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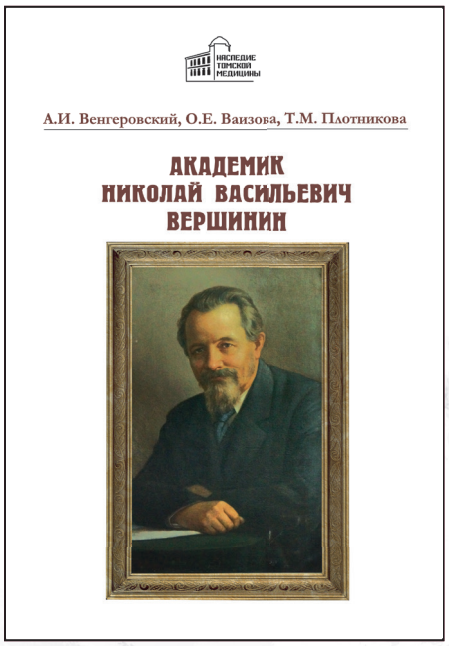

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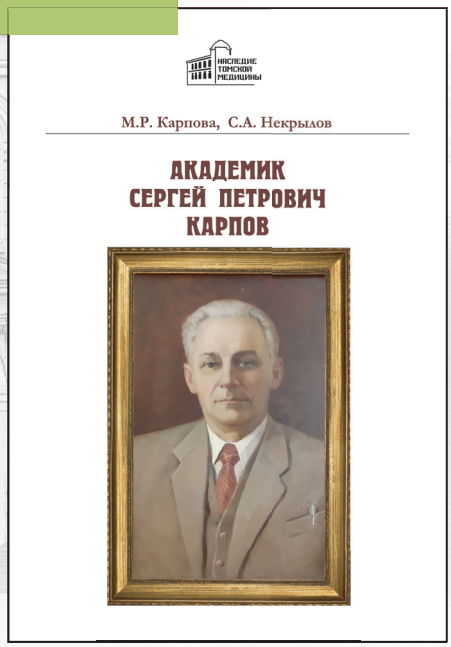

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Effect of COVID-19 infection in the third trimester of pregnancy on innate immunity parameters, association with obstetric and perinatal outcomes

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ABSTRACT

Aim. To analyze and compare parameters of innate immunity with obstetric and perinatal outcomes in patients with COVID-19 in the third trimester of pregnancy.

Materials and methods. The study included 2 groups: the main group encompassed patients with mild (subgroup 1, *n* = 31) and moderate (subgroup 2, *n* = 40) COVID-19 during the third trimester of pregnancy; the control group included women who did not have COVID-19 during pregnancy (*n* = 22). By the enzyme-linked immunosorbent assay (ELISA), we determined the level of anti-SARS-CoV-2 immunoglobulin (Ig)M and IgG, tumor necrosis factor alpha (TNFα), interleukin 6 (IL-6), and interferon gamma (IFNγ) in the blood plasma. Complete blood count was performed on the automated hematology analyzer. Expression of CD-14 and HLA-DR antigens in monocytes was analyzed on the flow cytometer. SARS-CoV-2 RNA in placenta samples was detected by the reverse transcription polymerase chain reaction (RT-PCR).

Results. A moderate course of COVID-19 in the third trimester of pregnancy was associated with lower levels of anti-SARS-CoV-2 IgG and IFNγ in the maternal blood and umbilical cord blood, as well as by lower expression of CD-14 and HLA-DR by monocytes compared to mild COVID-19. A mild course of the disease was characterized by an increase in the number of monocytes in the maternal blood. No differences in leukocyte and lymphocyte counts were noted. There were also no differences in birth weight and one-minute Apgar score. At 5 minutes, the Apgar scores for moderate COVID-19 were lower than those for mild infection. The moderate course of COVID-19 increased the risk of preterm birth, neonatal cerebral ischemia, intraventricular hemorrhage, and respiratory distress syndrome. No risk of intrauterine SARS-CoV-2 infection was detected.

Conclusion. The severity of COVID-19 in the third trimester of pregnancy is associated with dysregulation of the innate immunity, which determines the nature of obstetric and perinatal complications.

Keywords: COVID-19, innate immunity, cytokines, obstetric and perinatal outcomes

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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Conformity with the principles of ethics. All patients signed an informed consent to participate in the study. The study was approved by the local Ethics Committee at the Far Eastern Research Center for Physiology and Pathology of Respiration (Protocol No. 144 of 09.06.2023).

