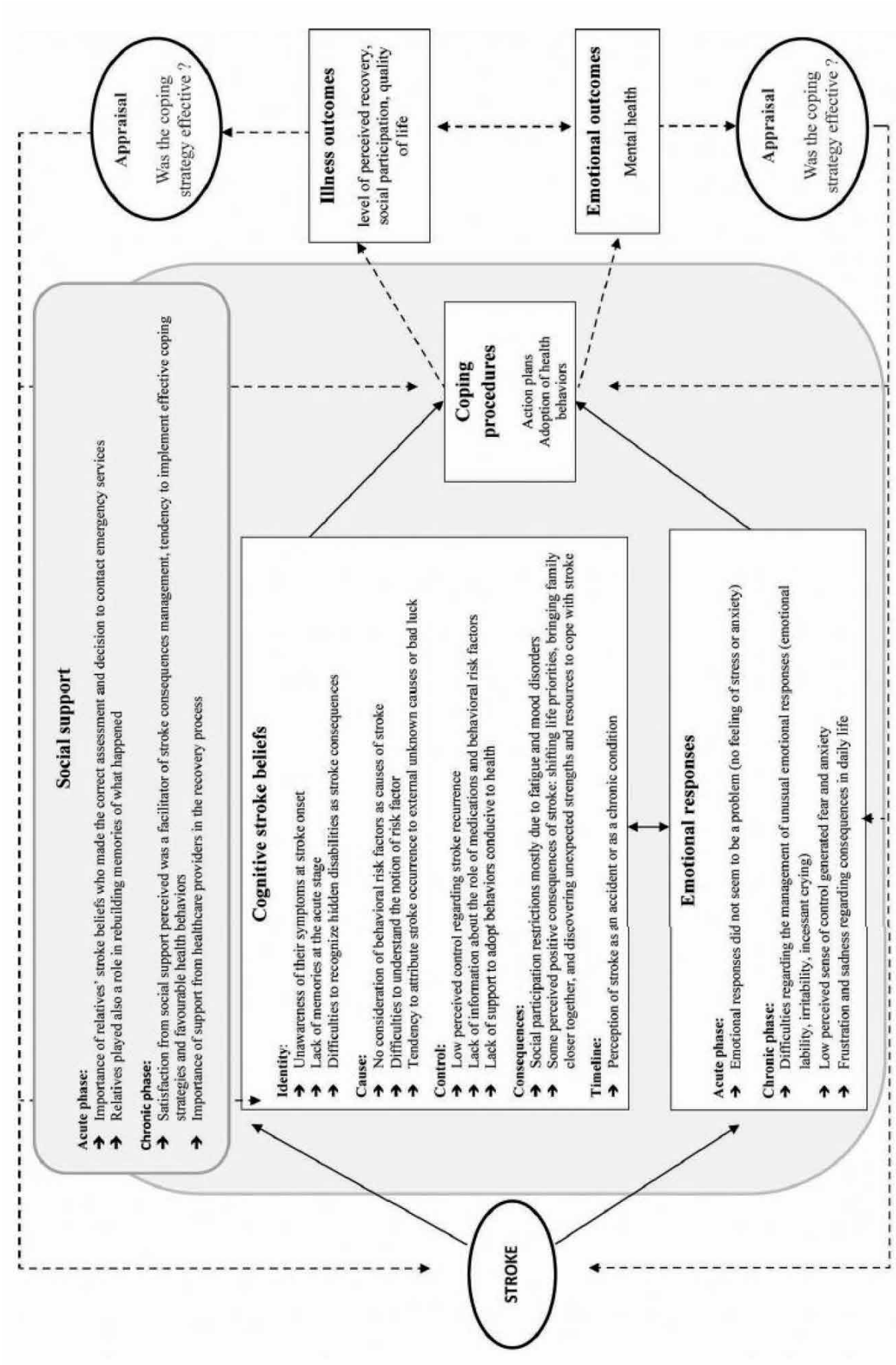

Fig. 2. Common sense-based model of self-regulation for stroke, according to C. Della Vecchia et al. (2019) [28]

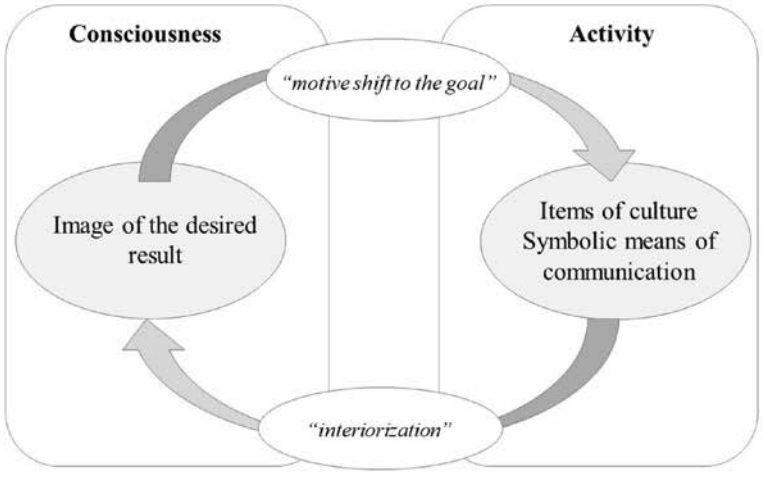

Fig. 3. Dialectical unity of consciouness and activity [27]

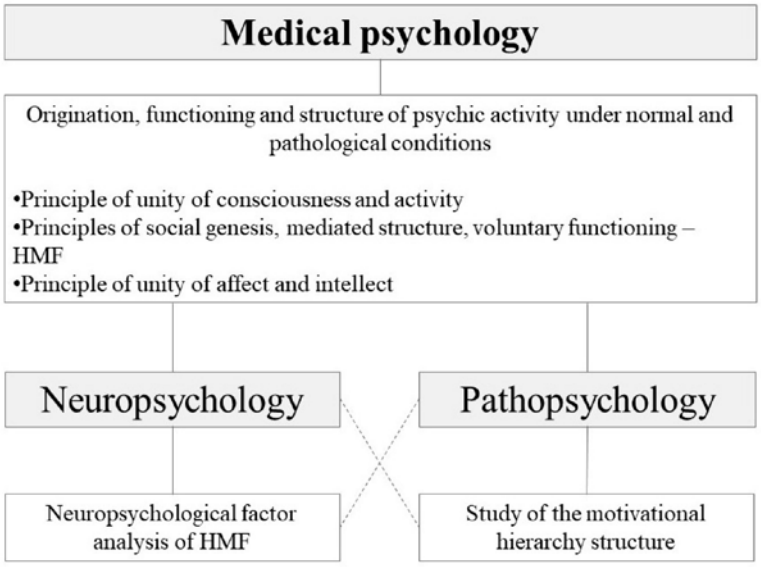

Fig. 4. Relationship between neuro- and pathopsychology within the second stage of MR of patients after stroke

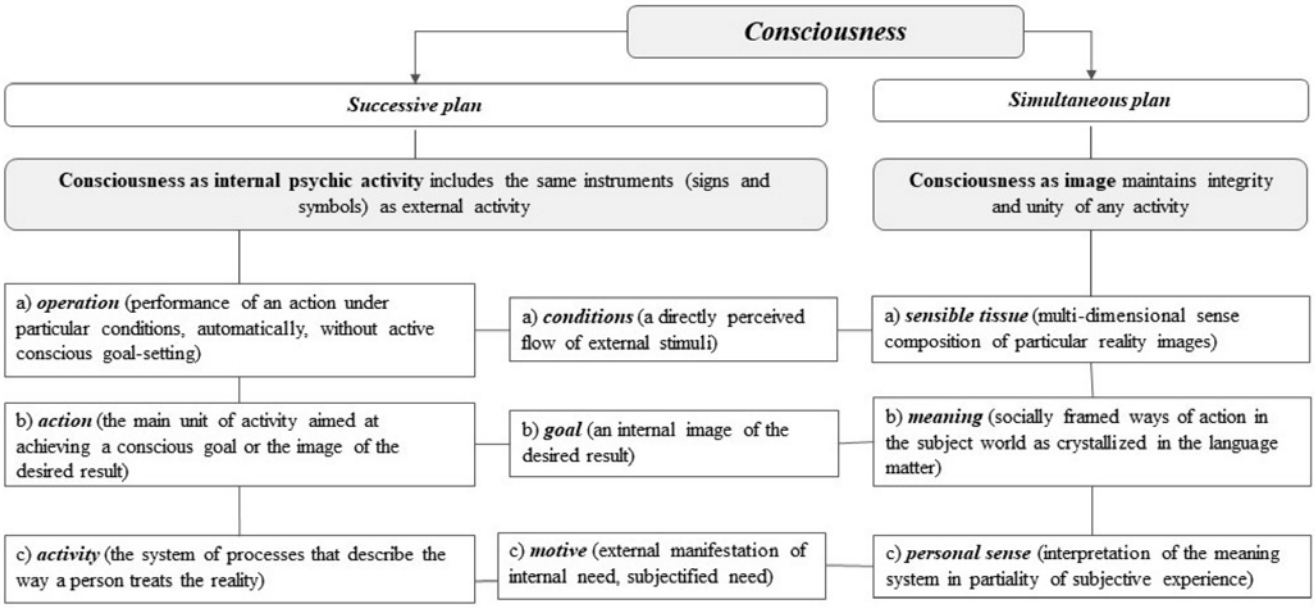

Fig. 5. The two plans of consciousness analysis according to the activity theory by A.N. Leontev [26, 27]

become a dummy", "My head does not work", etc.). Such general clichés result in negative emotional experiences, self-assessment, and anxiety regarding one's own mental status that adversely affect the RP. An interview with 57 adults in the early recovery period of stroke detected the presence of subjective cognitive complaints that were not associated with psychometrical assessment of cognitive processes [35]. The regression analysis of data has shown that subjective complaints served as the most important predictor of patient's involvement in social activity. Therefore, apart from neuropsychological work with speech, psycho-educational interventions aimed at enrichment of the vocabulary describing psychic functions and states are needed in order to support the RP.

A feeling or experience described with a certain term undergoes the process of the "secondary designation" fitting into a discursive net of myths around a disease that exist in the information space surrounding a person. The narrative to which a subjective perception of the disease refers guides their further mental activity. It determines the direction of an associative sign – symbolic process. Here stroke serves as one of the well-articulated pathologies extensively discussed in the modern media. Being a leading cause of disability, stroke is rightfully considered to be highly socially sensitive. As the world population grows, the incidence of stroke is continuously increasing as well (up to 12 million per year, according to the WHO in 2025) [3]. This contributes to the spread of myths, concerns, and fears in the information space around the topic of stroke that can have a negative effect, being actualized and emotively loaded when a person faces stroke [28] (Fig. 2). Instead, objective knowledge has a positive effect on the recovery process. A longitudinal study of 60 patients after stroke undergoing MR showed a relationship between qualitative knowledge and well-defined expectations from rehabilitation with more successful results at the time of discharge [36]. Thus, the work of a medical psychologist with a patient's IP is essential in the RP maintenance. Therefore, neuropsychologists deal with the recovery of general operational basics of semantic memory, speech, and executive functions. In the meantime, a pathopsychologist focuses on the conceptual part of the patient's perception of stroke and helps get rid of irrational and dysfunctional beliefs by using different psychotherapy methods.

3. *Personal sense.* The discursive structure developed from socially framed language means and meanings acquires a personal sense when subjected to psychic activity of an involved biased individual. A phenomenological study consisting of 73 interviews conducted with six patients in the late recovery period from stroke demonstrated that the participants tended to focus on the social side of their symptoms rather than on the state of a disturbed function itself [31]. As a rule, the patients interpreted a physical deficiency as a complete loss in an important area of live (for example, incapability of going to a pub with friends). Within this social side of their problems, such key topics as change of roles and isolation surfaced as well [31]. An ability to pursue the deep meaning of the disease is associated with peculiarities of motivational hierarchy and other personal traits of a patient. An example of a successful solution to the "meaning problem" [27] was provided by neurobiologist Jill Bolte Taylor [37]. In her autobiographic book "My Stroke of Insight: A Brain Scientist's Personal Journey", she described her personal experience of recovery from stroke as something that allowed her to gain new knowledge of the human nature and capabilities, to broaden the understanding of the subject matter of brain science and led to the insight and discovery of the internal source of harmony and peace of mind. Creating the conditions for such transformation of the personal sense of a disease is an important task of a medical psychologist within the second stage of MR of patients after stroke, since it is the deep personal sense that motivates a person to overcome difficulties and limitations [38, 39]. In this context, neuropsychological interventions are necessary for recovery of such HMFs as operational basis of reflection (namely, functions of programming, regulation and control, speech, memory, etc.). A pathopsychological component of rehabilitation consists in the creation of an environment encouraging reflection of subjective experience by a person relying on HMFs.

It is noteworthy that today the activity approach to consciousness and the cultural – historical concept of HMF are becoming increasingly important for contemporary neuroscience [40]. Theoretical and methodological concepts of Russian scientists get larger empirical support in present-day objective experimental studies of cognitive and affective processes [41–43]. In neurorehabilitation practice, this means relying on the International Classification of Functioning, *Disability, and Health* (ICF). The structure of the ICF seeks to describe the patient's condition in a complex, multi-factor fashion considering the influence of personal

and environmental factors. Defining the domains of activity and participation (d) along with body functions (b) makes it possible to draw some comparisons between the ICF concept and the ideas of the levels described in the activity theory by A.N. Leontev (Fig. 5) [44]. In turn, differentiation between realization and capacity as the two components of the assessment of activity and participation in the ICF corresponds to the ideas of the "zone of proximal development" by L.S. Vygotsky [45].

MECHANISMS OF INFLUENCE OF ILLNESS PERCEPTION ON THE REHABILITATION POTENTIAL OF PATIENTS AFTER STROKE AT THE SECOND STAGE OF MEDICAL REHABILITATION

In fundamental neuroscience, recent decades have brought a large number of studies on the interrelation between depressive patterns of thinking, emotions, and behavior and changes in the mechanisms of brain plasticity [46]. In the article published by Nature Publishing Group, Rebecca Price and Ronald Duman presented an integrative concept of neuroplasticity that summarizes the results of experiments on animals with clinical materials and studies involving healthy participants (Table 1). This model describes neuroplasticity as a multidomain neurobiological, psychological, and social construct [46]. Depression, low cognitive flexibility, and stereotype repertoire of behavior were associated there with malfunction of plasticity mechanisms in the nervous system disturbing normal energy processes and comprehensive integration of information they encode [47, 48]. On the other hand, high motivation, flexible thinking, and diverse behavior were associated with higher neuroplasticity [13].

The review of studies of the impact of neuroplasticity on stroke recovery published between 2000 and 2023 in such resources as PubMed, Embase, and the Cochrane Library confirmed its substantial prospects for the rehabilitation of patients after stroke [49] (Table 2). Despite that, according to the authors of the article, there are substantial individual differences in neuroplasticity both due to personal experience and to general sociocultural aspects.

Table 1

Neuroplasticity Markers across Levels of Analysis, according to R. B. Price and R. Duman (2020) [46]

Level of analysis	Dysfunctional state	Treatment goal state
Molecular/cellular	↓ Synaptic number and function, neuronal atrophy	↑ Synaptogenesis, ↑ neurotrophic factors
Neural network	↓ PFC – limbic circuit connectivity	↑ PFC – limbic connectivity and limbic system, ↑ regulatory control over limbic regions
Cognitive function	↓ Flexibility, ↓ cognitive control, ↓ goal-directed inhibition / excitation of lower-order functions	↑ Flexibility and cognitive control, ↑ goal-directed inhibition/excitation capacity
Affective information processing	Rigid negative biases in implicit information processing (e.g., attention, memory, interpretations, self-representations)	Unbiased and flexible information processing
Clinical/self-report	Perseverative negative thoughts, repetitive maladaptive behaviors, depression	Novel positive thoughts/perceptions, diversified behavioral repertoire, euthymic mood, improved function/engagement

Table 2

Key Neuroplastic Changes Associated with Stroke Rehabilitation, according to N. Aderinto et al. (2023) [49]

Neuroplastic changes	Description of changes	Neural structures involved
Dendritic remodeling	Structural changes in dendrites, including sprouting and arborization	Affected and unaffected brain regions
Synaptic plasticity	Strengthening or weakening of synapses based on activity and experience	Neurotransmitter systems, cortical and subcortical regions
Cortical reorganization	Changes in cortical maps and functional organization of brain regions	Motor and sensory cortices, association areas
Neurogenesis	Generation of new neurons in specific brain regions	Hippocampus, subventricular zone
Axonal sprouting	Formation of new connections or sprouting of existing axons	Corticospinal tract, other neural pathways

Thus, Nicholas Aderinto and his colleagues justified at the neurophysiological level the necessity of personalized interventions and interdisciplinary cooperation for the full use of the enormous potential of neuroplasticity in stroke recovery [49].

When analyzing the options for the application of knowledge about the mechanisms of neuroplasticity in clinical practice, J. Shaffer emphasizes that psychology enjoys unique opportunities for the maximization of the "revolution aimed to transform diagnostics and treatment of mental diseases and to overcome the absence of significant progress in control of underlying health conditions and mortality for the last 100 years" [50, 51]. Referring to the writings of Marian Diamond, one of the founders of studies on neuroplasticity, J. Shaffer describes the key aspects that ensure brain health maintenance. They include novelty and challenges, physical activity and proper nutrition, love, social acceptance, low stress level, as well as healthy sleep [51]. J. Shaffer states that positive psychology [52] can promote positive emotions, thus strengthening interaction with enriched environment and therefore potentially contribute to intensification of the neuroplasticity in their central nervous system (CNS) [13].

Many modern researchers of psychological factors of brain plasticity pay special attention to positive neurophysiological effects of meditation and mindfulness [53]. Thus Isaac N. Treves and his colleagues analyzed 68 correlation studies utilizing structural MRI, fMRI with tasks, fMRI at rest, and EEG. They presented a convincing body of evidence for the relationship between mindfulness and a) reduced reaction of the amygdaloid body to emotional stimuli, b) increased cortical thickness in frontal and insular areas of neocortex, c) increased differentiation of the brain default mode network. According to many researchers, the positive effects of meditation are as follows: 1) changes in the brain activation patterns on fMRI, 2) changes in visual evoked potentials (which reflects the impact of meditation on attention), 3) changes of amplitude and synchronicity of high-frequency oscillations (probably playing an important role in synchronization of brain large neuronal networks) [54]. It should be noted that from the neuropsychological point of view, meditation and mindfulness practices are effective methods of training executive functions enabled by the activity of the prefrontal cortex [55]. Such practices include inhibition of involuntary reactions to stimuli and prolonged attention focusing on the particular mental state. In terms of pathopsychology, they can be treated as development of a regulatory component of psychic activity and maintenance of the complex hierarchy of motives.

Therefore, IP as a complex system of interrelated subjective perceptions of the disease can be considered as a psychological component of the RP. Based on the properties of its elements, a person formulates their personal view on the prospects of recovery. Emotional processes corresponding to such perception are associated with particular physiological processes that affect the dynamics of neuroplasticity mechanisms in one way or another. Therefore, maintaining the RP during MR requires psychological therapy with IP aimed at strengthening of positive feelings facilitating neuroplasticity and contributing to recovery of CNS malfunctions. At the same time, such IP should refrain from supporting an infantile illusion of the omnipotence of patients [56] but rather provide for their objective acceptance of limitations and good adherence to the MR program.

CONCLUSION

The rehabilitation potential is a complex dynamic system influenced by many different factors, including psychological ones. Just as the action potential of a neuron depends on the integrity of its membrane complex, the rehabilitation potential of a person is determined to a great extent by the functions of their consciousness as the specific boundary between the outer and inner worlds [57]. According to the theoretical and methodological tradition of Russian medical psychology, the IP can be treated as a psychological component of the RP that is 1) social in origin, 2) mediated in structure, 3) accessible to voluntary control. During the second stage of MR of the patients after stroke, work on IP quality improvement for the RP support requires joint efforts of a neuropsychologist and a pathopsychologist. A neuropsychologist is responsible for the recovery of gnostic components of sensible tissue of consciousness and associative connections within the meaning system. A pathopsychologist works on building conditions for the optimization and regulation of affective components of sensible tissue of consciousness and search for the personal sense of the disease for a patient. Modern studies on the relationships between cognitive and emotional processes and neuroplasticity mechanisms provide biological foundations for the methods of work of a medical psychologist within rehabilitation from stroke. They elucidate the brain processes which

can be affected by carefully selected personalized psychological techniques in order to increase intentionality and awareness in the patient.

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Bogatova V.D. – conception and design; analysis and interpretation of the data. Kuznetsova A.M.Y. – analysis and interpretation of the data. Polyaev B.B. – critical revision of the manuscript for important intellectual content. Mikadze Y.V. – critical revision of the manuscript for important intellectual content. Gordeev M.N. – final approval of the manuscript for publication. Suvorov A.Y. – final approval of the manuscript for publication. All authors confirm that the authorship criteria match the ICMJE guidelines (all authors made a substantial contribution to conception, search and analysis of the data, and drafting of the article, as well as read and approved the final version of the manuscript before publication).

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Infantile Hemangioma: Modern Perspectives on Pathogenesis and Treatment

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ABSTRACT

Infantile hemangioma (IH) is a significant and common interdisciplinary issue in the pediatric population. The etiology of this condition is not fully understood. Several hypotheses exist regarding the origin of IH: the placental hypothesis, genetic mutations, and the influence of external factors. Treatment approaches for IH are diverse and include systemic pharmacotherapy, local treatment, and surgical intervention. Each of these methods has both advantages and limitations, making it impossible to use any single approach as a universal solution for all patients. The beta-blocker propranolol is currently recognized as the first-line medication. The authors of the lecture presented a literature review of modern approaches to selecting treatment for IH, as well as predictors of propranolol efficacy and its possible mechanism of action.