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Влияние перенесенной в третьем триместре беременности инфекции COVID-19 на показатели врожденного иммунитета, связь с акушерскими и перинатальными исходами

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РЕЗЮМЕ

Цель. Анализ и сопоставление данных врожденного иммунитета с акушерскими и перинатальными исходами при перенесенной в третьем триместре беременности инфекции COVID-19.

Материалы и методы. В исследование включены две группы: основная – с перенесенной в третьем триместре беременности инфекцией COVID-19 легкого (подгруппа 1, *n* = 31) и среднетяжелого течения (подгруппа 2, *n* = 40), контрольная – женщины, не болевшие COVID-19 в течение всей беременности (*n* = 22). В плазме крови иммуноферментным методом определяли уровень анти-SARS-CoV-2 иммуноглобулинов (Ig) классов M и G, содержание цитокинов фактора некроза опухоли альфа (TNFα), интерлейкина (IL) 6 и интерферона гамма (IFNγ). Клинический анализ крови осуществляли на автоматическом гематологическом анализаторе, экспрессию CD14- и HLA-DR-антигенов в моноцитах – на проточном цитометре, РНК SARS-CoV-2 в образцах плаценты – методом обратной транскрипции полимеразной цепной реакции.

Результаты. Среднетяжелое течение COVID-19 в третьем триместре беременности ассоциировалось с более низким уровнем в крови у матери и в крови пуповины новорожденных анти-SARS-CoV-2 IgG, IFNγ, а также экспрессии моноцитами CD14 и HLA-DR по сравнению с легкой формой заболевания. При легкой форме отмечено повышение количества моноцитов в крови матери. Различий в показателях лейкоцитов и лимфоцитов не выявлено. Также отсутствовали различия по массе тела новорожденных и оценке по шкале Апгар на 1-й мин. На 5-й мин показатели при среднетяжелом течении заболевания были ниже, чем при легкой форме инфекции. Среднетяжелое течение COVID-19 увеличивало риск преждевременных родов, развития церебральной ишемии мозга новорожденных, внутрижелудочковых кровоизлияний и синдрома дыхательных расстройств. Риск внутриутробной SARS-CoV-2 инфекции отсутствовал.

Заключение. Тяжесть течения COVID-19 в третьем триместре беременности связана с дисрегуляцией врожденного иммунитета, что определяет характер акушерских и перинатальных осложнений.

Ключевые слова: COVID-19, врожденный иммунитет, цитокины, акушерские и перинатальные исходы

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

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INTRODUCTION

Since March 2019, the world has been affected by the pandemic of novel coronavirus infection (COVID-19), which was listed as a public health emergency of international concern until May 2023 [1]. Viral mutations and new variants of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) are associated with a varying clinical course of the disease (ranging from mild to severe) linked to immune dysregulation [2]. It is reported that pregnant women with COVID-19, particularly those with the Delta variant of the disease, have an increased risk of hospitalization and developing severe disease compared to the general population [3].

According to other studies, pregnant and non-pregnant patients show similar risks of infection and developing severe manifestations of COVID-19 [4]. The role of COVID-19 in the development of placental insufficiency, preterm birth, and stillbirth has also been determined [5]. However, definitive conclusions can only be drawn after the end of the pandemic.

Myeloid cells (monocytes / macrophages) are believed to be directly involved in the pathogenesis of COVID-19. Studies have reported various responses of monocytes to SARS-CoV-2, determining the development of systemic inflammatory response syndrome [6], hyperactivation or a lack of response to type I interferons (IFN-I) in the blood and lung tissues in severe forms of the disease [7]. Authors noted dysregulation of innate immunity and decreased expression of human leukocyte antigen class II (HLA-DR) by monocytes, which is considered as a marker of immunosuppression and severity of COVID-19 [8].

Other studies have shown the differences in phenotypes of peripheral and lung myeloid cells with low expression of HLA-DR, dysfunctional blood monocytes, and hyperactive monocytes / macrophages of the respiratory tract producing proinflammatory cytokines in moderate and severe COVID-19 [9]. However, the effect of pregnancy on the development of innate immunity in mothers and their newborns following COVID-19 remains poorly studied, and research is limited in scope [10].

The aim of this work was to analyze and compare parameters of innate immunity with obstetric and perinatal outcomes in mothers with COVID-19 in the third trimester of pregnancy.

MATERIALS AND METHODS

The study was carried out in accordance with the principles of the Declaration of Helsinki and approved by the Bioethics Committee at the Far Eastern Research Center for Physiology and Pathology of Respiration (Protocol No. 144 of 09.06.2023). All participants signed a written informed consent to participate in the study. The clinical site for the study was the maternity unit of the Blagoveshchensk City Clinical Hospital. Laboratory studies were carried out at the Far Eastern Research Center for Physiology and Pathology of Respiration. From January 2022 to March 2023, 93 women at 35–40 weeks pregnant were examined: 71 women with mild (subgroup 1, $n = 31$) and moderate (subgroup 2, $n = 40$) COVID-19 during the third trimester of pregnancy (main group) and 22 women who did not have COVID-19 during the entire pregnancy (control group). All studies were conducted during the predominant circulation of SARS-CoV-2 Omicron strain.

Inclusion criteria for the main group were: singleton, spontaneous pregnancy; COVID-19 in the third trimester of pregnancy; clinical symptoms of a respiratory disease; CT (computed tomography) signs of viral pneumonia with a typical clinical presentation and relevant epidemiological history. Exclusion criteria were multiple pregnancies; pregnancy resulting from *in vitro* fertilization; exacerbation of chronic noninfectious diseases; presence of chronic nonspecific lung diseases; extrapulmonary foci of infections; specific bronchopulmonary diseases; developmental genital anomalies; detected sexually transmitted infections; progestogen support; immunodeficient conditions; smoking. All study participants were selected as cases and controls and were comparable in age and body mass index (BMI).

The age in subgroup 1 was 27.0 (25.0; 30.0) years ($p = 0.441$); the age in subgroup 2 was 27.0 (25.0; 30.0) years ($p = 0.465$), which had no significant difference compared to the control group – 28.5 (25.7; 31.0) years. BMI values in subgroup 1 were 24.7 (23.0; 29.1) ($p = 0.691$) and in subgroup 2 – 24.8 (21.7; 29.3) ($p = 0.669$), which also did not differ significantly from the control group – 24.6 (22.1; 25.0). In the main group, no significant differences in age ($p = 0.968$) and BMI ($p = 0.954$) were found.

Blood samples for the studies were taken at the time of hospitalization in the maternity unit by venipuncture into ethylenediaminetetraacetic acid (EDTA) vacuum tubes (China). Umbilical cord blood was collected from the central vein into EDTA vacuum tubes immediately after cord clamping soon after birth. Blood plasma was obtained by centrifugation

(15 min, 1,000 g). All plasma samples were stored at –70 °C until the analysis was started. Placental material was collected immediately after birth and placed in sterile containers. Sample preparation, extraction, and amplification of SARS-CoV-2 RNA were carried out by reverse transcription polymerase chain reaction (RT-PCR) on the DT-96 detection amplifier (DNA-Technology, Russia) using commercial reagent kits (DNA-Technology, Russia) in strict accordance with the manufacturer's instructions. The enzyme-linked immunosorbent assay (ELISA) was used to determine anti-SARS-CoV-2 IgM and IgG in paired plasma samples (SARS-CoV-2-IgM ELISA-BEST Kit, SARS-CoV-2-IgG quantitative-ELISA-BEST Kit, Russia), levels of tumor necrosis factor alpha (TNFα) (TNF alpha-ELISA-BEST Kit, Russia), interleukin 6 (IL-6) (Interleukin-6-ELISA-BEST Kit, Russia), and interferon gamma (IFNγ) (Interferon gamma-ELISA-BEST, Russia).

All studies were performed on the Multiskan FC microplate photometer (USA) in strict accordance with the manufacturer's instructions for commercial reagent kits. Clinical blood test was carried out on the automated hematology analyzer Mindray BC-5150 (Shenzhen Mindray Bio-Medical Electronics Co., Ltd., China). Flow cytometry of peripheral blood mononuclear cells was conducted on the BD FacsCanto II flow cytometer (USA). We used lysed blood (Invitrogen™ eBioscience™ 10X RBC Lysis Buffer, USA) containing FITC-conjugated monoclonal antibodies to surface antigens CD14 (M5E2) (BD Biosciences, USA) and HLA-DR (L243) (BioLegend, USA). The mononuclear cell pellet obtained after two washes in phosphate buffered saline (PBS) (Biolot, Russia) and centrifugation (5 min, 400 g) was resuspended and used to detect monoclonal antibodies.