Keywords: hemangioma, malformation, children, propranolol

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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Инфантильная гемангиома: современный взгляд на патогенез и лечение

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РЕЗЮМЕ

Инфантильная гемангиома (ИГ) – значимая и распространенная проблема междисциплинарного характера в детской популяции. Этиология данного заболевания до конца не известна. Существует несколько гипотез возникновения ИГ: плацентарная, в результате генетической мутации и воздействия внешних факторов. Подходы к лечению инфантильных гемангиом разнообразны и включают системную фармакотерапию,

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местное и хирургическое лечение. Каждый из них обладает как достоинствами, так и ограничениями, что делает невозможным использование одного из методов в качестве универсального для всех пациентов. Препаратом первой линии в настоящее время признан β-адреноблокатор пропранолол.

Авторы лекции представили литературный обзор современных подходов к выбору лечения инфантильных гемангиом, а также предикторы эффективности применения пропранолола и его возможный механизм действия.

Ключевые слова: гемангиома, мальформация, дети, пропранолол

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источники финансирования. Авторы заявляют об отсутствии источника финансирования при проведении исследования.

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INTRODUCTION

The aim of the lecture was to provide a literature review of data on the pathogenesis and treatment methods of infantile (neonatal) hemangioma (IH), as well as to present the results of current research on potential predictors of pharmacological therapy efficacy.

IH represents a benign vascular tumor formed through proliferation of vascular endothelial cells resulting from impaired local angiogenesis. Among all vascular pathologies, IH is the most prevalent, occurring in 5–10% of children during their first year of life [1]. The condition is more frequently diagnosed in girls, Caucasian children, and twins. It has been observed that IH develops particularly often in newborns with low birth weight and in preterm infants. This makes the pathology a significant concern in pediatric practice [2].

GENERAL INFORMATION

IH has a diverse localization, but in 60% of cases, it occupies the head and neck region [3]. Clinical manifestations usually begin in the first weeks of life. A distinctive feature of IH is the dynamic nature of the tumor. During the first six months of life, active proliferation is observed, during which hemangioma reaches its maximum size. At the age of 8–9 months, a stabilization phase occurs, characterized by slowing of the formation growth, followed by a regression stage, which can last from one to several years [4]. The average age of spontaneous involution cessation is 3.5 years [5]. To determine the treatment strategy, it is important to differentiate IH from other vascular anomalies presented in the ISSVA (International Society for the Study of Vascular Anomalies) classification of 2014 [6]. Differential diagnosis is made between vascular malformations and rarer forms of vascular tumors: congenital hemangioma, tufted angioma, and kaposiform hemangioendothelioma [2]. The diagnosis is made on the basis of anamnestic data and a specific disease pattern. Ultrasound and magnetic resonance imaging can be used as additional diagnostic methods [5]. In complicated cases, the immunohistochemical marker GLUT1 (glucose transporter 1) allows for differentiating IH from other vascular anomalies, except for verrucous venous malformation. This protein is responsible for glucose transportation and is expressed in any form of IH at all stages of its development [7].

There are three clinical forms of IH: nodular, appearing as a single locus (the most common form); segmental, limited to an anatomical segment, and multifocal, manifested by multiple hemangiomas with visceral lesions, more often of the liver. Depending on the depth of the skin lesion, the following forms are distinguished: superficial, affecting only the upper layers of the dermis and accounting for about 60% of all IHs; subcutaneous, located in the deep layers of the dermis and hypodermis, accounting for 15% of IHs; and mixed, accounting for about 25% in the profile of IHs [8].

Most hemangiomas are small asymptomatic masses that tend to regress on their own. However, about 10% of IHs can lead to local complications: ulcers, hemorrhage, necrosis, cosmetic defect and less often – to functional disorders of vital organs. Complications occur in the proliferation phase and are most typical of segmental forms of IH [9, 10]. Multifocal IH with liver damage can be accompanied

by severe thrombocytopenia and lead to thyroid hypofunction due to overexpression of type 3 iodothyronine deiodinase, inactivating thyroxine, and secretion of TTH-like factor thyrotropin [3]. Facial IHs are of particular concern because of possible complications in the eyes, as well as respiratory and swallowing disorders. Massive hemangiomas in this region may be accompanied by pathologies of the posterior fossa, cardiovascular, ophthalmic, and endocrine abnormalities as part of the PHACES syndrome (Posterior fossa, Hemangioma, Arterial lesions, Cardiac abnormalities, Eye abnormalities, Sternum). Among them, cerebrovascular abnormality is found in 72% of cases [11]. Voluminous IH in the sacrococcygeal and perineal region may be a symptom of LUMBAR syndrome (Lower body, Urogenital anomaly, Myelopathy, Bone deformities, Anorectal malformations, Renal anomalies), characterized by a combination of IH, urogenital anomalies, myelopathy, bone deformities, and renal anomalies. Thirty percent of patients with this syndrome have only one associated anomaly, and the risk of ulcers is about 70% [12]. These syndromes are mostly found in girls, with the prevalence of about 2% of all hemangiomas [13]. Very rare, but the most dangerous complications of IH can be disorders of vital functions. For example, hemangiomas of the upper respiratory tract can cause respiratory distress syndrome, mediastinal and liver tumors can form heart failure, and hemangiomas on the mucous membranes of the gastrointestinal tract can cause massive intestinal bleeding [8]. Voluminous hemangiomas after involution leave residual changes in the form of telangiectasias, fibrofatty changes, and skin atrophy, decreasing the quality of life of patients [14]. Despite the interdisciplinary nature of the problem, IHs often lack proper medical supervision and timely treatment, which worsens the prognosis of the disease and leads to the complications described above.

ETIOLOGY AND PATHOGENESIS

The etiology of this disease is not completely known and remains a subject of scientific research. There are several hypotheses of IH origin, including placental, following genetic mutations, and external factors.

The hypothesis of placental origin is supported by the expression of placental markers, such as laminin, merosin, GLUT-1, Lewis Y antigen, and Fc gamma receptor II (CD32) in the blood vessels of IH [15, 16]. According to this assumption, risk factors for IH include chorionic villus sampling and amniocentesis in pregnant women, in the course of which there may be transfer of mesenchymal cells of chorionic villi from the placenta into the fetal circulatory system and further into susceptible tissues, which represents the initiating stage of pathologic angiogenesis [17, 18]. According to a recent study by F. Moisan et al., endothelial cells of IH lack aquaporin-1 (AQP1), a water channel modulated during tumor cell migration and invasion, whereas endothelial cells derived from placental tissue are AQP1-positive [17].

It is believed that hemangiomas occur as a result of somatic mutation and clonal expansion of progenitor cells. The presence of the stem cell-specific SALL4 gene in IH cells supports this assumption [19]. Genetic predisposition to the development of hemangiomas is currently unconfirmed, but it is also one of the possible causes of IH development [7]. There are data on monozygotic twins with almost identical periorbital hemangiomas [20]. A number of scientific works in recent years have considered mutations and polymorphisms in genes regulating angiogenesis as possible causes of IH development [21, 22].

In the last decade, the hypothesis of the influence of external factors, such as hypoxia, increased estrogen level, and inflammation, has been widely spread [23]. Among them, the leading role is attributed to fetal hypoxia resulting from: multiple pregnancies and pregnancies complicated by preeclampsia, gestational diabetes mellitus, placental abnormalities or detachment, as well as late pregnancy, prematurity, and low birth weight [22, 24, 25]. It is not fully understood what mechanisms regulate the development of IH in hypoxia, but many researchers agree that the key process is the disturbance of vasculogenesis and the predominance of angiogenic factors over antiangiogenic ones [25, 27]. Vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) are currently considered to be the main angiogenic factors [24, 28]. Hypoxia as a root cause of IH development is supported by the data on the increased level of renin in premature and low birth weight infants, which promotes differentiation of tumor stem cells and leads to the growth of immature vascular tissue [25]. The results of the studies by R.M. Hyland and V. Praveen showed that retinopathy of prematurity and IH have a common mechanism of development – microvascular ischemia leading to neovascularization [27, 28].

The increased level of estrogen in the postpartum period and expression of its receptors

on the endothelium of IH microvessels affect further proliferation of endotheliocytes, probably due to the effect on FGF-2 production [7]. The prevalence of hemangiomas in girls also suggests a possible role of estrogen in the etiology of the disease, by stimulating angiogenesis through the expression of the angiogenic factor VEGF [29, 30]. For the same reason, it is thought that higher estrogen levels in pregnant women may affect the vascularization process in the fetus and lead to abnormal vascular proliferation [31]. It has been shown that estrogen concentration in peripheral blood increases in the proliferative phase of IH and decreases in the involution phase, and in the profile of hemangiomas, there are significantly more receptors to estrogen than in unchanged vessels [30]. Thus, in breast cancer, estrogen increases proliferation and migration of endothelial cells [31]. Another study demonstrated the effect of estrogen on the migration of mast cell progenitors in IH tissue, where activated mast cells secreted angiogenic factors VEGF and FGF-2 [32]. In addition, these cells secrete chymotrypsin, trypsin, and matrix metalloproteinases necessary for the destruction of extracellular matrix, providing space for IH vascular growth [31].

Despite the active study of etiology and pathogenesis, none of the existing hypotheses yet explains all the features of epidemiology, course, and clinical manifestations of IH.

TREATMENT

Treatment approaches to IH are diverse and include systemic pharmacotherapy, topical, and surgical treatment [33]. Each of them has both advantages and limitations, which makes it impossible to use one method for all patients. Currently, a need for a personalized approach based on individual patient characteristics, the clinical presentation of the disease, and potential risks associated with therapy is increasingly emphasized [34].

Topical treatment of IH with a nonselective β-blocker timolol is widely used for small superficial skin lesions. It is generally well tolerated and can produce excellent visual results [35, 36]. However, therapy with this drug has side effects, such as desquamation, erythema, local xerosis, ulceration, and risk of secondary infection [37].

Surgical treatments include cryodestruction, sclerotherapy, excision, and laser irradiation. Cryodestruction has been known for a long time, but with the advent of new techniques, its use has been limited. Exposure to liquid nitrogen at –195.6 °C is effective for superficial nodular hemangiomas. In case of more diffuse tumors with abundant blood supply, the method is not used due to a possible risk of cosmetic defects and hemangioma regrowth [38]. Sclerotherapy is used for small superficial masses with small feeding vessels. Its essence is to inject a sclerosant into the feeding vessel of hemangioma, which damages the endothelium and promotes thrombus formation. Disadvantages of this treatment method are risks of microcirculation disorder in tissues around hemangioma, up to necrosis of the tumor. Surgical excision is indicated in life-threatening conditions associated with the localization of IH or its complications and to eliminate residual changes in the tumor after involution, such as excess tissue. Laser irradiation is effective in eliminating residual skin pigmentation [39, 40]. Despite the effectiveness of topical and surgical treatments, they have a risk of complications, including hypopigmentation, burns, localized skin atrophy, and infection [41].

Systemic pharmacotherapy is used mainly for potentially dangerous forms of hemangiomas associated with their localization, size or structural features of the lesion area [42]. First-line drugs currently encompass β-blockers, which proved highly efficient in a multicenter randomized controlled trial in 2015. They include non-selective β-blockers acting on β1-, β2-, and β3-adrenoreceptors and selective drugs mainly blocking β1-adrenoreceptors [43]. Propranolol, a non-selective β-blocker, has become the gold-standard therapy for IHs due to its high efficacy and satisfactory tolerability [44]. However, cases of tumor resistance to therapy as well as tumor regrowth after treatment completion have been reported [25, 45]. Due to non-selectivity of the drug, side effects can develop, such as hypotension, bradycardia, bronchospasm, hypoglycemia, and electrolyte balance disorders.

The mechanism of β-blocker action in IH is not fully understood. Currently, researchers agree that the main role of propranolol is to trigger autophagy of hemangioma cells. However, the specific molecular mechanism and cellular stage of this process remain unexplored [46]. There is evidence supporting the effect of β-blockers on β-adrenergic receptors of IH mast cells, thereby promoting endotheliocyte apoptosis [47]. It was also suggested that β-blockers can increase the production of angiogenic factor VEGF-A. These studies are consistent with the conclusion that the level of VEGF-A in blood decreases in patients with IH upon propranolol

administration [48, 49]. To date, much attention has been paid to the role of glucose metabolism in angiogenesis of endothelial cells. Oxidative phosphorylation, pyruvate metabolism, and the tricarboxylic acid cycle potentially play a key role in the pathogenesis of IH. Propranolol in turn inhibits pyruvate kinase activity, suppressing glycolysis and thus inhibiting the growth of hemangiomas [50]. The mechanism of action of β-blockers in IH requires further study, as it may help develop new treatment options and identify early factors of successful therapy. In addition to β-blockers, other systemic drugs, such as glucocorticoids and metronomic chemotherapy with vinblastine / cyclophosphamide, are also used in the treatment of IH. These methods are usually used when first-line therapy is inefficient or in addition to β-blockers in the treatment of severe and complicated forms of hemangiomas [3].

PREDICTORS OF EFFECTIVE TREATMENT WITH PROPRANOLOL

A number of studies have identified early signs of effective treatment with β-blockers, namely propranolol. A reduction in heart rate by more than 20% from baseline at the initiation of propranolol therapy may be an early marker of a good response to treatment [51].

Initiation of treatment in the first six months of life is considered to be a determinant of successful treatment. This is due to the fact that overexpression of β1-adrenergic receptors is observed in the proliferative phase of hemangioma, which, according to the authors, contributes to the greatest efficacy of the drug during this period [52].

Platelet-derived growth factor PDGF-BB may be a potential marker of a response to propranolol treatment, as its level significantly decreases during therapy. However, these results were obtained on a sample of five patients and require additional studies [53]. Platelet-derived growth factor (PDGF) is a family of cytokines consisting of four isoforms: PDGF-A, PDGF-B, PDGF-C, and PDGF-D, which are produced mainly by platelets, endothelial cells, smooth muscle cells, and macrophages. The PDGF-BB isoform plays an important role in angiogenesis and tissue regeneration. Receptors to this protein are located in the vascular wall on fibroblasts and smooth muscle cells, through which a cascade of signals is initiated, leading to the activation of cell proliferation and the production of factors related to angiogenesis and extracellular matrix remodeling [54].

The level of cytokines IGF-1, IL-6, IL-8, PIGF, RANTES, and TGFβ1 decreases in patients with progressive IH growth and can be used as a predictor of hemangioma growth and be a point of application of immunotherapy as a new treatment approach [55]. It should be taken into account that the sample in this study was not representative and consisted of three patients.

The results of scientific works devoted to the role of VEGF in the process of hemangioma growth are rather contradictory. Most authors come to the conclusion that VEGF level determines the clinical course of IH [56, 57], while others point to the absence of significant changes in VEGF level depending on the stage of the disease, which casts doubt on its use as a predictor of a response to therapy [50]. VEGF is known to be a potent mediator of angiogenesis and vasculogenesis in fetuses, children, and adults. During embryogenesis, it regulates proliferation, migration, and growth of endothelial cells, thereby determining the density and size of blood vessels. After birth, VEGF maintains the integrity of endothelial cells and acts as a mitogen for micro- and macrovascular endothelial cells [58].

Structural features of the tumor detected by instrumental diagnostic methods may also be a prognostic factor for successful treatment with propranolol in IH. Hypervascularization of the tumor according to the results of color Doppler imaging before therapy and reduction of vascularization in the early period of treatment may be a harbinger of a good response. This is probably due to the fact that propranolol induces vasoconstriction and has an antiangiogenic effect, resulting in decreased vascularization of hemangioma. The prominent fatty component of hemangioma detected on magnetic resonance imaging before treatment was more common in patients with a poor response to therapy thereafter [24].

CONCLUSION

Infantile hemangioma is a significant and widespread problem of interdisciplinary nature in the pediatric population, represented by clinical forms ranging from superficial masses prone to self-resolution to extensive tumors leading to local complications or impairment of vital functions. The etiology of this disease is not fully understood. The leading role in the pathogenesis is attributed to the hypoxic state of the fetus. For a timely and correct choice of a treatment strategy for patients, it is important to differentiate this disease with other

vascular malformations. Diagnosis is usually made on the basis of anamnestic and clinical data. The choice of a treatment method should be based on clinical evaluation, including individual patient characteristics, localization and size of the lesion, taking into account possible risks and complications of the chosen approach. Monitoring the effectiveness of therapy, especially in complex cases, should be based not only on subjective clinical assessment, but also on objective imaging and laboratory control methods. Laboratory control may include monitoring of biomarkers related to tumor activity and the body's response to therapy. An integrated approach to the assessment of treatment dynamics facilitates timely correction of therapy, reducing the risk of complications and improving overall efficacy.

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Mechanisms of Recovery and Regeneration of Thermal Skin Damage Using Nanosecond Microwave Pulses

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ABSTRACT

The authors of this lecture performed a comprehensive analysis of possible mechanisms for stimulating reparative processes after thermal skin damage using nanosecond repetitive pulsed microwave (RPM) radiation. The study analyzes both thermal and non-thermal mechanisms of the biological action of electromagnetic radiation, with special emphasis on the molecular aspects of the effects of nanosecond microwave pulses. Special attention is paid to the role of membrane proteins, calcium-dependent signaling pathways, and extracellular matrix components in realizing the regenerative potential of low-intensity microwave exposure. The study reveals the complex relationships between the physical parameters of RPM radiation (intensity, frequency, duration of pulses) and the activation of key cellular processes that ensure accelerated healing without scarring. The work uses experimental data obtained on models of burn injuries in laboratory animals (Wistar rats) using spectrophotometric, hematological, and histological methods. RPM radiation is a promising physical factor for stimulating skin regeneration, acting through non-thermal mechanisms. The combination of RPMs with cell therapy and pharmacological agents can become the basis of new protocols for the treatment of burns and other skin injuries. Further research is aimed at developing personalized treatment regimens, taking into account phases of the injury.

Keywords: nanosecond pulses, microwave exposure, burn injury, skin regeneration, stem cells, calcium ions, extracellular matrix

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Механизмы восстановления и регенерации термических повреждений кожи посредством наносекундных микроволновых импульсов

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РЕЗЮМЕ

Авторы проанализировали возможные механизмы стимуляции репаративных процессов после термических повреждений кожи с помощью действия наносекундного импульсно-периодического микроволнового излучения (ИПМИ). Проведен анализ как тепловых, так и нетепловых механизмов биологического действия электромагнитного излучения, с особым акцентом на возможные молекулярные аспекты взаимодействия ИПМИ с клеточными структурами. Особое внимание уделено роли мембранных белков, кальций-зависимых сигнальных путей и компонентов внеклеточного матрикса в реализации регенеративного потенциала низкоинтенсивного микроволнового воздействия.

В работе раскрываются сложные взаимосвязи между физическими параметрами ИПМИ (интенсивность, частота, длительность импульсов) и активацией ключевых клеточных процессов, обеспечивающих ускоренное заживление без рубцевания. Использованы экспериментальные данные, полученные на моделях ожоговых повреждений у лабораторных животных (крысы линии Вистар), с применением спектрофотометрических, гематологических и гистологических методов.

ИПМИ представляет собой перспективный физический фактор для стимуляции регенерации кожи, действующий через нетепловые механизмы. Комбинация ИПМИ с клеточной терапией и фармакологическими агентами может стать основой новых протоколов лечения ожогов и других повреждений кожи. Дальнейшие исследования направлены на разработку персонализированных схем воздействия с учетом фаз раневого процесса.

Ключевые слова: наносекундные импульсы, микроволновое воздействие, ожоговая травма, регенерация кожи, стволовые клетки, ионы кальция, внеклеточный матрикс

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

Despite the significant number of studies on developing methods for the treatment of skin injuries (including thermal ones) using pharmacological approaches, surgical methods, and the impact of physical factors (in particular, low-intensity pulsed electromagnetic radiation) [1–6], the problem of effective stimulation of regeneration remains relevant in modern medicine. This necessitates the development of more effective methods that will allow to optimize the time and quality of tissue repair.

The aim of this lecture was to consider possible mechanisms and modern ways to repair damaged tissues, including after thermal injury.

EPIDEMIOLOGY OF BURN INJURIES AND THEIR SOCIAL AND PSYCHOLOGICAL CONSEQUENCES

According to statistical data, burns rank sixth in the profile of injuries in patients admitted to medical institutions of the Russian Federation, with the annual incidence exceeding 300 thousand cases. In about 20% of cases, thermal damage affects open areas of the body, which determines high importance of rehabilitation of post-burn deformities with subsequent prevention to ensure a satisfactory quality of life and psychosocial adaptation.

Clinically significant cosmetic defects following burn injuries can lead to persistent maladaptation, including disability, impaired social functioning, and the development of psycho-emotional disorders, which underscores the need for an integrated approach to rehabilitation of this category of patients [7].

FORMATION OF SCARS AFTER THERMAL INJURIES AND MODERN APPROACHES TO THEIR ELIMINATION

According to the study [8], final burn scar formation ends after an average of two months. The degree of hypertrophic scar severity is determined by a number of factors, including the depth and area of burn injury, individual regenerative abilities of the body, as well as timeliness and adequacy of medical care at early stages of the injury. In case of third-degree burns, the key task of treatment is to prevent the development of gross scar deformities, especially when injuries are localized in aesthetically significant areas (facial region, limbs) or on the joints, where excessive scarring can lead to contractures and limited mobility [7]. In cases of delayed repair or failure of natural epithelialization, surgical treatment is indicated, including autologous skin grafting, aimed at accelerating wound surface closure and minimizing the risks of blood loss and infectious complications.