Statistical analysis and data processing were carried out using the IBM SPSS Statistics version 23.0 software package (USA). The statistical analysis was performed using the Mann – Whitney test for paired comparisons. To compare three or more groups, the Kruskal – Wallis test was used. Quantitative variables were presented as the median (Me) and the interquartile range (Q_{25}; Q_{75}); categorical data were presented as proportions, frequencies, and percentages. The analysis of frequency differences in two independent study groups was conducted using the Fisher's exact test. With absolute frequencies in contingency tables being less than 10, the Yates' correction was used. The correlation analysis was conducted using the Spearman's rank correlation coefficient. Relative risks (RR) were analyzed using fourfold contingency tables with 95% confidence intervals (95% CI). Differences were considered statistically significant at a $p < 0.05$.

RESULTS

All women in the main group at the time of the study had a confirmed diagnosis of COVID-19: 43.7% were diagnosed with mild acute respiratory viral infection (ARVI) (subgroup 1), and 56.3% – with moderate disease with manifestations of pneumonia (subgroup 2) (RR = 1.27; 95% CI 0.96–1.69). The gestational age at the time of the disease in subgroup 1 was 35.0 (33.0; 37.0) weeks, and in subgroup 2 – 34.0 (32.0; 36.0) weeks ($p = 0.181$). The total time from the disease onset to delivery in subgroup 1 and subgroup 2 was 27.0 (18.0; 36.0) days and 32.0 (15.0; 48.0) days, respectively ($p = 0.286$).

In all women of the main group, SARS-CoV-2 IgM antibodies were absent in both maternal blood and umbilical cord blood (Table 1). However, the amount of IgG antibodies in subgroup 1 was 1.53 times higher than in subgroup 2. Intragroup paired comparisons did not reveal significant differences between the values of IgG antibodies in maternal blood and umbilical cord blood in subgroup 1 ($p = 0.992$) and subgroup 2 ($p = 0.371$). Further paired correlation analysis in the study subgroups revealed a significant association between the levels of IgG antibodies in maternal blood and umbilical cord blood in subgroup 2 ($r = 0.61$, $p = 0.0001$).

Blood test in women of the study groups revealed an increase in the average monocyte count by 1.16 times in subgroup 1 compared to the control group, while no significant differences were found compared to subgroup 2. No differences in leukocyte and lymphocyte counts were identified when comparing the main group and the control group.

The study of the proinflammatory cytokine profile in the blood of women showed that in subgroup 1, the values of TNFα were 1.72 times and 1.22 times lower than in the control group and subgroup 2, respectively. The level of IL-6 in subgroup 1 was 1.55 times and 1.35 times lower than in the control group and subgroup 2, respectively. The values of IFNγ in subgroup 1 were 1.9 times higher than in subgroup 2. In subgroup 2, the level of IFNγ did not change significantly compared to the control group. Significant differences in the levels of IL-6 and IFNγ were found in umbilical cord blood. In subgroup 1, the levels of IL-6 were

1.45 times lower than in subgroup 2 and did not significantly differ from those in the control group. The levels of IFNγ in subgroup 2 were reduced by 1.23 times and 1.11 times compared to subgroup 1 and the control group, respectively. Paired comparison of TNFα, IFNγ, and IL-6 values in maternal and umbilical cord blood revealed differences between the studied subgroups. The levels of TNFα in maternal blood were 2.02 times (p = 0.0001) and 1.8 times (p = 0.0001) lower than in umbilical cord blood. The levels of IFNγ were 1.1 times (p = 0.0001) and 1.67 times (p = 0.0001) higher, and the levels of IL-6 were 2.15 times (p = 0.0001) and 1.91 times (p = 0.0001) higher in subgroups 1 and 2, respectively.