Modern protocols for prevention of keloid and hypertrophic scars provide for comprehensive conservative therapy, which includes local treatment with silicone gels (*Kelo-Cote*, *Dermatix, Contractubex, Mederma*); intrascar injections of glucocorticoids (*Diprospan*, *Kenalog*), physiotherapy methods (electrophoresis with *Fermencol* or *Lidase*), ultrasound therapy with hydrocortisone, magnetic therapy, and balneotherapy as part of rehabilitation programs. The specified measures are most effective in the first 12–18 months after the injury, during the period of active scar formation. Taking this into account, optimizing duration of medical rehabilitation of patients with burns seems to be the key direction in reducing the risk of disabling consequences and improving the quality of life [8].

MODERN ASPECTS OF THE BIOLOGICAL ACTION OF REPETITIVE PULSED MICROWAVE RADIATION ON REGENERATIVE PROCESSES

Modern research demonstrates a growing interest in the study of the biological activity of various physical factors that are able to modulate regenerative processes. Special attention is drawn to nanosecond repetitive pulsed microwave (RPM) radiation, which has a pronounced impact on various levels of biological organization, from cellular structures to the whole body [9–11]. The conducted experimental studies allowed to reveal the key patterns of the biological action of RPM radiation. It was established that the nature and manifestation of the observed effects are strictly dependent on the impact parameters, including pulse repetition rate, radiation intensity, number of applied pulses, and total exposure duration [12].

The most important achievement of the conducted research was confirmation of the RPM ability to significantly accelerate the reparative processes in full-thickness skin lesions [13]. In addition, a pronounced reparative effect was revealed in experimental models of ulcers of gastric mucosa (ethanol-induced and neurogenic ulcers in laboratory animals), which indicates the prospects for a local application of RPM radiation in regenerative medicine [14].

Modern experimental data indicate a pronounced regenerative ability of RPM radiation in thermal skin lesions in laboratory animals (Wistar rats) [12]. Of particular interest is the combined use of RPM radiation with injection of stem cells from red bone marrow, demonstrating a synergistic effect that manifests itself in accelerated and complete wound healing with absence of keloid transformation of scar tissue with minimal severity of the inflammatory reaction [12].

The obtained results create the theoretical basis for development of innovative reparative therapy methods

for skin lesions. The supposed mechanism of observed synergism may include activation of proliferative processes with simultaneous stimulation of cell migration and differentiation, as well as induction of collagen remodeling and proliferation of endogenous fibroblasts. Transformation of granulation tissue into fibrous connective tissue, neogenesis of hair follicles (the appearance of characteristic bulbs), and ultimately complete restoration of the cytoarchitectonics of the dermis are histological confirmation of the effectiveness of the combined impact of stem cells and nanosecond RPM radiation (Fig. 1) [6, 12]. These changes are reliable markers of the completed regenerative process, which confirms the prospects for further study of combined techniques using physical factors and cellular technologies (Fig. 1).

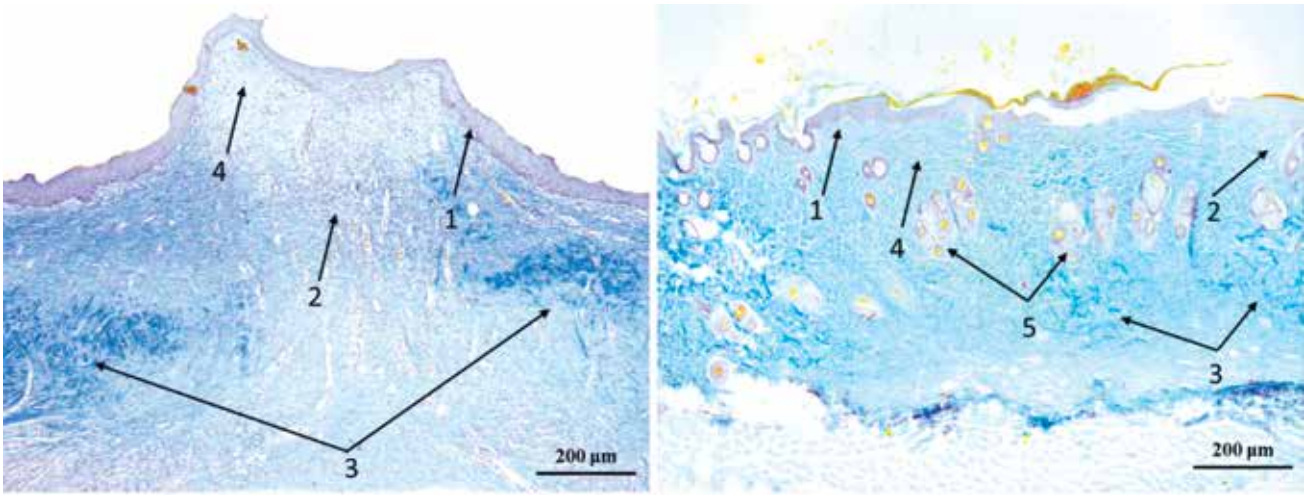

Fig. 1. Histological sections of skin regenerates of the Wistar laboratory rats on day 30 after modeling burn injury in the controls with self-healing without correction (left) [12] and after the combined use of nanosecond RPM radiation and the injection of cultured red bone marrow cells (right). *1* – newly formed epidermis; *2* – young granulation tissue; *3* – reticular dermis, represented by powerful collagen fibers of dense irregular connective tissue; *4* – papillary dermis layer, represented by fibroblasts of loose fibrous irregular connective tissue; *5* – hair follicles. Staining with modified Azan

In spite of the accumulated experimental material demonstrating the stimulating effect of RPM radiation on wound healing, there is still no unified concept explaining the mechanisms of interaction of RPM radiation with the structural components of the skin. Due to bioethical limitations, such studies are possible exclusively on animal models, which determined the aim of this work: to study the primary mechanisms of RPM energy absorption by damaged skin covers and to analyze the stimulating effect of nanosecond microwave pulses on regeneration in the experiment.

PHASE SPECIFICITY OF THE PHYSIOTHERAPY IMPACT DURING SKIN REGENERATION

To optimize therapeutic approaches using RPM radiation, it is necessary to have detailed understanding of the histological and molecular changes occurring in damaged skin at various stages of regenerative process, which sequentially replace one another with partial temporary overlap.

According to literature data, skin is a complex organ consisting of two main layers: epidermis and dermis (Fig. 2) [15]. The epidermal layer is characterized by pronounced structural organization, including pilosebaceous units that combine follicular structures and sebaceous glands associated with them as well as interfollicular epithelium. The dermal layer is morphologically subdivided into a superficial papillary zone and a deeper reticular layer, with the dermal papilla performing regulatory function in relation to the hair follicle cycle and the muscle attached to it ensuring motor activity of the hair.

The dermis contains numerous cellular elements (fibroblasts, immunocompetent cells), vascular and nervous structures, as well as dermal adipose tissue (Fig. 2, *a*). Hypodermis, represented by subcutaneous adipose tissue, is localized directly under the dermal layer. Repair of skin lesions starts immediately after injury and includes successive stages of hemostasis and inflammatory reaction (Fig. 2, *b*). The forming fibrin matrix not only prevents blood loss, but also

creates structural basis for migration of various cell populations. During the proliferative phase, it is possible to observe active migration and proliferation of keratinocytes, fibroblasts, and endothelial cells with parallel reorganization of the extracellular matrix (ECM) (Fig. 2, *c*). The remodeling phase is characterized by structural transformation of collagen fibers and elimination of temporary cellular elements (Fig. 2, *c*). It should be noted that, according to experimental data [16] with small excision injuries in laboratory mice, hair follicles do not fully regenerate, and the defect is replaced with scar tissue (Fig. 2, *c*), whereas with extensive injuries, after completion of epithelialization, it is possible to observe the phenomenon of wound-induced hair follicle neogenesis (WIHN).

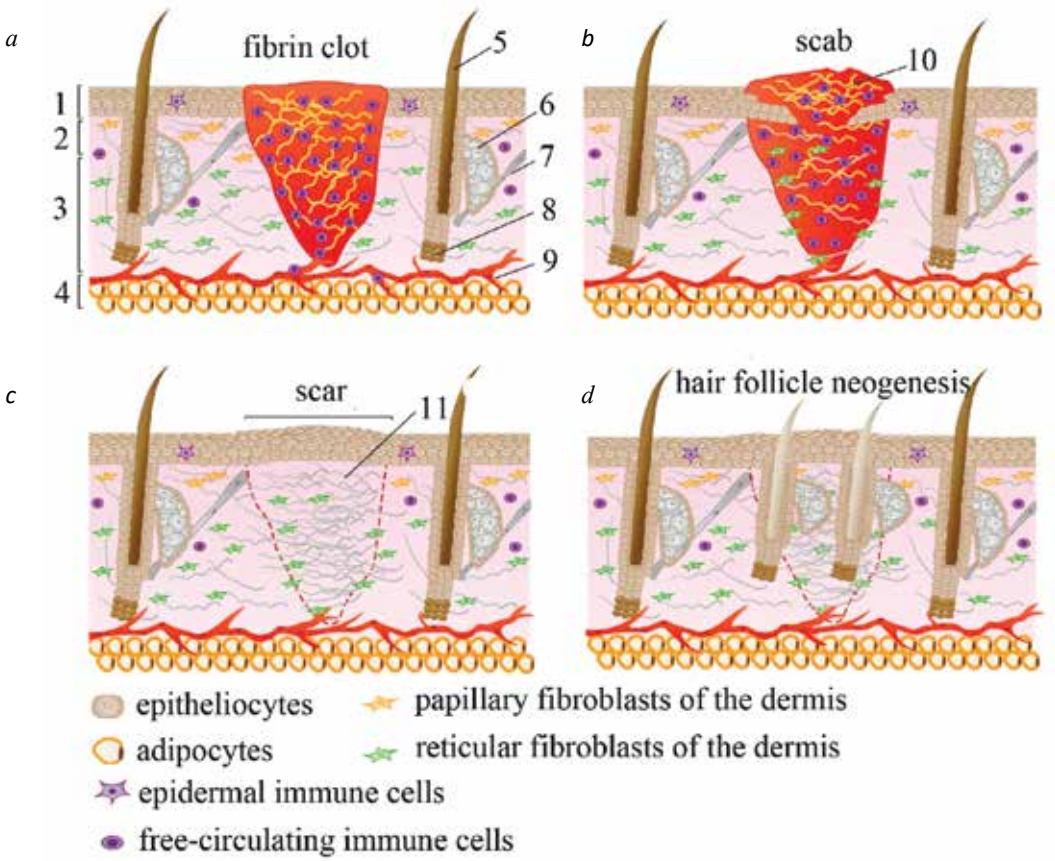

Fig. 2. Structural and functional arrangement of skin covers and dynamics of their repair and regeneration: from homeostatic mechanisms to phases of the wound healing process. The scheme integrates modern concepts of the histological structure of the skin and successive stages of its repair after injury *(a–d),* including the key cellular and molecular events. The dynamics of healing reflects: phases of hemostasis and inflammation (*a*) with formation of a fibrin clot and migration of immune cells; proliferative phase (*b*) with active re-epithelialization, angiogenesis, and formation of granulation tissue; as well as remodeling (*c, d*), demonstrating either scarring of small wounds (*c*) or regeneration with neogenesis of hair follicles and sebaceous glands in cases of extensive damage (*d*). The scheme emphasizes the role of dermal stem cells, the dynamics of ECM remodeling, and critical significance of cell – cell interactions at each stage [15, 16]. *1* – epidermis, *2* – papillary layer of the dermis, *3* – reticular layer of the dermis, *4* – white adipose tissue of the dermis, *5* – hair, *6* – sebaceous gland, *7* – muscle that pulls hair upright, *8* – hair bulb, *9* – blood vessels, *10* – fibrin filaments, *11* – components of ECM (collagen, elastin) [15, 16]

THE MECHANISM OF ABSORPTION OF NANOSECOND MICROWAVE PULSE ENERGY

Experimental data obtained on a burn injury model in Wistar rats show significant improvement in regenerative processes both with local 4-fold exposure to RPM radiation alone and with its combination with the injection of cultured red bone marrow cells. The effect is manifested through reducing the time of complete epithelialization and, with optimal exposure parameters, through scar-free skin regeneration. It is important to note that the effectiveness of exposure was strictly dependent on radiation parameters, such

as the repetition frequency of microwave pulses, their intensity, and total number [6, 12].

Fundamental research would suggest that initiation of reparative processes is related to the features of electromagnetic energy absorption by biological tissues [17]. The physical mechanisms of this phenomenon include generation of conduction currents (caused by the movement of ions under the effect of external radiation) and displacement currents (caused by oscillations of dipole molecules). In this case, the thermal component of the biological effect associated with the heating of tissues during the passage of induced currents is essential [18].

The complex histological organization of skin covers and underlying structures, characterized by pronounced electrophysiological heterogeneity, leads to spatial unevenness in the distribution of the induced electric field and, as a result, to local temperature gradients. The theoretical foundations of this phenomenon were laid in studies on bilayer lipid membrane (BLM) models in the 1980s [19, 20]. It was experimentally established that the difference in dielectric permittivity between the electrolyte (ε of solution) and the Teflon partition (ε of Teflon) leads to concentration of electromagnetic field in the area of the membrane-forming hole, where the specific absorption rate (SAR) can be 2–3 orders of magnitude higher than the values in the surrounding solution (Fig. 3) [19, 20].

A similar mechanism for focusing electromagnetic energy can be implemented in microvessels of the skin, where the heterogeneity of the electrical properties of tissues creates conditions for local thermal effects. It is worth noting that, according to the data [21], the key factor contributing to the acceleration of repair under extremely high-frequency electromagnetic exposure (EMR of EHF) is precisely the improvement of microcirculation in the perifocal area of damage.

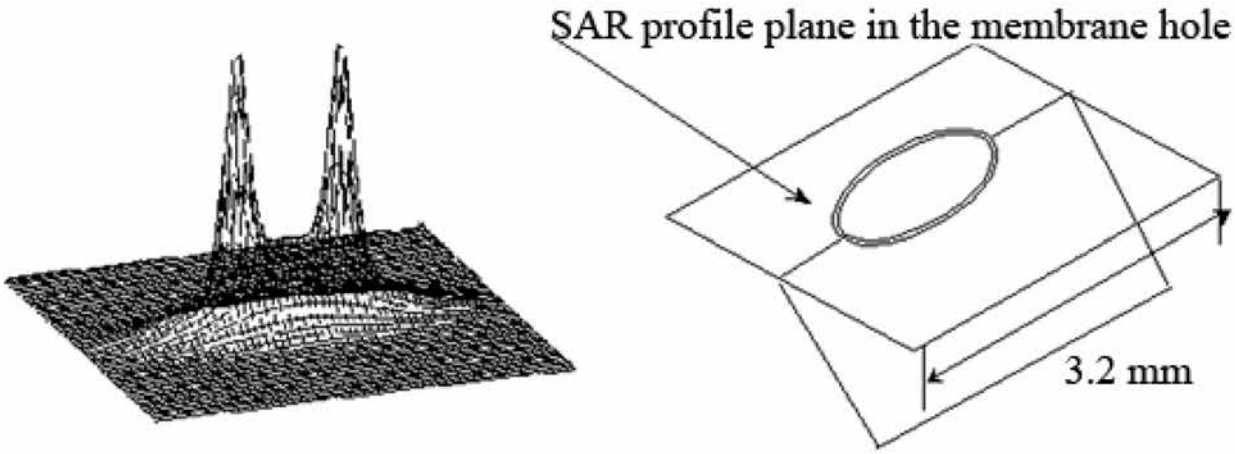

Fig. 3. Specific absorption rate in the solution along the edges of the bilayer lipid membrane. Note: the excess SAR in the solution filling the membrane (on the left) compared to the average overheating in the whole volume [19, 20]

A legitimate question arises about the applicability of the thermal effect model to RPM radiation. Experimental studies on the epididymal adipose tissue model in laboratory mice [22] demonstrated that exposure to 4,000 nanosecond pulses with peak power flux density of 1,500 W/cm^2 caused a temperature increase of no more than 0.08 °C. Taking into account electrophysiological heterogeneity of biological tissues, it can be assumed that even local overheating of blood in microvessels unlikely exceeds 10 times the value of the baseline temperature effect, which limits the maximum local temperature increase to about 0.2 °C. Such insignificant temperature gradient is physiologically incapable of inducing significant vasodilation and, as a result, cannot explain the observed improvement in the trophism of regenerating tissues.

These data allow to exclude with a high degree of probability the thermal mechanism as the main factor responsible for stimulation of reparative processes under the effect of RPM radiation and suggest existence of alternative, non-thermal mechanisms of biological action. One of the possible non-thermal mechanisms may be indirect activation of skin mast cells, leading to the release of histamine, which was demonstrated in experiments with low-intensity (up to 50 μW/cm^2) EMR of EHF [23].

It is known that histamine is able to modulate expression of heat shock proteins, which, in turn,

activates endothelial nitric oxide synthase (eNOS) contributing to an increase in NO production [24]. The combination of these processes ensures the development of a vasodilator effect and improvement of the microcirculatory channel in the perifocal area of the injury. The obtained data suggest that the mechanism of the stimulating effect of low-intensity nanosecond RPM on wound healing is a complex, multilevel process involving both intracellular signaling cascades and intercellular interactions, which requires further detailed research.

ANALYSIS OF NON-THERMAL MECHANISMS OF SKIN REGENERATION BY LOW-INTENSITY RPM

In the process of skin injury repair, the crucial role is attributed to the ECM and stem cells, which is a complex dynamic system ensuring the structural and functional integrity of tissue components (Fig. 4) [25]. The ECM performs multiple functions: it serves as a mechanical framework of connective tissue, mediates intercellular interactions, regulates transport of substances and cell migration, and also acts as a depot for growth factors, ensuring their controlled release in accordance with the phases of regeneration. The most important mechanism of stem cell regulation on the part of the ECM is realized through the maintenance of cellular polarity, orientation of the mitotic spindle, and control of the asymmetry of cell division [26]. By binding growth factors and interacting with cellular surface receptors, the ECM modulates the transmission of molecular signals and regulation of transcriptional activity, thereby determining the morphofunctional characteristics of regenerating tissue [27].

Fibroblasts, as the main effector cells, exercise strict control over synthesis and proteolytic degradation of the ECM components, which directly affects the processes of self-renewal, proliferation, and differentiation of stem cells. Involvement of the ECM in the formation of specialized stem cell niches, unique microenvironments that support the pool of progenitor cells, is of particular importance [28]. In these niches, transmembrane integrin receptors mediate transmission of signals from the ECM to stem cells, regulating their proliferative activity and mobility (Fig. 4) [28]. Integrins specifically interact with the key ECM ligands, including fibroblast growth factor (FGF), tumor necrosis factor (TNF α), interleukin (IL) 1, IL-6, and fibronectin fibers. Activation of integrin receptors initiates a cascade of intracellular signals, in particular through the PI3K/AKT pathway, that stimulates migration and endothelial differentiation of mesenchymal stem cells, significantly enhancing their reparative potential [29]. Thus, modulation of the dynamics of ECM remodeling and activation of components of stem cell niches can be considered as some of the key mechanisms of stimulation of wound healing under the impact of RPM radiation.

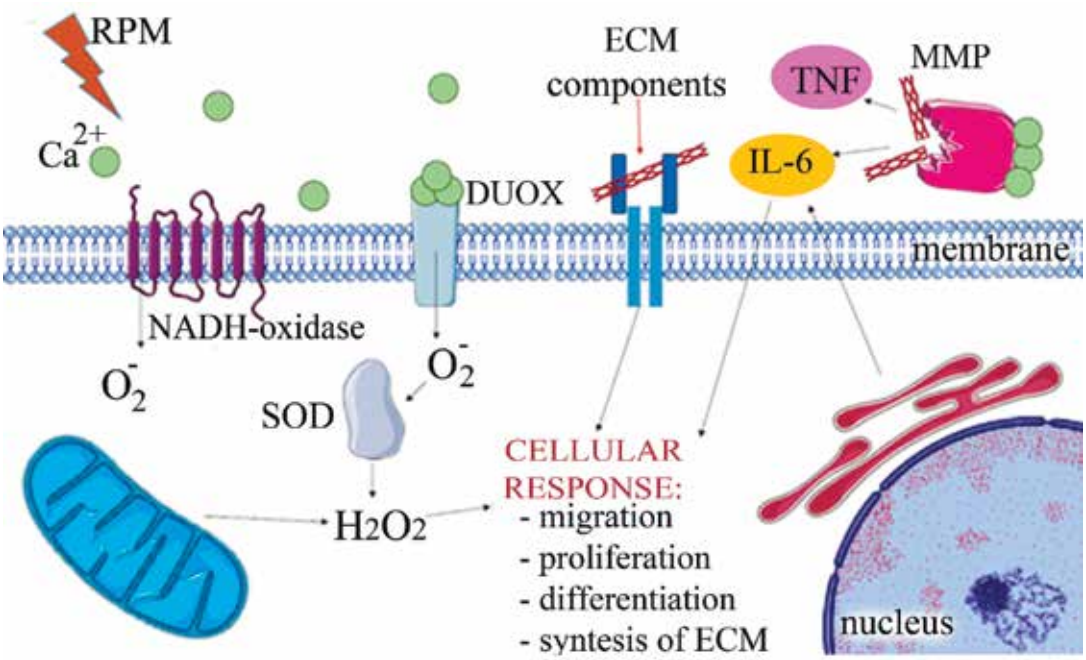

Fig. 4. Possible targets for the impact of nanosecond RPM, providing stimulation of skin lesion regeneration. Explanations are contained in the text

The impact of nanosecond RPM radiation is potentially able to modulate affinity of interaction of the ligands with integrin receptors, changing efficiency of signal transmission and functional activity of corresponding proteins. The similar mechanism can be realized through Ca^{2+}-dependent processes in accordance with the Adey model [30], which explains the frequency dependence of biological effects of electromagnetic actions. Under certain RPM parameters, calcium ions that stabilize the liquid crystal structure of cell membranes can dissociate from surface receptors, increasing fluidity of the membrane bilayer and conformational mobility of integrins. Microwave pulse energy is sufficient to transfer the key molecular components in the active state, that leads to a change in the efficiency of the total signal cascade.

Electromagnetic radiation of non-thermal intensity, which includes nanosecond RPM radiation, is able to influence intracellular signaling pathways through several interrelated mechanisms: changes in membrane permeability for Ca^{2+} ions; modulation of calcium binding to surface polyanionic structures; regulation of calcium interaction with calcium-binding proteins (calbindin, calretinin) [31] and matrix metalloproteinases (MMP) [32]. MMPs play the key role in ECM remodeling by controlling processes of cell adhesion, differentiation, and proliferation in the wound zone, which promotes minimization of scarring changes [28, 32]. Nanosecond RPM radiation can alter calcium-dependent activity of MMP, inducing controlled degradation of ECM components (collagen, elastin). This process leads to a decrease in the volume of scar tissue and creation of space for migration and differentiation of stem cells, which ultimately promotes full-fledged regeneration of all skin layers.

Calcium-activated dual oxidase (DUOX), a membrane enzyme involved in the generation of reactive oxygen species (ROS), may be an important molecular target of RPM radiation [33]. It can be assumed that the impact of RPM can modulate catalytic activity of DUOX, changing the kinetics of conversion of molecular oxygen in hydrogen peroxide, which acts as an important paracrine messenger in cellular signaling pathways. An increase in the local concentration of hydrogen peroxide in wound area initiates a cascade of physiological reactions: increased leukocyte recruitment, activated proinflammatory M1 macrophages, responsible for cytokine production, and anti-inflammatory M2 macrophages, promoting resolution of inflammation and re-epithelialization [33].

The ability of DUOX to take part in realization of "respiratory explosion" of immunocompetent cells, the key mechanism of antimicrobial protection in the wound, is of particular interest [33]. This assumption is consistent with the results of previous studies, which recorded changes in the levels of peroxides, lipid peroxidation products, and oxidative modification of proteins in the liver and blood of the laboratory animals (outbred mice) after the impact of RPM [34, 35].

Thus, it is possible to assume the existence of a DUOX-mediated mechanism in which RPM radiation, through activation of this enzyme and subsequent generation of hydrogen peroxide and other ROS, triggers a complex network of signaling pathways regulating key processes of wound healing: cell migration and proliferation, differentiation of cellular elements, neoangiogenesis, and mobilization of the stem cell pool. This mechanism may explain the observed acceleration of reparative processes under the impact of RPM radiation, although additional experimental studies are required to definitively confirm this hypothesis.

CONCLUSION

The performed analysis of possible molecular mechanisms of stimulation of reparative processes in burn injuries of the skin under the effect of RPM radiation of non-thermal intensity allows us to form comprehensive understanding of the multilevel regulation of regeneration processes. However, it is necessary to take into account that existing data do not exclude the presence of additional, yet unexplored, pathways of biological action of RPM, which can significantly contribute to the observed therapeutic effect of accelerated and high-quality healing without cicatricial complications.

Systematic study of correlation dependences between impact parameters (radiation intensity, pulse repetition rate, number of pulses per session, and total number of procedures) and dynamics of reparative processes, that would allow to optimize treatment protocols and maximize clinical effectiveness, acquires particular importance. Deep understanding of fundamental mechanisms stimulating regeneration of thermally damaged skin opens up prospects for the development of combined therapeutic approaches combining the effect of RPM radiation with cellular technologies (injection of mesenchymal stem cells) or pharmacological agents (cytokines, growth factors).

Early initiation of comprehensive treatment aimed at preventing functionally significant complications of burn injury, in particular hypertrophic scarring, using rational combinations of medical therapy, surgical methods (dermotension, autologous skin grafting), and physiotherapy procedures is of particular importance. Such a multimodal approach makes it possible not only to improve the quality of the regenerated skin, but also to significantly reduce rehabilitation time in patients with burn injuries.

Accumulated experimental data create the theoretical basis for the development of innovative methods to stimulate healing of superficial skin defects of various origins that can be widely used both in burn medicine and in the treatment of trophic ulcers, diabetic skin lesions, and other pathological conditions accompanied by violation of skin integrity. A promising direction of further research is detailed decoding of molecular and cellular mechanisms of synergistic action of RPM in combination with biologically active substances and cellular preparations, which will make it possible to create personalized treatment protocols taking into account features of pathological process in a particular patient.

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Proteomic Studies in Coronary Atherosclerosis

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ABSTRACT

Proteomic studies have made a significant contribution to the study of the pathogenesis of cardiovascular diseases, creating the basis for the development of new potential biomarkers for assessing the risk of developing diseases and their complications. We analyzed the main foreign and domestic publications over the past 15 years using the PubMed/Medline and RSCI/elibrary.ru databases and summarized the available data on proteomic studies in the field of atherosclerotic cardiovascular diseases and coronary atherosclerosis. In this literature review, priority was given to studies on the search for new proteomic biomarkers of coronary atherosclerosis, including proteomic markers of unstable atherosclerotic plaques. The data from our own proteomic studies on potential biomarkers of coronary atherosclerosis are presented.

Keywords: atherosclerosis, proteomics, mass spectrometry, biomarkers; macrophages

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Протеомные исследования при коронарном атеросклерозе

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РЕЗЮМЕ

Протеомные исследования внесли существенный вклад в изучение патогенеза сердечно-сосудистых заболеваний, создавая основу для разработки новых потенциальных биомаркеров оценки риска развития заболеваний и их осложнений.

Цель исследования: обобщить имеющиеся данные о протеомных исследованиях в области сердечно-сосудистых заболеваний атеросклеротического генеза и коронарного атеросклероза. Проведен анализ основных зарубежных и отечественных источников преимущественно за последние 15 лет по базам данных PubMed/Medline, РИНЦ/ELIBRARY.RU. Приоритет был отдан исследованиям по поиску новых протеомных биомаркеров коронарного атеросклероза, в том числе протеомных маркеров нестабильной атеросклеротической

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бляшки. Приведены данные собственных протеомных исследований потенциальных биомаркеров в области коронарного атеросклероза.

Ключевые слова: атеросклероз, протеомика, масс-спектрометрия, биомаркеры, макрофаги

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

In the modern world, the study of early atherosclerosis and its complications is one of the most relevant areas of scientific research on cardiovascular diseases (CVD). Proteomic studies have made a significant contribution to the study of the pathogenesis of CVD, creating the basis for the development of new potential biomarkers for assessing the risk of developing diseases and their complications.

Atherosclerosis is a chronic progressive inflammatory disease of large and medium-sized arteries characterized by the formation of atherosclerotic plaques. Atherosclerotic lesions occur as a result of abnormal lipid retention in the intima of the arterial wall, which leads to the production of cytokines and inflammatory mediators by vascular cells, attracting circulating monocytes to the lesion site [1, 2]. The progressive accumulation of lipids, lipoproteins, and inflammatory cells leads to the formation of a fatty strip, which subsequently evolves into an extensive lesion and atheroma [3]. The pathology develops slowly, and, as a rule, the symptoms of atherosclerosis do not appear for several years. However, the continued growth of the plaque reduces the lumen of blood vessels to the point where obstruction of coronary blood flow begins, which leads to stable angina pectoris. By itself, it rarely causes death, but in obstructive and non-obstructive atherosclerotic plaques, erosion or rupture can occur, resulting in clinical complications, such as ischemia, myocardial infarction, and death from cardiovascular events [4].

Modern research methods enhance the understanding of the processes responsible for the progression of atherosclerotic plaques. Thanks to the currently available proteomic methods, new potential biomarkers have been identified for predicting the risks of developing adverse cardiovascular events [5–7].

PROTEOMIC STUDIES OF ATHEROSCLEROTIC PLAQUES

To study the involvement of proteins in the pathological process of coronary atherosclerosis, it is important to investigate the specific relationships between proteins in the coronary arteries, protein expression, and concentration. The proteomic profile of the vascular wall in coronary atherosclerosis can help identify possible diagnostically significant protein structures or potential biomarkers of the disease and develop new approaches to the diagnosis of coronary atherosclerosis and its complications.

In the first large-scale proteomic study of human coronary artery proteins and coronary atherosclerotic plaques, 806 differentially expressed proteins were identified. Some of them were involved in the development of atherosclerosis, while others might be involved in the progression of the disease. All of them were divided into four groups: 1) extracellular matrix proteins, 2) lipid-binding proteins and proteins related to metabolism, 3) proteins related to inflammation, and 4) phagocytic ligands and receptors of apoptotic cells [8].

From the molecular biology perspective, coronary artery disease can be defined as a community of thousands of proteins that collectively alter cellular processes and lead to a characteristic remodeling of the local coronary artery environment. To characterize the proteome of human coronary arteries, samples of coronary arteries in two autopsy cases (men aged 64 and 69 years), divided into 20 segments, were studied using proteomic research methods. One hundred seventy-four differentially expressed proteins were detected in pathological and healthy intima.

The molecular functions of these proteins primarily included: binding (41.47%), catalytic activity (33.24%), transporter activity (9.41%), and structural molecular activity (7.06%) [9].

Serum amyloid P-component (SAP) is an acute phase protein which plays a significant role in the biological processes of the cardiovascular system, such as inflammation and fibrosis. Increased SAP expression was observed in hemorrhagic atherosclerotic plaques of the carotid arteries compared to fibrous plaques [10]. Annexin 5 is found in the vascular endothelium and has anti-inflammatory, anticoagulant, and anti-apoptotic effects due to binding of phosphatidylserine molecules [7]. It has been shown that the level of annexin 5 in the blood increases significantly after the destruction of the atherosclerotic plaque [11]. In a later study, at the stage of unstable atherosclerotic plaques of the coronary arteries, increased levels of SAP and annexin 5 were noted [12].

The human arterial proteome and features associated with early atherosclerosis of the coronary arteries and aortic samples (sectional material from 100 people aged 15–55 years, 200 arterial samples) were studied using mass spectrometry. Significant differences were found in the prevalence of mitochondrial protein, tumor necrosis factor α, insulin receptor, PPAR-α and -γ between coronary and aortic samples, between atherosclerotic and healthy tissues. It was shown that some biomarkers of tissue proteins indicating early atherosclerosis predict anatomically defined coronary atherosclerosis, thereby confirming the possibility of using human tissue proteomics for clinical and diagnostic purposes. The authors concluded that the human arterial proteome can be considered as a complex network, the architectural features of which vary significantly depending on the anatomical position and the presence or absence of atherosclerosis [13].

Cyclin dependent kinases (CDK) are serine / threonine kinases which phosphorylate the corresponding amino acid residues in proteins. There are 11 known CDKs, each of which is activated by one or more cyclins and other similar molecules after reaching their critical concentration. CDK9 is activated by cyclins T1, T2a, T2b, and K. With a decrease in intracellular cyclin concentration, reversible CDK inactivation occurs. In the study, patients with coronary atherosclerosis showed high concentrations of CDK9 compared to the control group. In addition, high enzyme values correlated with a high content of CD14 and monocytes/macrophages in the atherosclerotic focus. The authors suggest that CDK9 may be a potential biomarker of atherosclerotic inflammation [14].

In a proteomic study of atherosclerotic plaque homogenates obtained during endarterectomy in patients with carotid artery atherosclerosis, the authors identified a group of 33 proteins differentially expressing stable and unstable plaques. A steady increase in ferritin, SOD2, and fibrinogen (fragment D) and a decrease in the levels of glutathione transferase and SOD3 were found in unstable plaques. The mass spectrometry data were confirmed by Western blot analysis. The functional importance of the different isoforms of SOD is not yet clear. Increased fibrinogen levels (fragment D) may contribute to the instability of atherosclerotic plaques. In addition, positive correlations were obtained between the level of ferritin in the blood and in the homogenates of atherosclerotic plaques, which allowed the authors to consider ferritin as a potential marker of atherosclerosis progression [15].

Similar results were obtained by another group of scientists. When comparing the proteomic profiles of homogenates of stable and unstable atherosclerotic plaques obtained from the same person, it was found that the unstable plaques had high concentrations of ferritin and fibrinogen, while the stable atherosclerotic plaques were dominated by apoE, actin and L-lactate dehydrogenase B. The identified proteins, according to the authors, may be potential markers of complications of atherosclerotic lesions [16].

Four hundred sixty-three proteins were studied in the analysis of atherosclerotic plaques and blood plasma of patients with atherosclerosis (n = 34) who underwent carotid endarterectomy (n = 14), compared to the protein profile of healthy volunteers. Consistently high levels of thrombospondin-1, a protein that regulates cell interactions with one other and with the extracellular matrix, and vitamin D-binding protein were obtained. The data were obtained by liquid chromatography and mass spectrometry and confirmed by Western blot analysis [17].

A complex of proteomic research methods revealed 118 proteins differentially expressed in fibrous and hemorrhagic plaques. This allowed the authors to identify three biological processes associated with atherosclerosis (platelet degranulation, vascular autophagy, and negative regulation of fibrinolysis). The data from proteomic studies made it possible to identify new biomarkers (calponin-1, DJ-1, vascular

endothelial growth factor, and procollagen C protease enhancer) of plaque vulnerability [10].

In the study of cancer biomarkers, it was found that vascular smooth muscle cells have different and unusual morphology in the atherosclerotic plaque, which correlates with the proliferative state of the cells. Proteomic analysis revealed proteins associated with the formation of atherosclerosis, including mimecan (osteoglycin), Ras-1 suppressor protein (RSUP-1) and cathepsin D, which were simultaneously identified as biomarkers of cancerous tumors. In this case, the expression of mimecan and RSUP-1 was suppressed in the atherosclerotic plaque, while the expression of cathepsin D was increased [17]. Earlier studies also identified a decrease in osteoglycin expression in hemorrhagic atherosclerotic plaques, which, according to the authors, can lead to plaque instability [10]. On the other hand, there are studies with the opposite point of view. It was shown that the concentration of osteoglycin in the blood of patients with coronary artery disease increases. However, in patients with complex coronary lesions, its level was reduced, and it was suggested that osteoglycin plays a role in stabilizing coronary plaques [18]. In a study examining the prognostic value of certain biomarker proteins in patients with coronary artery disease, circulating osteoglycin (mimecan), whose expression is elevated in vulnerable atherosclerotic plaques, was named a promising biomarker of adverse cardiovascular events [19]. A study performed using proteomic methods confirmed the high content of mimecan in samples of stable fibrous and unstable necrotic – dystrophic atherosclerotic plaques in patients with coronary atherosclerosis [12].

Endothelial cells form a metabolically active barrier between the vascular lumen and the vascular wall. Oxidative stress and modifications of tubulin, a component of microtubules of endothelial cells, destabilize vascular integrity and increase permeability, leading to increased cardiovascular risk [20]. In rabbits with hyperlipidemia and atherosclerotic changes, the regulation of tropomyosin, actin, and keratin proteins in the tissues of the carotid artery and the middle cerebral artery was increased [21]. Mutations in the tropomyosin 1 gene can cause hereditary cardiomyopathy, left ventricular hypertrophy, or impaired diastolic function in the absence of hypertension and aortic stenosis [22].

In patients with coronary atherosclerosis, the proteomic profile of stable atherosclerotic plaques of the coronary arteries showed a significant increase in the content of proteins: actin, tropomyosin, vimentin, keratin, tubulin, and microfibrils of associated glycoprotein 4 (MAGP-4) [12].

Human serum albumin (HSA) is the main protein in human blood plasma. It has been demonstrated that a low concentration of HSA in the blood is a prognostic factor of atherosclerosis in blood vessels, regardless of traditional risk factors in patients with HIV infection. In addition, HSA has been shown to be associated with markers of systemic inflammation and hypercoagulation (interleukin 6, tumor necrosis factor α, C-reactive protein, fibrinogen, and D-dimer). The pathophysiological mechanism underlying this association is the ability of HSA to bind many ligands, including proatherogenic ones, thereby preventing their contribution to oxidative stress [23]. The unstable atherosclerotic plaque is characterized by overexpression of various proatherogenic factors and ligands, which possibly leads to the transfer of HSA from blood plasma to atherosclerotic foci. A proteomic study of unstable atherosclerotic plaques of the necrotic – dystrophic type confirmed the increased content of HSA and fibrinogen [12].

PROTEOMICS OF MACROPHAGES IN ATHEROSCLEROSIS

The most important components of the atherosclerotic plaque are immune cells, primarily macrophages [24]. Plaque macrophages are formed mainly due to the differentiation of circulating monocytes recruited from the bloodstream. These monocytes infiltrate the arterial wall during the transmigration process, which involves adhesion molecules and chemotactic factors. In addition, it has recently been shown that the resident population of macrophages can also be maintained by local proliferation. Macrophages are involved in all stages of atherosclerotic lesion, from onset to progression and rupture. In addition, macrophages contribute to inflammation, lipid accumulation, formation of the necrotic nucleus, and degradation of fibrous thickening leading to plaque rupture. However, macrophages are a heterogeneous and plastic population. It has recently been shown that they can also participate in the stabilization of atherosclerotic plaques and even contribute to their regression [24].

MACROPHAGE PHENOTYPES

In response to stimuli from the microenvironment, such as growth factors, cytokines, and chemokines, macrophages differentiate into different phenotypes. Previously, macrophages were divided into classical (M1) and alternative (M2) macrophages with

proinflammatory characteristics. In particular, cytokines, which include tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ) and granulocyte – macrophage colony-stimulating factor (GM-CSF), or bacterial products, such as lipopolysaccharides (LPS), direct macrophages towards the classical phenotype. Alternative macrophages are induced by cytokines, such as interleukins IL-4 and IL-13 or macrophage colony stimulating factor (M-CSF).

M1 macrophages produce high levels of proinflammatory cytokines, including IL-12, IL-23, IL-6, IL-1b, IL-8, and TNFα, and low levels of the anti-inflammatory cytokine IL-10. In addition, they exhibit increased microbicidal activity and release large amounts of reactive oxygen species and nitrogen-containing radicals.

In contrast to proinflammatory M1 macrophages, high levels of transforming growth factor β (TGFβ) and low levels of IL-12 and IL-23 characterize the M2 macrophage phenotype. M2 macrophages express the mannose receptor (CD206) in large numbers and promote wound healing through the process of efferocytosis, matrix remodeling, and fibroblast recruitment [25, 26]. The division of macrophages into M1 and M2 is based on *in vitro* observations and reflects extreme manifestations within a wide range of different macrophage phenotypes. Currently, this classification seems to be an overly simplified view of the complex heterogeneity of macrophage phenotypes, especially in the atherosclerotic plaque, where a diverse microenvironment is involved in the transformation of macrophages towards either the M1 or M2 phenotype. First of all, there are four subtypes in the M2 phenotype. M2a macrophages are induced by IL-4 or IL-13. They stimulate cell growth and tissue repair, and they are characterized by high endocytosis activity and increased expression of CC chemokine ligands – CCL17, CCL18, and CCL22. M2b macrophages are induced by immune complexes, IL-1b, and toll-like receptors and also modulate immune and inflammatory responses. In comparison with other M2 macrophages, they demonstrate the ability to produce both anti-inflammatory and proinflammatory cytokines, such as IL-10, IL-6, IL-1b, and TNFα [27].

The M2c phenotype or inactivated macrophages are induced by TGFβ, IL-10, and glucocorticoids. They secrete CCL16 and CCL18 in large amount and show high capacity for efferocytosis [28]. Finally, M2d macrophages obtained after stimulation with agonists of the toll-like receptor and the adenosine A2A receptor produce vascular endothelial growth factor in high concentrations and IL-12 and TNFα in low concentrations. In addition, unlike other M2 phenotypes, this subgroup does not show high levels of the CD206 receptor [29].

Various subpopulations of macrophages associated with the presence of hemoglobin and erythrocytes have been identified in hemorrhagic areas of human atherosclerotic plaque. M(Hb) macrophages express high levels of CD206 and CD163, the scavenger receptor for the hemoglobin/haptoglobin complex, which is necessary for effective hemoglobin clearance after intracellular hemorrhage [30]. After the digestion of red blood cells, the released heme group can stimulate the polarization of macrophages into the Mhem phenotype, followed by the activation of transcription factor 1. This activation leads to the expression of heme oxygenase-1 (HO-1), the liver X-receptor (LXR)-α and the ATP-binding cassette transporter ABCA1, which demonstrates an atheroprotective effect and prevents the formation of foam cells [31, 32].

THE ROLE OF MACROPHAGE PHENOTYPES IN THE ATHEROSCLEROTIC PLAQUE

The development of the atherosclerotic plaque, as well as its activity, is associated with an increase in the total number of resident macrophages in the plaque. In particular, the number of M1 and M2 macrophages increases with plaque growth, and the total number of macrophages in an unstable plaque is greater than in a stable one [33, 34]. Since each macrophage phenotype exhibits different properties and demonstrates different functions, the predominance of a particular phenotype can have a strong effect on plaque development, stabilization, or regression. It has been shown that macrophages localized in the shoulder of the plaque (a site prone to rupture) mainly exhibit a proinflammatory phenotype and express M1 markers, whereas macrophages located in the fibrous cap express both M1 and M2 markers [33]. Thus, if M1 macrophages located in the fibrous cap are involved in destabilizing the plaque by producing MMP, M2 macrophages can partially stop such a destabilizing effect by releasing profibrotic factors, such as fibronectin, insulin-like growth factor, and TGFβ, which helps stabilize the plaque [35]. Therefore, there is predominance of M2 macrophages in stable plaques. In contrast, M1 macrophages predominate in rupture-prone plaques [34–36].