Table 1

Parameters of innate immunity in maternal and umbilical cord blood in the study groups, *Me* (Q_{25}; Q_{75})

Parameter	Main group		Control group	p
	Subgroup 1	Subgroup 2		
Peripheral blood				
Anti-SARS-CoV-2 IgG, BAU / ml	168.0 (104.0; 216.0)	110.0 (56.0; 197.2)	–	p_3 = 0.029
TNFα, pg / ml	30.0 (22.0; 47.9)	42.3 (27.1; 61.8)	51.5 (36.9; 58.5)	p_1 = 0.001; p_2 = 0.485; p_3 = 0.004
IL-6, pg / ml	20.9 (17.7; 29.5)	27.0 (17.9; 64.3)	31.9 (18.4; 49.2)	p_1 = 0.034; p_2 = 0.900; p_3 = 0.042
IFNγ, pg / ml	4.0 (2.8; 5.0)	2.1 (2.0; 2.6)	2.7 (2.0; 6.1)	p_1 = 0.780; p_2 = 0.074; p_3 = 0.0001
Leukocytes, 10^9 / l	8.75 (7.36; 9.82)	8.1 (7.0; 9.3)	8.2 (7.5; 9.6)	p_1 = 0.950; p_2 = 0.498; p_3 = 0.582
Lymphocytes, 10^9 / l	21.5 (15.0; 25.2)	16.4 (3.4; 21.6)	18.5 (15.9; 20.5)	p_1 = 0.279; p_2 = 0.260; p_3 = 0.164
Monocytes, 10^9 / l	7.34 (6.1; 8.7)	6.7 (5.1; 8.2)	6.5 (4.6; 7.2)	p_1 = 0.044; p_2 = 0.480; p_3 = 0.194
CD14, %	78.9 (73.5; 83.4)	55.1 (49.8; 63.3)	94.5 (92.8 ;97.8)	p_{1_3} = 0.0001
HLA-DR, %	78.3 (74.0; 83.2)	52.9 (48.5; 60.7)	95.2 (92.8; 98.4)	p_{1_3} = 0.0001
Umbilical cord blood				
Anti-SARS-CoV-2 IgG, BAU / ml	142.0 (102.0; 240.0)	109.0 (25.3; 194.0)	–	p_3 = 0.037
TNFα, pg / ml	60.5 (58.6; 81.3)	76.0 (65.2; 89.5)	85.1 (74.8; 90.0)	p_1 = 0.006; p_2 = 0.236; p_3 = 0.064
IL-6, pg / ml	9.7 (7.6; 11.0)	14.1 (10.9; 23.6)	7.9 (4.8; 35.0)	p_1 = 0.657; p_2 = 0.358; p_3 = 0.0001
IFNγ, pg / ml	4.3 (3.3; 5.7)	3.5 (2.6; 4.0)	3.9 (3.1; 5.7)	p_1 = 0.619; p_2 = 0.007; p_3 < 0.0001
CD14, %	77.7 (74.5; 82.7)	55.6 (50.7; 59.7)	96.4 (92.6; 98.2)	p_{1_3} = 0.0001
HLA-DR, %	78.6 (73.2; 83.1)	58.6 (50.9; 66.1)	95.4 (93.8; 96.7)	p_{1_3} = 0.0001

Note. Here and in Table 2–4: p_1 – statistical significance of differences between subgroup 1 and the control group; p_2 – statistical significance of differences between subgroup 2 and the control group; p_3 – statistical significance of differences between subgroup 1 and subgroup 2.

Significant paired correlations in subgroup 1 were found between the levels of TNFα (r = 0.78, p = 0.0001), IL-6 (r = 0.72, p = 0.0001), and IFNγ (r = 0.84, p = 0.0001) in maternal blood and umbilical cord blood. In subgroup 2, a correlation was found between the levels of IFNγ in maternal blood and umbilical cord blood (r = 0.60, p = 0.0001). Investigating the antigen composition of monocytes in maternal blood revealed that in subgroup 1, the expression of CD14 was 1.2 times lower than in the control group and 1.43 times higher than in subgroup 2. In subgroup 2, the number of monocytes expressing CD14 was 1.71 times smaller than in the control group. The analysis of HLA-DR expression in subgroup 1 revealed a decrease by 1.21 times compared to the control group and an increase by 1.48 times compared to subgroup 2. In subgroup 2, the HLA-DR values were 1.8 times lower than in the control group. In subgroup 1, a decrease in the circulation of CD14 by 1.24 times was noted in umbilical cord blood compared to the control group and an increase by 1.4 times compared to subgroup 2. In subgroup 2, monocytes were characterized by lower levels of CD14 (by 1.73 times) compared to the control group.