Atherosclerotic plaques from the human carotid artery were analyzed using real-time PCR and

Western blotting. When analyzing the cellular contents and distribution of macrophages of the M1 and M2 phenotypes, it was shown that in stable plaques, the expression of CD68 was 3 times lower, the expression of ABCA1 was 2.7 times lower, and the expression of CD206 (M2 marker) was 2 times higher than in unstable plaques. In addition, it was shown that M2 macrophages in stable plaques were found in relatively large numbers (42 ± 5% of the total population of macrophages), while in unstable plaques, they accounted for only 23 ± 3% [37]. Based on this, it is possible to hypothesize that the balance between M1 and M2 macrophages, as well as their distribution in the plaque, can strongly influence the fate of atherosclerotic lesions.

A comprehensive study of various macrophage phenotypes and their prevalence in the plaque may be important for predicting a clinical outcome and preventing fatal events in CVD. Since proteins are the main active factors in most biological processes, a proteomic profile can become an effective tool for identifying complex molecular pathways in multifactorial diseases, including atherosclerosis.

PROTEOMIC ANALYSIS OF MACROPHAGES IN THE ATHEROSCLEROTIC PLAQUE

The atherosclerotic plaque is a complex structure consisting of several cell types with different phenotypes. The nature of changes in the plaque strongly depends on intercellular interactions. Thanks to proteomic analysis of the plaque, it is possible to obtain a wide range of proteins involved in the development of atherosclerosis. The presence of a large number of proteins produced by macrophages in the atherosclerotic plaque also confirms the important role of these cells.

Analysis of 35 atherosclerotic plaques from human coronary arteries using direct tissue proteomics with LC-MS/TMS allowed for the identification of 806 proteins, which provided the first full-scale proteomic map of human coronary atherosclerotic plaques. It has been shown that among these proteins, annexin I is expressed in resident macrophages in the inner lining of the vascular wall, which exhibit the foam cell phenotype. [8]. In addition, in this study, the authors showed that the method of direct tissue proteomics is comparable to laser capture microdissection, and using this method it is possible to determine the absolute number of specific cytokines and growth factors in coronary arteries found in low concentrations.

A study was conducted using LC-MS/TMS analysis on extracts of stable areas and areas after rupture from newly isolated plaques from the human carotid artery. During the analysis, several proteins and biological pathways associated with plaque rupture were identified, such as plaque loss of basement membrane proteins, extracellular proteolysis, inflammation, and decreased cell matrix adhesion, which were confirmed in ruptured plaque extracts from the human carotid artery [38].

Macrophages play a crucial role in the development and progression of atherosclerotic plaques. They represent a complex heterogeneous population of several phenotypes, which are characterized by different and often opposite functions. The ability to determine the overall profile of each phenotype is an attractive goal for developing therapeutic strategies aimed at stopping disease progression and stimulating regression. Proteomics provides an effective tool that includes various high-performance and constantly evolving methods that can help understand the diversity of cells present in the atherosclerotic plaque and their behavior. The proteome is a rich source of potential biomarkers that may be useful for characterizing the progression of atherosclerosis and identifying diagnostic and therapeutic targets aimed at plaque stabilization and/or regression [39].

THE MALDI METHOD AND THE PROTEOMIC PROFILE OF BLOOD SERUM IN CORONARY ATHEROSCLEROSIS

In our study of the proteomic profile of blood serum in coronary atherosclerosis, we used the traditional matrix-activated laser desorption/ionization (MALDI) method based on the NCBI database with protein separation by 2D electrophoresis. Blood serum samples from patients with coronary artery disease and coronary atherosclerosis and the “no coronary artery disease” group were examined. The study of proteins was carried out on pools (mixtures) of blood sera. Groups of protein fractions were identified, the protein content of which varied by more than 1.5 times between the experiment and the control ($p < 0.05$). Most of the proteins, the level of which varies in serum samples of patients with coronary atherosclerosis, can be attributed to acute phase proteins and transport proteins: ceruloplasmin, transthyretin, retinol-binding protein 4, hemopexin and proteins − components of the complement system C3, C4, and C9. The level of kininogen and transcription regulators, zinc finger protein 133, and

B-cell CLL/lymphoma 6 member B protein, also decreased in the blood of patients. An increase in the level of the following proteins in the blood serum pool of patients with coronary atherosclerosis was revealed: hemopexin, transthyretin, retinol-binding protein 4, complement system proteins C4, C9, and C3 (chain B) (Table 1) [40].

It is known that in atherosclerosis and CVD, the complement system is activated [41]. Our study revealed an increase in the content of complement components C3 (chain B), C4, C9 and a decrease in the level of complement component C3 (chain C) in the serum pool of patients with coronary atherosclerosis. A comparison of the positions of these proteins on the gel with their theoretical molecular weight indicates that, apparently, we discovered some isoforms of C3 (C chain). To date, there is no data on the relationship between serum concentrations of various isoforms of component C3 chains and atherosclerosis.

Table 1

Mass Spectrometry Identification of Serum Proteins [40]

Spot No.	NCBI Id	Protein name	Mass, kDa	pI	sc, %	score	Δ
1.1	gi|386789	hemopexin	51512	6.57	40	70	+1.7
1.2	gi|386789	hemopexin	51512	6.57	26	72	+2
1.3	gi|386789	hemopexin	51512	6.57	26	74	+1.6
1.4	gi|386789	hemopexin	51512	6.57	28	78	+5.7
2.1	gi|180249	ceruloplasmin	97637	5.29	29	97	–1.6
2.2	gi|47125416	ceruloplasmin	24668	8.52	57	113	–3.8
2.3	gi|47125416	ceruloplasmin	24668	8.52	52	69	–2.0
3.1	gi|545478558	zinc finger protein 133 isoform f	70201	9.43	33	70	–11
3.2	gi|545478558	zinc finger protein 133 isoform f	70201	9.43	31	68	–10
4	gi|62898910	kininogen 1	47823	6.29	36	74	–1.8
5.1	gi|78101271	complement component C3c, chain C	39463	4.79	58	102	–3.5
5.2	gi|78101271	complement component C3c, chain C	39463	4.79	43	79	–2.4
6	gi|2258128	complement component 9	61728	5.42	26	91	+2
7	gi|78101270	complement component C3c, chain B	21482	5.84	45	114	+2.9
8	gi|401871713	complement component C4 chain C	33052	6.37	45	70	+1.4
9.1	gi|212374952	transthyretin, variant V20s, chain A	13741	5.35	89	176	+4.6
9.2	gi|377656323	transthyretin, chain A	12869	5,33	81	82	–2.7
9.3	gi|377656323	transthyretin, chain A	12869	5.33	81	83	+3.6
9.4	gi|2098255	transthyretin, chain A	13829	5.35	59	67	+3.7
10	gi|305677614	retinol binding protein 4, chain A	20018	5.24	77	105	+9

Note: Δ – the change in the concentration of proteins in the serum of patients with coronary atherosclerosis relative to the control, sc – sequence coverage.

Transthyretin and retinol-binding protein are functionally interacting proteins that form the vitamin A transport complex. In our study, three isoforms of transthyretin (9.1, 9.3, and 9.4) were detected, the concentration of which increased in the serum of patients, and one isoform (9.2) with a reduced concentration (Table 1). The sum of staining intensities of all the detected isoforms showed that the total concentration of transthyretin increased in the sera of sick people. Isoforms 9.2 and 9.4 have the same isoelectric point, and, apparently, 9.4 is a monomer, and 9.2 is a multimeric form. Thus, the monomeric form of transthyretin predominated in the sera of patients with atherosclerosis. Transthyretin is synthesized in the liver. In plasma, it is in the form of a homotetramer weighing 55 kDa and consisting of subunits weighing 13.8 kDa; it provides transport of thyroxine and retinol. Improper assembly of the tetramer, including due to point mutations, can lead to the formation of amyloid fibrils, which often occurs in affected arteries [42]. The concentration of the trimeric form of transthyretin is negatively correlated with the risk of developing CVD [43].

In plasma, transthyretin binds to retinol-binding protein, forming a complex that functions as a vitamin A transport system. The concentration of retinol-binding protein 4 is associated with cardiovascular risk factors associated with insulin resistance and coronary artery disease, therefore, this protein can be a marker of metabolic complications, atherosclerosis

and is used to assess coronary artery disease [44]. In addition, it is known that in patients with coronary atherosclerosis, increased levels of retinol-binding protein 4 correlate with the severity of the disease [45].

Hemopexin, an acute phase glycoprotein that binds hemoglobin and free heme, protects the body from possible oxidative damage. It is known that iron accumulates in atherosclerotic plaques and affected areas of the arteries and in a catalytically active form can cause proatherogenic events, such as the production of reactive oxygen species and lipid peroxidation [46]. Therefore, some researchers consider hemopexin as a protective protein in this process, although its role in atherosclerosis is not fully understood [47]. We have revealed an increase in the concentration of four isoforms of hemopexin in the sera of patients with coronary atherosclerosis [40].

Ceruloplasmin is a specific copper-containing plasma glycoprotein that belongs to acute phase proteins. Ceruloplasmin has pro- and anti-inflammatory properties, so its role in atherosclerosis is controversial. There is evidence that a reduced level of ceruloplasmin may be an unfavorable prognostic sign in patients with coronary artery disease when combined with high concentrations of C-reactive protein, a well-known marker of the acute phase of inflammation [48]. However, there are studies that associate high levels of ceruloplasmin with heart failure [49]. Analysis of proteins decreasing in the blood serum pool of patients with coronary atherosclerosis showed a decrease in the level of one isoform and two fragments of ceruloplasmin [50].

THE MRM METHOD IN STUDIES OF THE RELATIONSHIP OF SERUM PROTEIN CONCENTRATIONS WITH UNSTABLE ATHEROSCLEROTIC PLAQUE IN CORONARY ATHEROSCLEROSIS

The MALDI method of identifying proteins by their peptide maps has become a true standard in proteomic research. However, further improvements in methods and instruments have made it possible to analyze complex mixtures and achieve higher accuracy. Modern quantitative proteomic analysis, used for the identification and determination of biological molecules based on mass spectrometry, is a method with accurate quantitative simultaneous determination of a large number of proteins in various biological samples. Multiple reaction monitoring (MRM) with internal standard peptides labeled with stable isotopes is the most widely used method for the absolute quantitative analysis of target proteins in the field of proteomics [51].

We examined blood serum samples from 40 men, group 1 (St) – patients who, according to the histological analysis, had only stable atherosclerotic plaques (AP) in their samples; group 2 (Unst) – patients who, according to the histological analysis, had only unstable APs in their samples [52]. Protein concentrations in the blood serum samples were measured using the PeptiQuant Plus Proteomics Kit (Cambridge Isotope Laboratories, USA), the proteins were identified by the MRM method on an ultra-high resolution triple quadrupole time-of-flight mass spectrometer with electrospray ionization combined with a high-performance liquid chromatograph. As a result of the comparative analysis, proteins were isolated, the concentration of which was statistically significantly different in the studied groups ($p < 0.05$) (Table 2).

Serum samples of patients with unstable APs exhibited increased concentrations of the proteins attractin, complement factor H, fibrinogen, and fibulin-1, as well as reduced levels of proteins involved in the development of the inflammatory process and the body's immune response, such as ceruloplasmin, hemopexin, haptoglobin, afamin, complement components (C3, C7, C9), and complement factor B. At the same time, there was a reduced level of proteins involved in the coagulation cascade and fibrinolysis and proteins functionally related to them (α-2-antiplasmin, α-2-macroglobulin, heparin cofactor 2, coagulation factor XII, prothrombin, plasminogen, PAI-1, vitronectin). Multifactorial logistic regression analysis confirmed the association of instability with the concentration of attractin (OR = 1.045; $p = 0.027$), afamin (OR = 0.988; $p = 0.001$), hemopexin (OR = 0.997; $p = 0.020$), haptoglobin (OR = 0.967; $p = 0.001$), and components of the complement system. In addition, multifactorial logistic regression analysis showed an association of instability with an increased concentration of fibulin-1 (OR = 1.008; $p = 0.05$) in patients with unstable APs.

PROTEOMIC PROFILE OF ATHEROSCLEROTIC PLAQUE TISSUE AT DIFFERENT STAGES OF DEVELOPMENT IN CORONARY ATHEROSCLEROSIS

To study the involvement of proteins in the pathological process of coronary atherosclerosis, it is important to investigate the specific relationships

between proteins at different stages of development of atherosclerotic lesions in the coronary arteries. In addition to changes in known lipid and inflammatory molecules, certain proteins can influence the development of atherosclerotic lesions into unstable plaques. Temporal analysis of the proteomic profile of the vascular wall in coronary atherosclerosis can help identify possible diagnostically significant protein structures or potential biomarkers of the disease and develop new approaches to the diagnosis of coronary atherosclerosis and its complications.

We examined tissue samples of atherosclerotic plaques containing intima media of the coronary arteries. All samples were obtained from patients who had undergone coronary artery endarterectomy during the operation for intraoperative indications. Samples from male patients were taken for the study with similar clinical characteristics. According to the results of the morphological and histological analysis, all samples were classified as stable or unstable atherosclerotic plaques. A mix of atherosclerotic plaque homogenates at different stages of development were prepared for proteomic analysis.: 1) mix of stable atherosclerotic plaque homogenates at the stage of lipidosis and fibrosis (StL), 2) mix of stable atherosclerotic plaque homogenates at the stage of fibrosis and calcification (StF), 3) mix of unstable atherosclerotic plaque homogenates of necrotic – dystrophic type (Unst). Moreover, the proteomic profiles of every atherosclerotic plaque were determined. The proteins were identified by MALDI mass spectrometry using tryptic mass maps with the Mascot search algorithm. To find differences, a quantitative comparison criterion was used for the difference of at least 1.5 times between the average values of the staining intensity of protein spots (in relative fluorescence units (RFU)) in the groups corresponding to 3 stages (Table 3).

Table 2

Quantitative Mass Spectrometry Identification of Proteins in the Blood, *M* ± *SD* [52]

No.	Protein name	Protein concentration, fmol/μl		*p*
		Group (St)	Group (Unst)	
1	serum albumin	374440.00 ± 61793.83	354465.00 ± 58076.57	0.140
2	ceruloplasmin	1891.77 ± 511.66	1646.48 ± 418.60	0.021
3	α-1-acid glycoprotein	18027.10 ± 7298.18	13287.65 ± 4678.42	0.001
4	α-1- antichymotrypsin	6224.75 ± 3299.37	4545.25 ± 2367.32	0.011
5	α-1- antitrypsin	27696.0 ± 7929.29	23672.0 ± 9887.34	0.048
6	hemoglobin (subunit α)	4785.9 ± 2342.02	4204.15 ± 2608.95	0.297
7	haptoglobin	589.55 ± 261.55	479.60 ± 194.18	0.036
8	hemopexin	1973.6 ± 247.48	1756.55 ± 310.65	0.001
9	serotransferrin	19999.50 ± 3002.74	18329.50 ± 3243.45	0.019
10	retinol-binding protein 4	1237.08 ± 287.36	1372.42 ± 413.33	0.093
11	transthyretin	510.13 ± 179.43	640.23 ± 456.87	0.098
12	afamin	330.12 ± 117.85	264.59 ± 73.53	0.004
13	apolipoprotein A-I	21096.25 ± 6127.08	21626.0 ± 3662.74	0.640
14	apolipoprotein B-100	276.24 ± 79.53	211.04 ± 68.84	0.0001
15	apolipoprotein C-I	5019.2 ± 1251.40	5069.6 ± 1353.80	0.863
16	apolipoprotein L1	590.28 ± 158.45	501.25 ± 200.51	0.031
17	complement component C1q (subunit B)	75.86 ± 31.96	67.07 ± 17.17	0.129
18	complement component C1q (subunit C)	117.84 ± 36.25	120.22 ± 35.42	0.768
19	complement component C1r	230.49 ± 51.37	210.20 ± 70.84	0.147
20	complement component C1s	47.18 ± 10.83	48.99 ± 22.84	0.652
21	complement component C3	590.51 ± 137.97	516.46 ± 139.39	0.019
22	complement component C7	73.23 ± 19.38	61.94 ± 11.18	0.002
23	complement component C9	167.05 ± 66.10	138.93 ± 56.85	0.045
24	complement factor B	4951.7 ± 1358.16	4215.7 ± 1135.39	0.010
25	complement factor H	530.54 ± 79.29	577.37 ± 84.59	0.014
26	attractin	48.43 ± 9.97	55.17 ± 17.14	0.035
27	fibrinogen, α-chain	143.55 ± 4.79	261.0 ± 21.88	0.001
28	fibrinogen, γ-chain	55.3 ± 32.9	113.04 ± 72.14	0.001
29	fibulin-1	660.54 ± 98.04	713.33 ± 131.49	0.045

Table 3

Comparison of the Amount of Protein for Pools / Individual Gels in Atherosclerotic Plaque Homogenates, RFU, ×10^5 [53]

No.	Protein name	MIX / INDIVID		
		StL (n = 5)	StF (n = 5)	Unst (n = 5)
1–3	serum albumin	4.3 / 5.2	12.3 / 7.7	46.3 / 22.6
4–6	vimentin	101 / 12.9	2.4 / 5	4.1 / 13.2
7	tubulin (β chain)	2.5 / 7.6	1.4 / 3.1	1.1 / 4.3
8–10	actin (α cardiac muscle 1) actin (aortic smooth muscle)	84 / 98.3*	29.2 / 43.5	33.4 / 32.1
11	actin (cytoplasmic)	91.3 / 92.6	18.4 / 53.1	37.7 / 33.4
12–14	fibrinogen (β chain)	1.3 /–	3.2 / 19.2	2.9 / 331.2
15	tropomyosin (β chain)	40.3 / 37.9	2 / 17.0	2 / 12.9
16	tropomyosin (α1 chain)	– / 7.8	–/ 2.7	– / 4
17–19	microfibril-associated glycoprotein 4	22.4 / 39.4	4.5 / 54.9	3.2 / 26.7
20	mimekan	26.5 / 44.0	126.5 / 63.7	55.4 / 49.3
21	annexin A5	2.8 / 8	0.7 / 16.1	2.2 / 8.4
22	keratin (type I cytoskeletal 9)	6.4 / 23.8*	–/ 7.5	1.7 / 2
23–24	serum amyloid (P-component)	25.3 / 40	5.9 / 48.8	22.2 / 61
25	peroxiredoxin-2	– / 1	– / 4.7	– / 2.5

Note. MIX – average data for pools of plaque homogenates; INDIVID – average data of individual plaque homogenate gels; maximum values are shown in bold; * statistically significant differences, $p < 0.05$.

A comparison of individual atherosclerotic plaque gels showed the existence of significant individual differences in the staining intensity of specific spots between plaque samples within the same stage. As the plaque develops from stable at the stage of lipoidosis and fibrosis to unstable one, these differences increase. This fact did not allow us to detect statistically significant differences between the groups of samples at different stages in most cases. Isoforms of the same protein, differing only in point pI, were grouped together. Summary data on the comparison of the amount of protein (RFU) in the corresponding spots in the StL, StF, and Unst groups are shown in Table 3. At the stage of lipoidosis and fibrosis of stable atherosclerotic plaques, the content of cytoskeletal proteins increased: actin, tubulin, tropomyosin, keratin, and vimentin. At the stage of fibrosis and calcification of stable atherosclerotic plaques, a significant increase in the level of proteins responsible for regulating cell migration and proliferation and involved in redox homeostasis of cells was found: microfibril-associated glycoprotein-4, mimecan, annexin A5, and peroxiredoxin-2. Unstable atherosclerotic plaques (necrotic – dystrophic type) were characterized by high levels of serum albumin, fibrinogen, serum amyloid (P-component), and a maximum content of vimentin [53].

The results of these studies represent a potential proteomic platform for further study of plaque instability in coronary atherosclerosis. The potential role of the studied proteins in the development of coronary atherosclerosis, as well as their prognostic value as biomarkers of atherosclerotic plaque instability, should be the subject of further research.

CONCLUSION

Attempts to clarify the molecular mechanisms underlying cardiovascular diseases have been made repeatedly, however, these diseases still remain some of the leading causes of death worldwide. Atherosclerosis is the morphological basis of coronary artery disease and its complications. Cardiovascular proteomics is a new field in which significant progress has been made over the past few years in identifying new candidate biomarkers for the diagnosis and obtaining information on the molecular pathophysiology of cardiovascular diseases. The human blood plasma proteome reflects the physiological state of the cardiovascular system and has been used for decades to study plasma biomarkers in a standard analysis designed to diagnose and monitor cardiovascular diseases.

Diagnosing patients with high-risk atherosclerotic plaques before clinical manifestations remains a difficult task and requires an improved approach to predicting the onset of symptoms. The development of proteomic technologies has made it possible to

analyze proteins associated with the development of the disease. These changes reflect the molecular and cellular mechanisms and may make it possible to predict the dynamics of the disease.

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Gene Ontology for Genomics and Biology

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ABSTRACT

The aim of the lecture was to consider the role of gene ontology (GO) and the GO Consortium in shaping the knowledge base for genomics, proteomics, and biology. GO organizes and continually updates data on the molecular functions and biological processes in which genes and their products are involved.

The structure of GO, the features of GO term hierarchy and the connections between them, as well as the elements of each term are considered. The features of services for working with basic knowledge and various ways to access civil defense data are given. In addition to term characteristics, GO pays great attention to annotations – statements that link a gene product to a certain ontology term. The annotation process captures the action and location of a gene product using terms, providing a reference and a type of evidence.

The areas of application of GO related to the analysis of genomics and proteomics data are considered. The main approaches used by researchers are functional annotation of genes and pathway enrichment analysis. Analysis of large volumes of data (for example, when assessing gene expression) allows to gain knowledge about the involvement of genes and their products in various processes, extract biological meaning, and evaluate the features of molecular mechanisms in various diseases. The increasing role of GO in the formation of new knowledge in the relevant field is shown.