The analysis of HLA-DR expression in umbilical cord blood monocytes in subgroup 1 showed a decrease by 1.21 times compared to the control group and an increase by 1.34 times compared to subgroup 2. In subgroup 2, the HLA-DR values were 1.63 times lower compared to the control group. In paired comparisons of the average CD14 and HLA-DR values in maternal blood monocytes and umbilical cord blood, no significant differences were found for subgroup 1 (p = 0.576 and p = 0.468, respectively) and for subgroup 2 (p = 0.968 and p = 0.05, respectively). Significant paired correlations in subgroup 1 were found between the parameters of maternal blood and umbilical cord blood for CD14 (r = 0.63, p = 0.0001) and HLA-DR (r = 0.48, p = 0.007).

It is worth noting that in the studied subgroups, none of the placental samples showed the presence of SARS-CoV-2, indicating the absence of a risk of its vertical transmission to the fetus.

Table 2 presents pregnancy outcomes in the studied groups. Full-term births occurred in all women in subgroup 1 and in 87.5% of women in subgroup 2. The gestational age at the time of delivery in subgroup 1 was 39.0 (38.0; 40.0) weeks and did not have significant differences from the control group – 39.0 (38.0; 40.0) weeks (p = 0.756); however, it was significantly higher than in subgroup 2 – 38.0 (37.0; 39.0) weeks (p = 0.034). The differences between subgroup 2 and the control group were also statistically significant (p = 0.027). Preterm births (PB) (O60.1) occurred in 12.5% of women in subgroup 2. Natural childbirth delivery (NCD) took place in 93.55% of women in subgroup 1 and in 87.5% of women in subgroup 2, while cesarean delivery (CD) – in 6.45% and 12.5% of women, respectively. Indications for elective CD were: mismatch between the pelvic size and the fetal head size, uterine scar after CD, incompetent cervix, breech presentation of the fetus with anticipated weight of more than 3,600 grams, and placenta previa. Premature rupture of membranes (PROM) (O42) occurred in 12.9% of women in subgroup 1, which was significantly less often than in subgroup 2 – 27.5%. The study showed that moderate severity of COVID-19 increased the risk of PROM (RR = 2.13 (95% CI 1.17–3.87)) compared to subgroup 1.

The average birth weight of newborns did not significantly differ between the subgroups and compared to the control group. The condition of the newborns was assessed by the Apgar score at 1 and 5 minutes (Table 3).

Table 2

Pregnancy outcomes in women of the study groups

Parameter	Main group				Control group		p
	Subgroup 1		Subgroup 2				
	abs.	%	abs.	%	abs.	%	
NBD	29	93.55	35	87.5	21	95.45	$p_1 = 0.552$; $p_2 = 0.049$; $p_3 = 0.158$
CD	2	6.45	5	12.5	1	4.54	$p_1 = 0.746$; $p_2 = 0.069$; $p_3 = 0.595$
PB	–	–	5	12.5	–	–	
PROM	4	12.9	11	27.5	2	9.1	$p_1 = 0.498$; $p_2 = 0.002$; $p_3 = 0.022$

Table 3

Birth weight and Apgar score in newborns delivered by mothers of the study groups, *Me* (Q_{25}; Q_{75})

Parameter	Main group		Control group	p
	Subgroup 1	Subgroup 2		
Birth weight, g	3,300.0 (3,190.0; 3,550.0)	3,295.0 (2,817.0; 3,737.0)	3,200.0 (3,040.0; 4,000.0)	$p_1 = 0.550$; $p_2 = 0.768$; $p_3 = 0.503$
Apgar score:				
– at 1 minute;	8.0 (8.0; 9.0)	8.0 (8.0; 9.0)	8.0 (8.0; 9.0)	$p_1 = 0.735$; $p_2 = 0.628$; $p_3 = 0.806$
– at 5 minutes	9.0 (9.0; 10.0)	9.0 (8.0; 9.0)	9.0 (9.0; 10.0)	$p_1 = 0.798$; $p_2 = 0.007$; $p_3 = 0.003$

No significant differences in the Apgar scores at 1 minute were found either between subgroups 1 and 2 or between the control group and the study subgroups. However, a decrease in the Apgar score at 5 minutes was determined in newborns delivered by mothers in subgroup 2 compared to those delivered by mothers in subgroup 1 and the control group. Cerebral ischemia (CI) (P91.0) was diagnosed in 6.45% of newborns in subgroup 1 and in 21.9% of babies in subgroup 2 (Table 4). Newborns of mothers in subgroup 2 had a higher risk of CI, RR = 3.83 (95% CI 1.63–9.01) compared to those delivered by mothers in subgroup 1. Respiratory distress syndrome (RDS) and intraventricular hemorrhages (IVH) were diagnosed only in newborns delivered by mothers in subgroup 2.