Keywords: bioinformatics, gene ontology, functional annotation, biological process, molecular function, cellular component, GO annotation, gene function

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Генная онтология для геномики и биологии

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РЕЗЮМЕ

Цель исследования – рассмотреть роль генной онтологии (GO) и Консорциума GO в формировании базиса знаний для геномики, протеомики и биологии. Генная онтология позволяет систематизировать и постоянно обновляет данные о молекулярных функциях и биологических процессах, в которых участвуют гены и их продукты.

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Рассмотрена структура GO, особенности иерархии терминов GO и отношения между ними, элементы каждого из терминов. Приведены особенности сервисов, обеспечивающих возможности работы исследователей с базой знаний с помощью различных способов доступа к данным GO. Помимо характеристик терминов в GO большое внимание уделяется аннотациям – утверждениям, связывающим продукт гена с конкретным термином онтологии. Процесс аннотации фиксирует действие и локализацию генного продукта с помощью терминов, предоставляя ссылку и вид доказательств.

Рассмотрены направления применения генной онтологии, связанные с анализом данных геномики и протеомики. Основные подходы, используемые исследователями, – это функциональная аннотация генов, анализ обогащения путей. Анализ больших объемов данных (например, при оценке экспрессии генов) позволяет получить знания о вовлеченности тех или иных генов и их продуктов в различные процессы в организме, извлечь биологический смысл и оценить особенности молекулярных механизмов при различных заболеваниях. Показана возрастающая роль GO в формировании новых знаний в соответствующей области.

Ключевые слова: биоинформатика, генная онтология, функциональная аннотация, биологический процесс, молекулярная функция, клеточный компонент, GO аннотация, функция гена

Конфликт интересов. Автор декларирует отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Автор заявляет об отсутствии финансирования при проведении исследования.

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INTRODUCTION

Modern genomics research provides the opportunity to work with large volumes of data. However, it is essential to address the challenge of extracting a biological meaning from the obtained information. The primary question is how to map sequence data onto known results of gene functional analysis and how to derive new insights into gene functions. Ontology tools offer a solution – providing a consistent framework for describing a specific subject area, in this case, genomics and molecular biology [1].

From a computer science perspective, an ontology is a model used to represent objects, their properties, and the relationships between them [2]. Within a given subject area, this model comprises a set of concepts (terms) with definitions and attributes, along with a corresponding set of axioms and inference rules [3]. Typically, knowledge bases for a subject area include repositories containing general knowledge about classes of concepts, their properties, and relationships, as well as knowledge bases about individual objects, their properties, and links to other objects (e.g., specific data). Both components are interconnected within the knowledge base [1].

Applied to genomics, the development of a gene function ontology involved creating a controlled vocabulary of terms with clear definitions and establishing a hierarchical structure of relationships between these terms. A major advantage was that this ontology enabled the study of functional aspects of genomes from different organisms and facilitated the annotation of functions for novel sequences [4].

During the development of the gene ontology, special attention was given to questions that needed to be described in terms of: the location of gene expression within the organism and the subcellular localization of the gene product; the timing of gene expression (in the context of organismal ontogenesis); the function of the gene product and its position within the hierarchy of biological processes; genes regulating the activity of the given gene product. Initially, these challenges were addressed in the context of the Drosophila genome database, FlyBase [4]. The concepts of molecular function and biological process were employed as primary dimensions for the classification. The first gene ontology developed in this manner was described in the article by M.T. Ashburner et al. [5].

Using the first comparison of two complete eukaryotic genomes – the yeast *Saccharomyces cerevisiae* and the worm *Caenorhabditis elegans* – it was demonstrated that a significant proportion of genes in these organisms were orthologs. Furthermore, 12% of the worm genes encoded proteins whose biological significance can be inferred from their similarity to orthologs in yeast (which represents 27% of the yeast genes). It was found that such proteins participate

in biological processes common to all eukaryotes (for example, DNA replication, transcription, and metabolism). A subsequent comparison of the genomes of yeast, worms, and fruit flies also confirmed the presence of homologs among them [6]. It was also shown that genes and proteins involved in fundamental biological processes were highly likely to be orthologous, a fact already confirmed in mammals and model organisms (e.g., yeast) [7–12].

Since a high degree of similarity and functional conservation was subsequently demonstrated across the genomes of different species, this finding opened new opportunities for the automated transfer of biological annotations from experimentally studied model organisms to less characterized species, thereby facilitating the development of the gene ontology.

THE GENE ONTOLOGY CONSORTIUM

Initially, the Gene Ontology (the Gene Ontology Consortium, GO Consortium) was a collaborative project involving three model organism databases: FlyBase16, Mouse Genome Informatics17, 18 (MGI), and Saccharomyces Genome Database19 (SGD). Subsequently, other organism databases joined the consortium [5].

The Consortium goal to create a structured, precisely defined, common, and controlled vocabulary to describe the roles of genes and gene products in any organism remains unchanged, despite progress in implementation and the dynamic development of the terminology system. Each element in GO is linked to other types of information, including gene and protein databases, such as SwissPROT [13], Gen-Bank [14], DDBJ [15], PIR [16], MIPS [17], YPD and WormPD [18], Pfam [19], SCOP [20], and ENZYME [21], facilitating continuous updating and refinement of knowledge about these entities [5].

The core GO terminology describes genes and their products within three categories (aspects). A *Biological Process* encompasses one or more ordered collections of molecular functions, often involving a chemical or physical transformation. Examples of high-level, general biological process terms (Fig. 1) include *GO:0065007 biological regulation* or *GO:0050896 response to stimulus*; low-level terms, such as *GO:0042770 signal transduction in response to DNA damage* or *GO:0072331 signal transduction by p53 class mediator*. A *Molecular Function* defines the biochemical activity of a gene product.

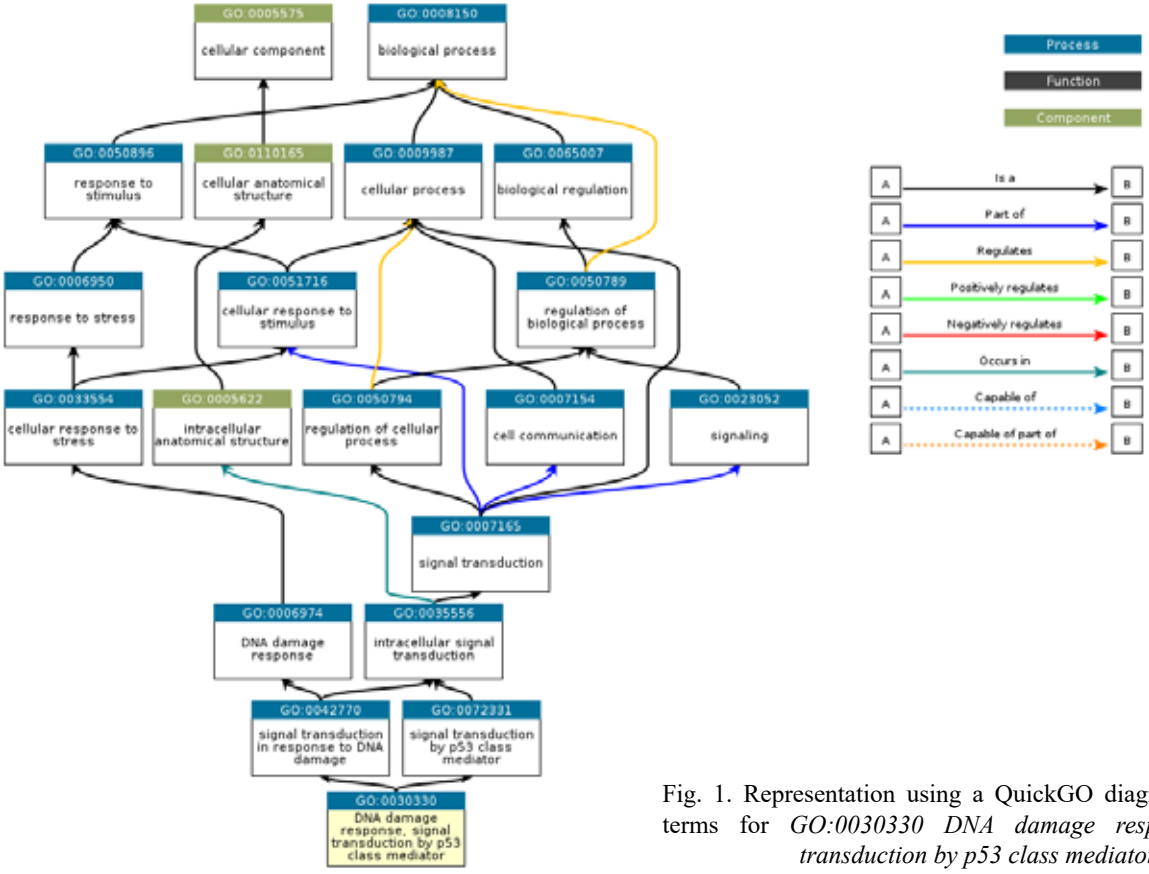

Fig. 1. Representation using a QuickGO diagram of parent terms for *GO:0030330 DNA damage response, signal transduction by p53 class mediator*

General examples include "*enzyme*", "*transporter*", or "*ligand*", while more specific terms include "*adenylate cyclase*" or "*Toll receptor ligand*". A *Cellular Component* indicates the location of gene product activity within a cell; examples are "*ribosome*", "*nuclear membrane*", or "*Golgi apparatus*" [5].

As of March 2025, the ontology contains 26,037 terms in the *Biological Process* aspect, 10,154 terms in the *Molecular Function*, and 4,023 items in the *Cellular Component* [22].

The GO structure is a graph in which each term represents a node, and the connections between nodes represent edges. It also possesses hierarchy, meaning that a child term is more specific than its parent (Fig. 1), and nodes may have multiple parent teams. GO also includes definitions and categories describing the relationships between terms. Commonly used relationships include: *is a*; *part of*; *has part*; *regulates*, *negatively regulates*, and *positively regulates* [23].

Features of the relationships between terms:

The term *"is a"* constitutes the fundamental structure of GO. If A *is a* B, it implies that node A refers to a subtype of node B. For example, mitotic cell cycle *is a* (subtype of) cell cycle.

The term *"part of"* represents part – whole relationships, used only if B is necessarily a part of A, and the presence of B implies the presence of A.

The term *"has part"* refers to the part – whole relationships from the parent's perspective. It is used only when A always contains B as a part; meaning A necessarily has B as a part. If A exists, B will always exist.

The term "*regulates*" describes relationships where one process directly influences another, i.e., regulates it. It denotes necessary regulation; if both A and B are present, B always regulates A, although A may not always be regulated by B [23].

All terms (except the root terms representing each aspect) have a subclass relationship to another term: for example, *GO:0023052 signaling is a* (refers to) *GO:0050789 regulation of biological process* (Fig. 1).

Currently, various methods consistent with graph-based terminology are employed for referencing and representing logical relationships in the GO. A node corresponds to a GO term; a parent node refers to a term closer to the root of the graph, and a child node – to a term closer to terminal nodes. For *is_a* and *part_of* relationships, the parent node refers to a more general GO term, and the child node – to a more specific term [23]. The direction of the relationship is indicated by an arrow (in Fig. 1, the black *is_a* arrow points from *GO:0023052 signaling* to *GO:0050789 regulation of biological process*), while dashed lines denote implied relationships, i.e., those not explicitly stated in the ontology.

Nodes (i.e., terms) in the GO graph can have any number and types of relationships with other nodes. Similar to hierarchies, such as a family tree or species taxonomy, a node can be connected to multiple more specific (child) nodes and may also have multiple parent (more general) nodes, maintaining various relationships with each parent [5, 23]. For example, in Fig. 1, the term *GO:0051716 cellular response to stimulus* has two parents: it is a subtype of *GO:0050896 response to stimulus* and simultaneously a subtype of *GO:0009987 cellular process*.

Each GO term consists of the following elements:

Accession: a unique seven-digit identifier prefixed by GO; e.g., GO:0005739, GO:1904659, or GO:0016597).

Name: the human-readable name of the term, e.g., mitochondrion.

Ontology: indicates the category to which the term belongs, specified as *molecular_function* (MF), *biological_process* (BP), and *cellular_component* (CC).

Synonyms.

Alternate IDs.

Definition: textual description of the term with reference to the source.

Comment.

History.

Chem. react.: participation in chemical reactions.

Subset.

If information for a particular element is unavailable, it is indicated as *None*. It is mandatory to specify how the term relates to other terms in the ontology [5].

In addition to terms, GO also includes annotations – statements linking a gene product to a specific ontology term. Collectively, annotations associated with a gene provide a comprehensive characterization of its biological role. As of 2025, GO contains 8,683,287 annotations: 3,064,581 for the Biological process, 2,791,054 for the Molecular function, and 2,791,054 for the Cellular component [22].

The Evidence and Conclusion Ontology (ECO) describes various evidence types arising during scientific research that support claims. These include: evidence from experimental studies; phylogenetic evidence, based on analysis of gene functions in phylogenetic branches and inference of gene relationships;

computational evidence; author statements (with or without evidence); and inferences from literature [25]. ECO also denotes whether annotation was performed manually or automatically. Users can select data associated with specific types of evidence. As of 2025, GO contains 3,971,399 phylogenetic evidence entries, 1,049,834 experimental evidence entries, and 2,460,718 automated entries [22].

As discussed, the GO annotation process captures gene product activity and localization using terms, providing references and evidence types (including evidence codes). The annotation format is standardized – a gene association file – where each line corresponds to an association between a gene product and a GO term, including evidence codes, references, and additional information [23].

GO resources also comprise software supporting the knowledge base functionality, web access to the ontology and annotations, and analytical tools [23]. Furthermore, GO functions as a dynamic ontology, continuously revising, expanding, and updating its content in accordance with accumulating biological knowledge.

SERVICES

In addition to maintaining the knowledge base and annotations, a significant role in supporting the GO is attributed to the development and enhancement of ontology tools. Currently, GO data can be assessed through various means, including web portals, downloadable files, and APIs.

Web interfaces allow users to access GO data via standard web browsers. The most popular services are described below.

AmiGO (http://amigo.geneontology.org) [24] is the official tool for working with gene ontologies and annotations complied from model organism databases, UniProtKB, InterPro, and other sources – a total of 37 to date. The tool provides functionalities, such as uploading user data for analysis and multiple search modes [25]. AmiGO is a product of the GO Consortium and serves as the official distribution channel for GO datasets.

QuickGO (http://www.ebi.ac.uk/QuickGO) is part of the Gene Ontology Annotation (GOA) project at the European Bioinformatics Institute (EBI) [26], developed by the European Molecular Biology Laboratory (EMBL-EBI), offering extensive capabilities for query-based data retrieval.

DAVID (https://davidbioinformatics.nih.gov/) is a database for gene annotation and visualization that facilitates functional analysis to interpret large gene expression datasets in the context of GO terms [27, 28].

Blast2GO (https://www.blast2go.com/) performs comprehensive genome analysis, assigning gene ontology terms by integrating gene sequence similarity data with functional annotations and predicting molecular functions [29].

PANTHER (https://www.pantherdb.org/) is a curated database of gene and protein families used to infer gene product functions; it forms an integral part of GO resources [30].

One of the most important applications of GO is gene set enrichment analysis. This approach, utilizing available annotations, enables identification of certain GO terms significantly represented (or underrepresented) within a specific gene set. The GO website provides direct linkage to the enrichment analysis tool from the PANTHER classification system [31].

GO data files contain up-to-date and historical ontology results and annotations. In some cases, it is more practical to extract raw data directly from these files using various tools. The most commonly used file types are ontology files and association files.

There are three different versions of GO, arranged in ascending order of complexity – go-basic, go, and go-plus [23]. Go-basic is the basic version of GO, where annotations can propagate upward through the graph. The relationships between elements are described as is_a, part_of, adjustable, negatively_ regulates, and positively_regulates (explained in the previous section of the article). Since many outdated tools still use this version, it remains available.

Go is the main version of GO, which includes additional relationship types within the hierarchy, such as has_part (has a part) and comes_in (is a part of), connecting elements that are not linked in go-basic. Go-plus is the most comprehensive version of GO, encompassing more relationships than the previous versions, as well as connections to external ontologies, including ChEBI [32], Uberon Anatomy [33], and Plant Ontology [34].

In modern research, it is often essential to analyze large gene sets, including by applying gene annotation methods. However, interpreting such results is a challenging task due to the large volume of data and the complexity of relationships between elements. To address this, visualization methods for large lists of GO terms are commonly employed.

In some cases, research involves characterizing dozens or hundreds of genes whose expression may

vary under different conditions within an organism. From the perspective of elucidating the mechanisms underlying these processes, it is important to determine whether these genes participate in the same metabolic or signaling pathways and biochemical processes. These gene sets are subject to statistical enrichment tests to identify relevant functional categories [35].

GO term enrichment analysis may produce long and redundant lists of significant terms, complicating interpretation. Visualization methods help to reveal the biological meaning by summarizing the main trends in the data. This typically involves grouping similar GO terms [35].

Visualization tools include browsers for interactive viewing (such as AmiGO and QuickGO), network construction software that – while not GO-specific – can display any type of graph, including GO or its subsets (Cytoscape [36], Gephi [37], and Pajek [38]). Additionally, Cytoscape plugins specialized in processing groups of GO terms have been developed, including EnrichmentMap [39], BINGO [40], and GlueGo [41]. Other visualization approaches represent GO term groupings as tag clouds (with colored and sized text) or hierarchical tree maps (REVIGO [42], GOSummaries [43]).

Processing of GO terms and GO annotations can be performed using Python as well as other programming languages – Java, R, Perl, and Matlab. The Gene Ontology Consortium website provides a list of available software libraries at:

ftp://ftp.geneontology.org/pub/go/www/GO.tools_by_type.software.shtml.

APPLICATION

GO plays a significant role in advancing biomedical research and acquiring new biological knowledge. The primary applications of GO relate to the analysis of genomics and proteomics data through the following approaches.

Functional annotation enables the classification of genes into three categories (biological process, molecular function, and cellular component), facilitating the assessment of their biological roles. This approach allows for the identification of genes involved in specific metabolic pathways, signal transduction, and other processes, thereby providing a deeper understanding of their functions and the functions of their corresponding protein products in cellular and organismal contexts. GO annotations are widely used to identify genes associated with particular diseases and to elucidate disease mechanisms. By highlighting key genes within pathways implicated in pathological processes, researchers can pinpoint potential therapeutic targets. Such analyses have been conducted across various molecular mechanisms in humans [44–49], animals [50–52], and plants [53–56]. GO annotation has become indispensable for analyzing large-scale gene expression data. Clustering and classification of gene expression profiles enable the identification of biologically meaningful trends dependent on experimental conditions [57].

Pathway enrichment analysis employs GO terms to map genes onto specific biological pathways (e.g., cell cycle, apoptosis, immune response, etc.) and to identify genes active at different stages within a pathway. This information aids in uncovering potential disease mechanisms at the cellular level [58–61]. This approach statistically assesses the representation of particular biological pathways or processes within defined gene sets, facilitating the functional interpretation of gene expression datasets. By using GO terms, one can detect biological processes or molecular functions that are significantly enriched under varying experimental conditions, thereby assisting in the elucidation of the biological significance of gene expression changes [62–65].

Comparative functional analysis using GO annotations enables the comparison of gene functions across different species via consistent GO terms. This approach supports the evaluation of homologous gene functions and tracking of evolutionary relationships. Furthermore, GO terms allow for the prediction of gene functions based on sequence homology in species with poorly annotated genomes [66–69].

CONCLUSION

The Gene Ontology and gene annotation system described above is continuously evolving, updating its knowledge base and adapting to new experimental findings. GO is an open, publicly accessible project, designed to make the annotation of homologous gene and protein sequences across multiple organisms as flexible and dynamic as possible by employing unified vocabulary. Concurrent referencing of various external knowledge bases and meticulous curation of annotations ensure comprehensive coverage of currently known data in this domain. Gene Ontology and gene annotation systems provide the research community with powerful tools to analyze large volumes of genomics and proteomics data and identify and interpret trends in biological processes under diverse organismal conditions, including the

identification of therapeutic targets. Overall, Gene Ontology serves as a foundational knowledge system that dynamically supports the interpretation and revision of biological, genetic, and proteomics data, thereby offering new possibilities for future research.

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Smart E-textiles for Monitoring Health Parameters: Application in Clinical Medicine, Sports, and Social Sphere

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ABSTRACT

Modern scientific advancements promote the development of effective wearable biomedical technologies that will improve the quality of life and personalize approaches to prevention, therapy, and rehabilitation. E-textiles or smart clothing technology are attracting significant attention due to the opening of new horizons for monitoring medical and biological health indicators.

The review provides a critical analysis of possible modern applications of smart textiles for monitoring physiological health indicators. The article discusses the advantages, current limitations, and future prospects, as well as related research and development needed to implement practical solutions in the field of e-textiles. Smart clothing can be used to collect and process data on human body movement and bioelectric potentials or chemical signals in real time for various medical and everyday purposes. Wearable devices are a promising biomedical technology due to their innovative potential for modernizing healthcare, professional sports, and social sphere, providing continuous and non-invasive recording of physiological parameters.

Continuous scientific and technological progress in the development of materials with improved biocompatibility and durability, optimization of analytics based on artificial intelligence, and more accurate assessment of big biomedical data will accelerate the implementation of smart textiles and their integration with other digital applications for intelligent health monitoring. Further development of wearable e-textiles will contribute to improving both their diagnostic efficiency and the quality of life of users.

Keywords: e-textile; textile electrode; health monitoring; wearable technology; medical and biological indicators; smart clothing

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Интеллектуальная одежда для мониторинга медико-биологических показателей здоровья: применение в клинической медицине, спорте и социальной сфере

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РЕЗЮМЕ

Современные научные достижения стимулируют разработку эффективных носимых биомедицинских технологий, которые позволят повысить качество жизни и персонализировать подходы к профилактике, терапии и реабилитации. Технология интеллектуальной, или «умной, одежды» привлекает значительное научное и практическое внимание благодаря открытию новых горизонтов для мониторинга медико-биологических показателей здоровья.

В обзоре представлен обобщенный критический анализ современных возможностей использования интеллектуальной одежды для регистрации физиологических параметров организма и мониторинга здоровья. Обсуждаются преимущества, текущие ограничения и будущие перспективы, а также связанные с ними исследования и разработки, необходимые для реализации практических решений в области электронных текстильных изделий для медицинского мониторинга. Интеллектуальная одежда может использоваться для сбора и обработки данных о биомеханике человеческого тела, биопотенциалов или химических сигналов в режиме реального времени для различных медицинских и повседневных целей. Носимые устройства являются перспективной биомедицинской технологией из-за ее инновационного и многообещающего потенциала для модернизации системы здравоохранения, профессионального спорта и социальной сферы, обеспечивая непрерывную и неинвазивную регистрацию медико-биологических показателей состояния человека.

В перспективе научно-технологический прогресс в разработке материалов с улучшенной биосовместимостью и долговечностью, оптимизация аналитики и более точная оценка больших массивов биомедицинских данных позволят ускорить внедрение носимых электронных текстильных изделий и их интеграцию с другими цифровыми приложениями для интеллектуального мониторинга здоровья и достижения целевых стратегий профилактики и терапии. Дальнейшее развитие технологии «умной одежды» повысит ее диагностическую эффективность и в целом качество жизни пациентов и пользователей.

Ключевые слова: интеллектуальная одежда, мониторинг здоровья, носимые биомедицинские технологии, медико-биологические показатели, «умная одежда»

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

Due to the heterogeneity and large volume of recorded medical and biological information, the need has gradually emerged to develop and implement advanced, intelligent and at the same time accessible technologies capable of collecting, storing, analyzing, and transmitting data [1]. Among such innovative medical technologies are wearable systems for monitoring physiological indicators, implemented through interdisciplinary solutions and scientific and technological integration of digital healthcare, microelectronics, wireless communications, analytics, and textile production [2, 3].

Adding electrodes and sensors to textiles and clothing is a promising way to record physiological parameters [4]. This technology is gaining recognition for its potential in diagnostics and long-term monitoring of daily activities and recording the dynamics of various vital signs. Due to scientific research and technological advances, wearable electronics are now becoming a sought-after product in the healthcare and sports medicine sectors. Mass production and integration of sensors into textiles open up new possibilities for monitoring health, fitness, and overall performance in clinical settings, daily life, and sports training [5].

Previously, sensors for recording medical and biological signals were placed directly on the body or in special compartments in textiles. The modern concept of wearable biomedical technologies "Wearable 2.0" provides complete integration of wearable electronics into clothing. In the scientific community, such systems are known as Smart clothing (SC), Intelligent textile, Electronic textiles (e-Textiles) or Wearable textile electrodes [6–8].

The aim of this review was to critically and constructively analyze the results of research to determine the potential of using Smart Clothing for monitoring and recording biomedical parameters. The advantages, current limitations, and future opportunities are discussed, as well as the related research and development required to implement practical solutions in the field of e-Textiles and their application in clinical medicine.

ALGORITHM FOR WORKING WITH SOURCES: SEARCHING AND SELECTING PUBLICATIONS

To achieve the stated goal and prepare the review, a search for publications was performed in the PubMed, Scopus, and RSCI databases using search queries and keywords. The systematic search included articles published over the past 10 years (2015–2025). The query and analysis language was English. In particular, the following keywords and their combinations were used: Smart clothing, e-Textile, Wearable technology,

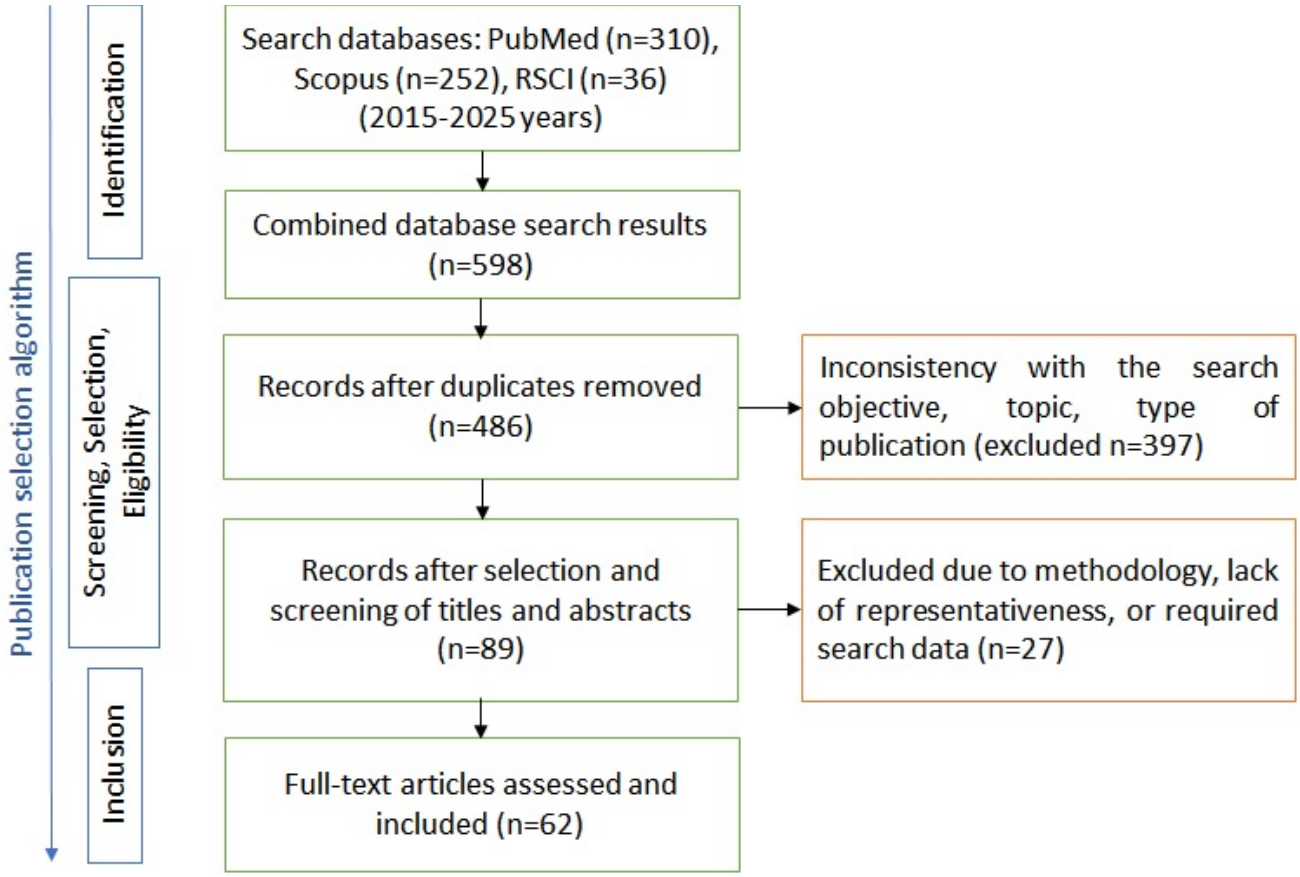

Fig 1. Flow chart for selecting publications

Textile electrode, Smart textile technology, Wearable electronic devices, Biosensors, Health monitoring, Digital health. The review was guided by the recommendations of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses and relied on the search methods described in the RELISH consortium. To expand the systematic search, the operators AND/OR were used to combine keywords. According to the stated search objective, abstracts of reports, meeting minutes, books, conference materials, and clinical cases were not used to analyze the results. The generalized analysis of the technology application included publications of the research results that adequately presented the initial data – characteristics and parameters of the development and use of SC for non-invasive recording and monitoring of medical and biological indicators of the human physiological state. At the initial stage, 598 articles were identified across all databases. The selection was performed based on titles and abstracts. After removing duplicates and publications that did not correspond to the topic and aim of the study, data were obtained, and full texts of 62 articles were analyzed in accordance with their methodology, results, and conclusions (Fig. 1). To comprehensively describe the possibilities of using SC, identify the advantages, limitations, and future prospects of the technology, and expand the critical view of the problem posed, the general analysis and references include data from original studies, technological developments, validation and observational clinical trials, meta-analyses, and research in related areas.

DEVELOPMENT AND APPLICATION AREAS OF SMART CLOTHING

The development of flexible, stretchable electrodes is a key component of electronic functional wearable textiles capable of sensing, transmitting, and analyzing data. Most clothing materials worn by humans every day are made of flexible textile fabrics consisting of one or more types of natural or artificial fibers, with the main requirements for them being the ability to provide comfort and physiological, including sensory, properties for the consumer [9, 10]. At the same time, the fabrics must be wear-resistant during cleaning (washing) and repeated use of clothing. However, the implementation of such requirements is significantly complicated when rigid or fragile electronic systems are integrated with textile materials, which is the main current limitation in the design of SC. Therefore, the general trend in the development of prototypes of electronic textiles is aimed at solving the problem of minimal disruption to user comfort when wearing such clothing and the ability to detach the electronic components. Another challenge is the precise positioning of the appropriate electrodes over the target area in order to perceive biosignals with the highest quality and without interference and artifacts [11, 12].

Potential areas of application of SC can be divided into three groups (Fig. 2): for clinical use in healthcare (the main direction) – registration and monitoring of physiological parameters, medical conditions, dynamics of the therapeutic process or rehabilitation treatment; application in sports – monitoring the physical status and performance, expanding the rehabilitation potential of athletes; for the social sphere – to facilitate any non-professional and everyday activity of patients, users, and especially persons with disabilities [13, 14]. Equally, electronic textile products can be used in the above-mentioned sectors, depending on their basic functionality and consumer choice (which determines a more pronounced demand for the technology from the fitness/sports and digital medicine industries). At the present stage, the focus is shifting towards the design and development of technically advanced high-performance SC, which is not only comfortable and interactive, but also offers significant added value in terms of functionality. E-Textiles are a promising asset because technologically advanced prototypes are adaptable and have the potential to be used in both the medical or sports sectors as well as in the social area due to the global population aging and the growing number of people with disabilities [15, 16].

TYPES OF SENSORS AND RECORDED BIOMEDICAL SIGNALS

E-Textiles require electronic components to interact with stimuli, such as mechanical, electrical, or chemical signals of the body's state. Various SC characteristics vary depending on the integrated electronics or technology platform, which creates new functionality for traditional clothing and provides additional value [17, 18]. It is likely that the most effective prototypes of e-Textiles being developed will eventually scale up into commercial products and significantly increase the market size, especially in the healthcare and sports sectors. The market for wearable e-Textiles is currently projected to grow at a rate of over $1 billion per year [5, 19]. Over the past 5–10 years, advances in nanotechnology and manufacturing

techniques have led to tremendous changes in the field of flexible electronics and the development of SC with the inclusion of additional functions to increase their commercial value, increasing the demand for the development of systems aimed at monitoring biomedical parameters of the human body's state [20–23].

Since the early days of SC development, various ways of integrating electronics into textiles have been demonstrated [24]. The first-generation e-Textiles included a rigid component (electronic device, electrode) attached or sewn onto the surface of the garment or embedded in special compartments or pockets. Such products are typically larger in size and less durable under constant use and maintenance. Subsequent development and manufacturing transformation have shifted the focus to more user-friendly e-Textiles with wearable technologies integrated into the garment itself. In the second-generation SC, conductive threads and biomedical sensors are actually woven into the textile structure to produce fully functional wearable e-Textiles. Thus, modern prototypes of smart textiles are characterized by full integration of textile and electrodes [25]. In the future, this technology will increasingly develop towards the Internet of Things (IoT) and deep integration with artificial intelligence, human-machine interfaces, and cloud technologies [7, 26].

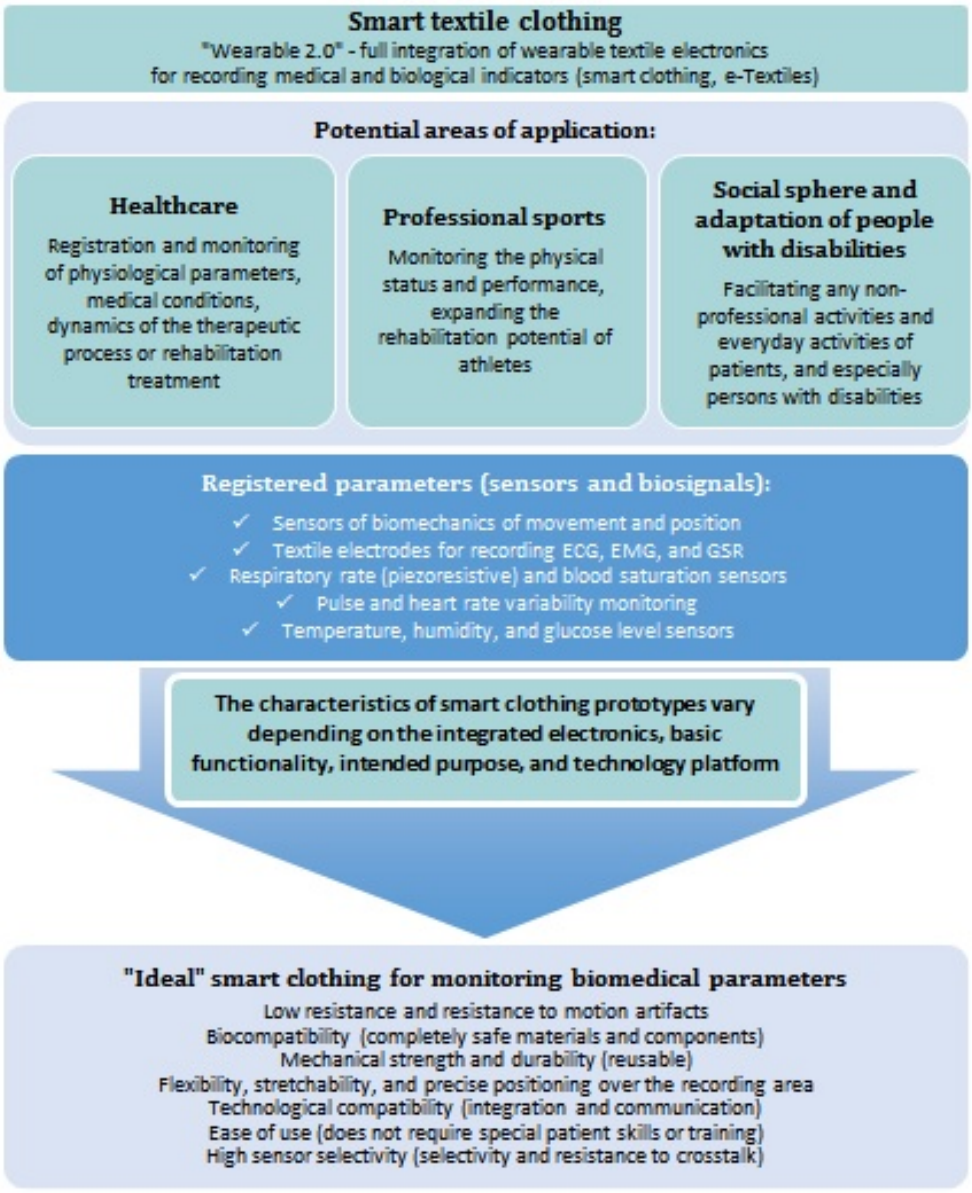

Fig. 2. Smart e-Textile technology for monitoring biomedical parameters

Sensors that can perceive medical and biological signals are conventionally classified into physical, electrical or chemical depending on the application purposes [27]. Various types of specialized electrodes are used to record physiological parameters, which can be either non-invasive – placed on the skin surface, or invasive. At the present stage, textile fabrics serve as a basic platform for integrating various electronic subsystems into them or on them. The quality and reliability of the recorded signal and the sensor itself integrated into the SC depend on the constituent components of the sensor unit [28, 29]. Sensors vary significantly in their intended purpose and can be, for example, biomechanical, temperature or biopotential sensors. Thus, well-known motion and position sensors are an accelerometer, magnetometer, and gyroscope (separately or in combination in one unit). Electrical potentials of cardiomyocytes and heart rate variability are among the most common biosignals recorded for monitoring human health in clinical practice, as well as in sports medicine and social area [30, 31]. Classic silver chloride-based hydrogel electrodes are not suitable for long-term wear, so dry textile-based electrodes have been developed for wearable sensor units. Such sensors are mainly manufactured by applying conductive ink (paste) to textiles using industrial or laboratory printing [32, 33]. The materials and manufacturing process of electrodes for recording electrical biopotentials of muscles and indicating muscle activation patterns (EMG) correspond to those described for ECG recording. Piezoresistive sensors sensitive to changes in resistance and capacitance integrated into textiles are used for real-time respiratory rate monitoring [34, 35]. Prototypes of SC with sensors for temperature and humidity changes or recording blood oxygen saturation and glucose levels are also being developed [14, 36, 37]. Such wearable electronics allow for monitoring health of patients and their clinical status over a long period of time. Therefore, e-Textiles for monitoring physiological signals can be an effective tool for early detection and monitoring of cardiovascular diseases, cerebrovascular events, sleep disorders, neurodegenerative and psychophysiological disorders, as well as for monitoring the rehabilitation process and daily activity [38–42], which determines broad opportunities for the application of the technology in clinical medicine, sports, and social sphere.

PROSPECTS OF DIGITAL MEDICINE

With technological advances and the need to improve the quality of healthcare services while reducing existing costs, a great amount of generated biomedical data has high added value in areas such as clinical decision-making support, chronic process monitoring, anti-aging and personalized medicine, cognitive well-being, staff productivity optimization, and multi-level management in healthcare. With the global shift to personalized, preventive, and evidence-based models of healthcare delivery in clinical practice, there is a growing demand for the use of safe and long-term monitoring, analysis, and storage of large volumes of information. Wearable technologies open new horizons and are recognized as a critical element of digital medicine, as they provide advantages for the cost-effective and safe use of modern medical technologies [43–46].

Systems developed as clothing with integrated electronics offer technology capable of non-invasive tracking and analysis of physiological biopotentials and biomechanical data, which opens up additional opportunities for improving preventive medicine, early diagnosis, and timely correction of physical and mental health [47, 48]. In particular, wearable technologies with integrated motion sensors and surface EMG can potentially be used in patients with motor disorders, neuromuscular and musculoskeletal dysfunction, during rehabilitation after acute cerebrovascular events, fibromyalgia, multiple sclerosis, neurodegenerative diseases or sleep – wake cycle disorders, and in general for monitoring daily activity. Electronic textiles sensitive to chemicals, electrophysiological skin response, and biopotentials open up prospects for continuous recording of vital signs for the early detection of pathological changes and effective monitoring of a wide range of chronic and age-associated diseases, including cardiovascular, metabolic, and psychophysiological disorders [1, 49–55].

Thus, modern technologies in healthcare, including wearable devices, can become a key element of telemedicine, remote monitoring, and rehabilitation treatment of patients, providing opportunities for personalized medical services outside of traditional clinical settings [56–58]. Moreover, the increasing prevalence of chronic diseases and global population aging only increase the need for the implementation of innovative technologies to improve the quality of medical care and direct the transformation of healthcare towards the widespread use and accelerated clinical testing of Smart clothing [59, 60].

SYSTEMATIC ANALYSIS OF TECHNOLOGY

The systematic review demonstrates three main directions in the research of SC for monitoring

biomedical parameters: technical development of prototype devices, validation trials for widespread use by clinicians and specialized researchers who require valid and reliable measurements, and observational clinical studies [3, 21, 25, 34, 61–63].

Technological Features and Methods of Manufacturing

Due to their high electrical conductivity and biocompatibility, metals (silver or copper) are often used in SC prototyping to manufacture conductive elements and sensors. Although there are many types of fabrics that can be technologically combined with metals, their durability during repeated use and maintenance of products remains a limitation and requires further scientific development and practical improvement [64, 65]. Polystyrene sulfonate, polypyrrole, and polyaniline belong to the class of organic polymers that, due to the peculiarities of their chemical structure, are electrically conductive and therefore are of interest for the development of electronic textiles. The main limitations of such polymers are their low mechanical stability and poor flexibility, which potentiates the search for composites with other elastic polymers to increase strength and reduce brittleness [66, 67]. Carbon-based materials are in demand in the field of biosensors due to their biocompatibility, flexibility, thermal and chemical stability. Graphite or graphene can be produced in large quantities and are relatively inexpensive, so commercialization of such electrodes for wearable biomedical technologies is quite feasible, but improvements in developments are required to ensure a uniform, stable coating of the material on textiles [68, 69].

Simple and inexpensive methods used to fabricate SC include dyeing, roll-to-roll coating, spray-coating, and dip-coating. In particular, in observational clinical studies and evaluation of electronic textiles prototypes, the use of such methods for depositing carbon conductive materials showed that e-Textiles retained mechanical strength and stability after 20–30 washing cycles and allowed for recording high-quality biosignals [70, 71]. Modern technologies using computer graphics, electrospinning methods, or industrial printing make mass production of SC possible. Electrospinning produces ultra-thin nanofibers with a diameter from several micrometers to hundreds of nanometers, which demonstrate good mechanical strength and high specific area, and the resulting textile electrodes have a porous structure, which improves air permeability, electrical conductivity, and adhesion to the skin required to obtain high-quality EMG and ECG signals. In laboratory printing, various types of conductive inks are tested on textiles, including metal nanoparticles, conductive polymers, and carbon materials. It is worth noting that the conductive materials used for printing must not only firmly bond to the fabric surface, but also be stable and not lose conductivity when stretched and repeatedly used in wearable technology [61, 72–75].

Advantages of Technology

1. Smart textile solutions provide a simple, convenient, and understandable platform, while containing non-invasive sensors.

2. Increased compliance – integration of electronic systems into clothing promotes patient adherence and better implementation of diagnostic recommendations.