Table 4

Incidence in newborns delivered by mothers of the study groups, persons

Parameter	Main group				Control group, 22		p
	Subgroup 1, 31		Subgroup 2, 40				
	abs.	%	abs.	%	abs.	%	
CI	2	6.45	9	22.5	–	–	0.003
IVH	–	–	5	12.5	–	–	
RDS	–	–	7	17.5	–	–	

Note. Statistical difference between the parameters of subgroup 1and subgroup 2 – p.

DISCUSSION

Studies have shown that COVID-19 can alter innate immunity in pregnant women not only during the acute phase of the disease but also after recovery. Our results showed that COVID-19 infection in the third trimester of pregnancy induced a sustained antibody and cytokine response at the time of delivery and caused a significant decrease in transplacental transfer of IgG antibodies with a more pronounced negative proinflammatory effect of TNFα and IL-6 and a reduced proinflammatory effect of IFNγ in moderate infection, which is in line with the available data [11].

Mild infection was associated with higher levels of IgG antibodies and reduced levels of TNFα and IL-6 in maternal blood and umbilical cord blood. In moderate infection, reduced IFNγ levels were also noted, apparently due to increased circulation of IL-6 and insufficient production of antiviral antibodies [12]. Maternal levels of IgG antibodies and proinflammatory cytokines were correlated with values in umbilical cord blood; the strength of the correlation was determined by the severity of COVID-19 in the third trimester of pregnancy.

We also detected no significant differences in lymphocyte and leukocyte count in maternal blood regardless of the severity of COVID-19 amidst the variability of the proinflammatory cytokine profile, indicating their dysfunction. Nevertheless, the predominance of the inflammatory cytokine profile in the blood of mothers with past COVID-19 should be considered in the context of significant fluctuations in parameters during full-term and preterm labor [13]. The study of the monocyte response to COVID-19 in the third trimester showed an increase in their count in maternal blood in mild COVID-19 compared to moderate disease. The percentage of classical CD14 monocytes and monocytes expressing HLA-DR in maternal blood and umbilical cord blood was reduced according to the severity of COVID-19 infection, determining complex immune dysregulation and forming temporary immunosuppression [14]. The decrease in the HLA-DR expression on cell membranes of CD14 monocytes was likely linked to the inhibitory effect of IL-6 [15]. Maternal levels of CD14 and HLA-DR were correlated with the ones in the umbilical cord blood. Regarding obstetric and perinatal outcomes, moderate COVID-19 in the third trimester increased the risk of preterm births, which is consistent with the data of systematic reviews and meta-analyses [16].

However, according to some reports, Omicron variant infection of pregnant women did not increase the risk of preterm birth compared to the Delta variant [17], though these findings require confirmation. PROM in moderate COVID-19 occurred 2.13 times more frequently than in mild infection, potentially increasing the risk of neonatal infection and associated complications. However, no SARS-CoV-2 nucleic acid was detected in any of the placental samples obtained from women with COVID-19 in the third trimester of pregnancy, which is consistent with the available data [18].

In assessing the condition of newborns, no differences in body weight and 1-minute Apgar score were found, although 5-minute scores in moderate infection were lower than in mild forms of the disease, which is consistent with research data and possibly indicates lower adaptive capacity of the newborn [19]. The risk of CI in newborns delivered by mothers with moderate COVID-19 increased by 3.83 times compared to mild infection. In 12.5% of newborns, IVH was diagnosed, and 17.5% of babies had RDS.

Therefore, the dysregulation of innate immunity in maternal blood and umbilical cord blood established in the study, the extent of which was associated with the severity of COVID-19 in the third trimester of pregnancy, contributes significantly to the development of obstetric complications and associated disorders in newborns, altering their individual adaptive response to infection.

CONCLUSION

We showed that the severity of COVID-19 in the third trimester of pregnancy was associated with the complexity of immune dysregulation characterized by reduced levels of SARS-CoV-2 IgG antibodies and proinflammatory IFNγ in maternal and umbilical cord blood, as well as with the decreased expression of CD14 and HLA-DR by monocytes. This may indicate the development of temporary immunosuppression. Parameters of innate immunity and cytokine response in the maternal blood were correlated with the ones in the umbilical cord blood. Moderate severity of COVID-19 increased the risk of preterm births, neonatal cerebral ischemia, intraventricular hemorrhage, and respiratory distress syndrome. No risk of vertical SARS-CoV-2 transmission to the fetus was detected.