3. Multifunctionality – several sensors can be embedded into one platform (garment) in different target areas, which eliminates the use of several separate devices and allows for measuring a wide range of signals.

4. Reusable dry textile electrodes as part of the SC are more cost-effective for long-term monitoring.

5. In the future, invisible and seamless integration of miniature electronic components will ensure a close and positioned contact of biosensors with the recording area, which will reduce measurement errors caused by cross-talk, displacement, and discomfort of rigid and large sensors.

Identified Limitations of the Technology

Despite the interest of basic and applied research in the development and scaling of SC for biomedical purposes, the analysis revealed a number of current limitations.

1. Decreased signal quality and accuracy depending on environmental conditions and external interference, sampling frequency, contact quality.

2. Lack of uniform standards for the production process, methodology, and legislation in the development and validation of products.

3. Energy efficiency – the need for a reusable energy source and integration with batteries, which negatively affect the size, comfort, and flexibility of products.

4. Limited contact area and difficulty in accurately positioning sensors without displacement, high sensitivity of sensors to motion artifacts.

5. Comfort and safety: ensuring long-term use without discomfort or irritation.

6. Wear resistance and limited service life.

7. The need for specialists to analyze large volumes of recorded information and identify suitable data for transferring scientific results into clinical practice, as well as systematic validation of products.

8. Ethical and privacy issues – strong encryption and secure solutions to protect patient information.

Prospects and Future Possibilities of the Technology

Validation studies and clinical trials of prototypes have identified the following characteristics of an "ideal" e-Textile product for recording medical and biological parameters [2, 9, 11, 27, 33, 68, 76–78]: biocompatibility; flexibility, and stretchability with precise positioning over the recording area; resistance to motion artifacts; mechanical strength; ease of use for the patient; technological compatibility, integration, and communication with other equipment; selectivity of integrated biosensors.

The solution to the described problems and limitations requires further research and the creation of additional opportunities for improving the SC. To improve the quality of recorded biosignals, reliability, durability, and the level of integration of wearable technologies, continuous development of new materials and components is required. The interaction of electronic textiles with other innovative technologies adds new functions and contributes to the development of the concept of next-level wearable biomedical devices in the field of SC personalization. Thus, the use of AI tools and digital technologies to improve analysis algorithms will allow for the development of personalized diagnostic and treatment plans, predicting the progress of rehabilitation or the training process [79–81]. In general, with an interdisciplinary approach and the simultaneous participation of various stakeholders, including technology developers and manufacturers, health and professional sports experts, as well as end users and government regulation, it is possible to effectively transform scientific achievements in the field of electronic textiles into consumer-oriented, economically in-demand, and sustainable solutions. Therefore, additional efforts to create industry-wide standards and optimize validation and implementation processes will play a decisive role in the development of wearable biomedical technologies.

CONCLUSION

Current advances in the development of wearable textile electrodes demonstrate a great potential of the technology for non-invasive monitoring of physiological parameters and subsequent effective and widespread implementation in practical healthcare for the prevention, therapy, and rehabilitation. However, further research is needed to overcome a number of problems and current limitations with maximum implementation of the potential. New steps in the further scientific and technical development of the technology include not only the development of new materials and components, but also systematic validation, improvement of manufacturing processes, and increased accessibility for users and patients. Future research should focus on the development of materials with improved biocompatibility and durability, optimization of analytics, and more accurate evaluation of large arrays of biomedical data. A comprehensive consideration of these areas will accelerate the implementation of e-Textiles and their integration with other digital applications for intelligent health monitoring, which will generally improve the diagnostic efficiency of the technology and the quality of life of patients and users in real conditions.

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Osteogenesis Imperfecta in a Family with a Novel Variant in the *COL1A1* gene and Gonadal Mosaicism: a Clinical Case

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ABSTRACT

Osteogenesis imperfecta (OI) is a clinically heterogeneous genetic disorder whose primary clinical manifestations include susceptibility to recurrent pathological fractures and progressive skeletal deformities. In clinical practice, cases are observed where the proband's parents show no overt signs of the disease despite having multiple affected children. This may suggest gonadal mosaicism – a condition in which the causative variant is present only in a subset of parent's germ cells.

Gonadal mosaicism remains an understudied inheritance mechanism in monogenic diseases, complicating genetic counseling and reproductive risk assessment. In OI, this phenomenon may account for sporadic cases or recurrent births of affected children to clinically healthy parents.

This article presents a case report of a family in which the proband and his younger sister were diagnosed with *COL1A1*-associated OI, while the parents and other children showed no disease manifestations. Based on clinical and genetic data, the likelihood of gonadal mosaicism in one parent is discussed, along with considerations for differential diagnosis, patient management strategies, and family genetic counseling.

This case highlights the importance of molecular genetic testing not only for the probands but also for their parents to clarify the inheritance mechanism and predict the risks of disease recurrence in the family.

Keywords: osteogenesis imperfecta; gonadal mosaicism; *COL1A1*

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Несовершенный остеогенез в семье с новым вариантом в гене *COL1A1* и гонадным мозаицизмом: описание клинического случая

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РЕЗЮМЕ

Несовершенный остеогенез (НО) – генетически гетерогенное заболевание, основными клиническими проявлениями которого являются склонность к рецидивирующим патологическим переломам и прогрессирующая деформация скелета. В клинической практике встречаются случаи, когда у родителей пробанда отсутствуют явные признаки заболевания, несмотря на наличие нескольких пораженных детей, что может указывать на гонадный мозаицизм – состояние, при котором каузативный вариант присутствует только в части половых клеток родителя.

Гонадный мозаицизм остается недостаточно изученным механизмом наследования моногенных заболеваний, что создает сложности в генетическом консультировании и оценке репродуктивных рисков. В случае НО этот феномен может объяснять спорадические случаи или рекуррентные рождения больных детей у клинически здоровых родителей.

В статье представлен клинический случай семьи, в которой у пробанда и его младшей сестры диагностирован *COL1A1*-ассоциированный НО, тогда как родители и другие дети не имеют признаков заболевания. На основании клинических и генетических данных обсуждается вероятность гонадного мозаицизма у одного из родителей, а также рассматриваются вопросы дифференциальной диагностики, тактики ведения пациентов и медико-генетического консультирования семьи.

Это наблюдение подчеркивает важность молекулярно-генетического тестирования не только пробанда, но и его родителей для уточнения механизма наследования и прогнозирования рисков повторных случаев заболевания в семье.

Ключевые слова: несовершенный остеогенез, гонадный мозаицизм, *COL1A1*

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Авторы заявляют об отсутствии финансирования при проведении исследования.

Соответствие принципам этики. Все лица подписали информированное согласие на участие в исследовании. Исследование одобрено локальным этическим комитетом Федерального государственного бюджетного научного учреждения «Медико-генетический научный центр имени академика Н.П. Бочкова» (протокол № 4/1 от 19.04.2021).

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INTRODUCTION

Osteogenesis imperfecta (OI), also known as brittle bone disease, is a connective tissue disorder characterized by bone fragility and susceptibility to fractures. Individuals with OI typically present with low bone mass, susceptibility to long bone fractures, vertebral compression, various bone deformities, scoliosis, and growth deficiency. OI can also lead to a range of extra-skeletal manifestations, including blue sclerae, hearing loss, dentinogenesis imperfecta, basilar invagination, and cardiovascular and pulmonary abnormalities [1].

The estimated prevalence of OI is approximately 0.4–1.1 per 10,000 individuals based on population survey data and patient registries [2-4].

Approximately 90% of cases are caused by heterozygous pathogenic variants in the *COL1A1* and *COL1A2* genes, which encode type I collagen (α1 and α2 chains, respectively) [1, 5]. The remaining 10% of OI cases are caused by pathogenic variants in "non-collagen" OI genes with autosomal recessive (*P3H1, CRTAP, SPARC, TENT5A, KDELR2, BMP1, TMEM38B, CREB3L1, SERPINH1, PHLDB1, WNT1, SP7, PPIB, MESD, SERPINF1, FKBP10, CCDC134*), X-linked recessive (*MBTPS2*), and autosomal dominant (*IFITM5*) inheritance patterns (according to the OMIM® database) [6].

Type I collagen is the primary protein component of the extracellular matrix in bones, skin, and tendons and is predominantly secreted by osteoblasts, dermal fibroblasts, and tenocytes. The triple helix of type I collagen consists of two α1 chains and one α2 chain, which plays a crucial role in the stability of the entire collagen molecular structure. Generally, variants in the *COL1A1* gene result in a more severe clinical phenotype than those in the *COL1A2* gene. This can be explained by the molecular stoichiometry of type I collagen, which implies the presence of a mutant chain in 75% and 50% of collagen triple helices due to defects in the α1 and α2 chains, respectively [5, 7].

According to observations by S.M. Pyott et al., in 32% of families with asymptomatic parents and a first child with OI, the disease recurred in subsequent children [8]. The recurrence of OI in siblings is attributed to an autosomal recessive inheritance pattern or parental gonadal mosaicism, which is estimated to account for 5–8% of all OI cases [9–12].

Mosaicism is a genetic phenomenon in which an individual has two or more cell populations with distinct genotypes. In the context of OI, this means the causative variant is present only in a specific proportion of the cell pool. Gonadal mosaicism is of particular clinical interest – a specific form of mosaicism where the genetic abnormality is confined to a subset of germ cells (gametes), while somatic cells remain genetically normal. This circumstance is of fundamental importance for genetic counseling, as a clinically healthy parent with gonadal mosaicism can transmit the pathogenic variant to their offspring, explaining the occurrence of sporadic OI cases in families with no relevant medical history. The risk of transmitting the variant varies depending on the proportion of affected gametes in the gonads, creating significant challenges for assessing reproductive risks. The mechanism underlying mosaicism involves postzygotic sequence variants occurring during early embryonic development, highlighting the necessity of using modern molecular genetic diagnostic methods to accurately determine the nature and prevalence of mosaicism in each specific case.

CASE REPORT

Proband: A 7-year-old male. Family history: the parents are of mixed ethnic background (mother IS Tatar, father IS Kazakh). He has an elder brother and two elder sisters (all clinically healthy), as well as a younger sister (2.5 years old) who has bluish sclerae and experienced a lower leg fracture before the age of 1. The mother's obstetric – gynecological history includes 8 pregnancies (5 deliveries, 3 abortions). The current pregnancy of the mother (resulting in the proband) was her seventh, complicated by anemia and toxemia. Perinatal history: the proband was delivered at term during the mother's fourth spontaneous vaginal delivery. His birth weight was 3,450 g, birth length was 52 cm, and the Apgar score was 8/8. A cephalohematoma was noted at birth. Developmental history: the proband's motor, mental, and speech development were age-appropriate.

Presenting complaints: multiple fractures (6 episodes) and impaired vision. History of fractures: at 11 months: fracture of the left tibia; at 1 year and 4 months: fracture of the right tibia; at 2 years: consolidated fracture of both bones of the right lower leg; at 3 years and 5 years: repeated fractures of the right lower leg; at 5 years and 8 months: closed displaced fracture of both bones of the left lower leg. At the age of 6 years and 1 month, the diagnosis of osteogenesis imperfecta was first established clinically, and therapy with bisphosphonates was initiated.

INSTRUMENTAL AND LABORATORY FINDINGS

Bone densitometry (in the context of bisphosphonate therapy) revealed reduced bone mineral density (BMD) in the lumbar spine (L1–L4) with a Z-score of –2.1. Total body BMD was not reduced (Z-score –0.3).

Blood biochemistry: a single episode of low ionized calcium level at 1.02 mmol / l (reference range 1.13–1.32 mmol / l) and vitamin D deficiency at 24.6 ng / ml. Other parameters of calcium – phosphate metabolism (alkaline phosphatase, total calcium, inorganic phosphorus, parathyroid hormone) were within normal limits.

Radiography of the lower leg bones: the bones showed a moderate valgus deformity; rarefaction of

the bone structure with horizontal sclerotic bands in the metaphyses was observed.

Pure tone audiometry: no hearing pathology was detected.

PHENOTYPIC FEATURES

Anthropometric data: head circumference – 51 cm (–0.85 SD), height – 116 cm (–0.89 SD), weight – 20 kg (BMI –0.52 SD). Facial phenotype: synophrys, blue – grey sclerae, long eyelashes, flattened nasal bridge. Other characteristics: significant joint hypermobility, widely spaced nipples (nipple hypertelorism), pes planovalgus (flat feet), and impaired/abnormal posture.

Clinical course: following the initiation of bisphosphonate therapy, no new fractures have been recorded. A positive trend was observed, evidenced by improved bone mineral density on follow-up densitometry of the lumbar spine after one year, with an increase in the Z-score (L1–L4) to –1.5. The total body Z-score was –0.2.

To verify the clinical diagnosis, quad whole-genome sequencing with the analysis of the Arthrogryposes panel was performed for the proband, his younger sister, and both parents. Sequencing was conducted in collaboration with Biotechnology Campus LLC as part of the 100,000 + Me national genetic initiative.

DNA analysis (isolated from peripheral blood lymphocytes) of the patient and family members was performed on a DNBSEQ-T7 genetic analyzer by the paired-end (PE150) method. Sample preparation was performed by the PCR-free method with enzymatic fragmentation (MGI).

A previously unreported heterozygous variant of uncertain significance was identified in exon 39 of the *COL1A1* gene (chr17:50189527del). This variant led to a frameshift (NM_000088.4: c.2679del, p.(Pro895LeufsTer213)). The identified variant was not present in the control population of the Genome Aggregation Database (gnomAD v3.1.2). Heterozygous variants in the *COL1A1* gene were described in patients with type II OI (OMIM: 166210), Caffey disease (OMIM: 114000), Ehlers – Danlos syndrome, type 1 arthrochalasia (OMIM: 130060), type I OI (OMIM: 166200), {Bone mineral density variation QTL, osteoporosis} (OMIM: 166710), combined OI and Ehlers – Danlos syndrome 1 (OMIM: 619115), type IV OI (OMIM: 166220), and type III OI (OMIM: 259420). The depth of coverage at the variant position was 37x. Based on the totality of this evidence, the variant was classified as likely pathogenic according to the Guidelines for the Interpretation of Human DNA Sequence Data Obtained by Massively Parallel Sequencing (MPS) Methods [13].

The same variant was also identified in a heterozygous state in the proband's younger sister but was not detected in either parent. These findings suggest parental gonadal mosaicism. Investigation of paternal semen samples is currently planned to test for the presence of the identified variant.

CONCLUSION

The analysis of this clinical case of OI, associated with a novel *COL1A1* variant and suspected gonadal mosaicism, highlights the diagnostic challenges and complexities in genetic counseling for this condition. The identification of a likely pathogenic variant in the proband and his sister, despite the absence of clinical manifestations in the parents, underscores the necessity for comprehensive genetic testing of all family members, including screening for mosaic forms, which are reported in 5–8% of OI cases [9, 10].

The presumption of gonadal mosaicism significantly alters the assessment of reproductive risks. The empirical average recurrence risk for a family with a child affected by an autosomal dominant disease exceeds the general population risk and is estimated at 1–3%. However, in individual cases, the actual risk of disease recurrence is influenced by the proportion of affected gametes, posing significant challenges for genetic counseling [14]. An important aspect is the application of high-coverage next-generation sequencing (NGS) methods to detect low-level mosaicism. Paternal gonadal mosaicism can be identified through molecular genetic analysis of spermatozoa [15]. A distinct challenge remains the detection of maternal gonadal mosaicism due to the inability to routinely study oocytes. For couples with a high risk of gonadal mosaicism (e.g., those with multiple affected children), the use of *in vitro* fertilization with preimplantation genetic testing for monogenic disorders (PGT-M) or invasive prenatal diagnosis are potential options.

This observation emphasizes the importance of developing standardized approaches to diagnosing mosaic forms of OI, including the application of advanced sequencing techniques, and the need to establish patient registries for such cases to better understand the clinical significance of varying levels of mosaicism. The obtained results broaden the understanding of the genetic heterogeneity of OI and highlight the critical role of interdisciplinary collaboration in managing these patients. This is essential for refining diagnostic

algorithms, optimizing therapeutic strategies, and improving the quality of genetic counseling for families with suspected mosaic forms of the disease.

Prospects for future research include investigating the correlation between the level of mosaicism and clinical manifestations, developing new pathogenetic treatments, and refining management protocols for patients with rare forms of OI.

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К участию приглашаются научные и научно-педагогические работники, специалисты в области общей патологии и патологической физиологии, аспиранты и молодые ученые.

Формат конференции – гибридный (очный + онлайн).

Формы участия в конференции: пленарные доклады ведущих ученых (20-25 минут), устные доклады участников (15 минут), стендовые доклады, публикация материалов (тезисы) в сборнике РИНЦ.

В рамках конференции будет организована секция молодых ученых с конкурсом на лучший доклад.

КЛЮЧЕВЫЕ ДАТЫ

- Регистрация участников и направление материалов тезисов до 1 мая 2026 года
- Информирование участников о включении в программу до 15 мая 2026 года
- Направление видеопрезентации стендового доклада в оргкомитет до 25 мая года
- Публикация предварительной программы конференции до 25 июня 2026 года
- Публикация сборника тезисов до 20 сентября 2026 года

РЕГИСТРАЦИЯ

Регистрация необходима на каждый доклад/тезисы. Заявки на участие и тезисы принимаются до 1 мая 2026 г. Заявки на участие могут быть поданы только через автоматическую систему регистрации участников:

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1. Программа Power Point (или другие программы для создания презентаций).
2. Фон слайдов – белый; шрифт текста – черный. Размер слайдов – широкоформатный 16:9.
3. В оформлении использовать таблицы, графики, фото, изображения, диаграммы и прочие визуальные средства передачи информации.
4. Текстовую информацию записать как пояснения к слайдам на аудиосопровождение к презентации.
5. После записи аудиокомментариев к слайдам проверить качество воспроизведения звука: громкость, отсутствие шумовых помех.

Оргкомитет оставляет за собой право исключить из программы стендовые доклады, не соответствующие техническим требованиям, и стендовые доклады, полученные после 25 мая 2026 г

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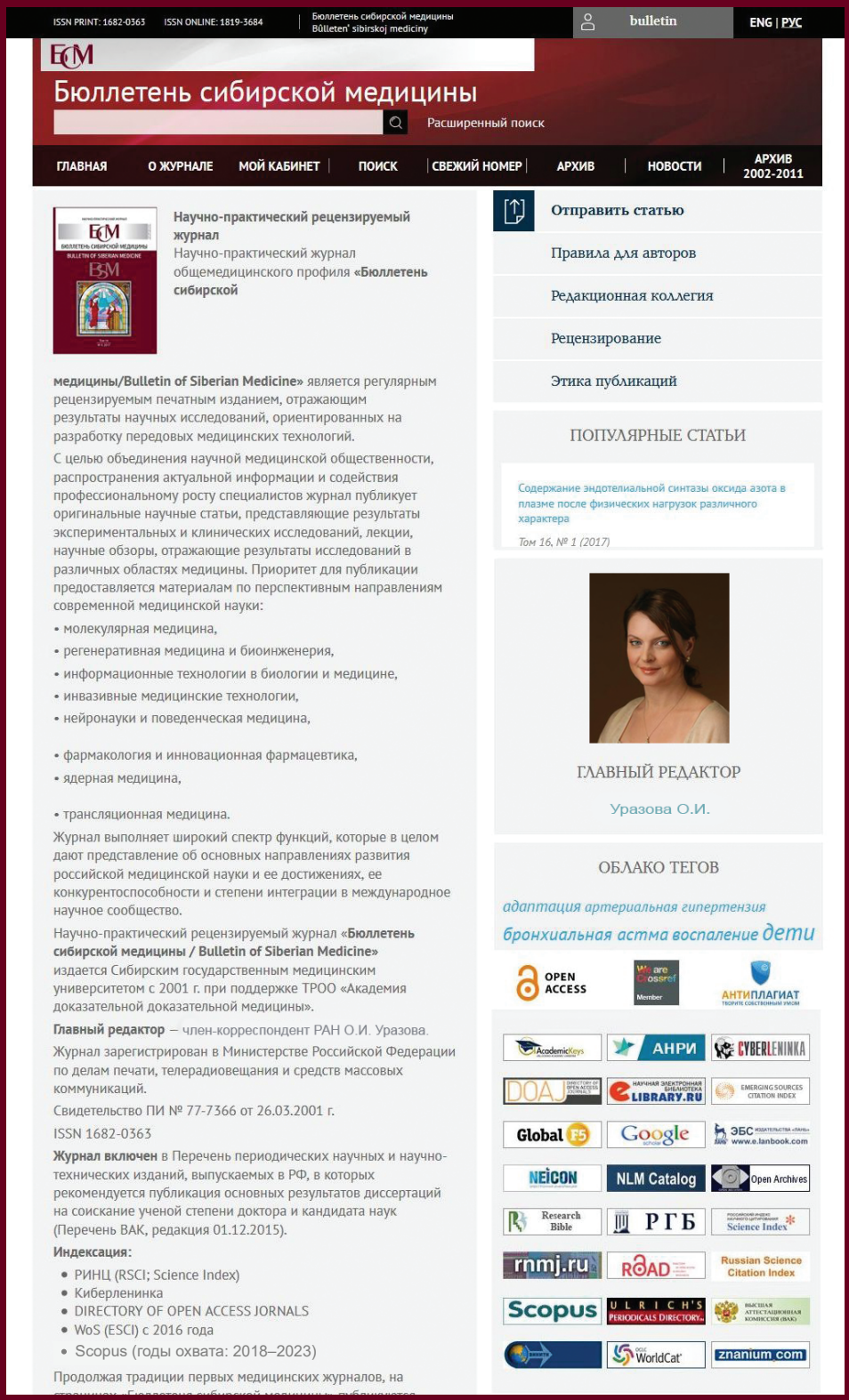

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