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Andrievskaya I.A. – conception and design, drafting of the manuscript, final approval of the manuscript. Lyazgiyan K.S. – analysis and interpretation of the data, statistical processing of the research results. Zhukovets I.V. – drafting and editing of the article. Ustinov E.M. – collection and processing of the material, carrying out of studies.

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Food allergen sensitization patterns in psoriasis patients

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ABSTRACT

Background. Psoriasis is a chronic relapsing systemic disease characterized by inflammation in the skin. Etiology of psoriasis remains elusive, since there are many factors triggering a pathological process in the skin. Data on the frequency of allergies in patients with psoriasis are extremely few and contain conflicting results in the literature, which determines the relevance of the study. Researchers described coexisting atopic dermatitis (AD) and psoriasis (PS), which does not exclude common causes and mechanisms leading to skin damage.

Aim. To study and conduct a comparative analysis of food allergen sensitization patterns in patients with psoriasis and atopic dermatitis.

Materials and methods. A prospective study included patients with psoriasis (group 1, $n = 51$) and atopic dermatitis (group 2, comparison group, $n = 20$) aged 18–57 years. A control group (group 3, $n = 19$) encompassed apparently healthy sex- and age-matched individuals. Specific allergy testing included allergy history and determination of sensitization patterns by analyzing serum concentrations of total immunoglobulin E (IgE) and allergen-specific IgE (sIgE) to food allergens using ELISA test systems (Alkor-Bio, Russia) on the Thermo Scientific Multiskan FC microplate photometer. The calculation and analysis of the obtained data were carried out using the Statistica 8.0 software package.

Results. The concentration of total immunoglobulin E in the blood serum for PS patients was 57.9 [31.6; 135.1] IU / ml, for AD patients – 210.4 [56.2; 1,000.0] IU / ml, and for the control group – 45.1 [23.4; 144.0] IU / ml, respectively, $p_{1,2} = 0.005$; $p_{2,3} = 0.001$; $p_{1,3} = 0.4$. Food allergen sensitization was determined significantly more often in the group of AD patients compared to the group of PS patients: 95.0 ($n = 19$) vs. 37.2% ($n = 19$), respectively, $p_{1,2} = 0.005$. In the group of AD patients, sensitization to chicken eggs, tomatoes, and peanuts was found significantly more frequently than in the group of PS patients and in the control group. Sensitization to beef, buckwheat, and potatoes was significantly more common in the group of PS patients than in the controls.

Conclusion. Following the study of the serum concentration of allergen-specific IgE (sIgE) to food allergens, we revealed food allergen sensitization not only for AD patients, but also for PS patients. However, in our study, sensitization patterns to the studied allergens have their own characteristics depending on the specific disease.

Keywords: psoriasis, atopic dermatitis, food allergy, sensitization, allergen-specific IgE

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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Conformity with the principles of ethics. All patients signed an informed consent to participate in the study. The study was approved by the Ethics Committee at Research Institute of Medical Problems of the North (Protocol No. 12 of 10.12.2013).

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Сенсибилизация к пищевым аллергенам больных псориазом

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РЕЗЮМЕ

Псориаз – хроническое рецидивирующее системное заболевание, характеризующееся воспалительным процессом в эпидермисе. Вопрос об этиологии псориаза остается открытым, так как существует множество триггеров для запуска патологического процесса в коже. Данные о частоте возникновения аллергии у больных псориазом крайне немногочисленны и содержат противоречивые результаты в литературе, что определяет актуальность исследования. Известно о сочетании атопического дерматита и псориаза, что не исключает общности причин и механизмов, приводящих к поражению кожи.

Цель. Изучить и провести сравнительный анализ спектра сенсибилизации к пищевым аллергенам больных псориазом и атопическим дерматитом.

Материалы и методы. Проведено проспективное исследование больных псориазом (ПС, 1-я группа, n = 51) и атопическим дерматитом (АД, 2-я группа, группа сравнения, n = 20) в возрасте 18–57 лет. Контрольная группа (3-я группа, n = 19) включала практически здоровых людей, сопоставимых по полу и возрасту с больными. Специфическое аллергологическое обследование включало сбор аллергологического анамнеза, установление спектра сенсибилизации путем определения концентрации общего иммуноглобулина Е (IgE) и аллерген-специфических IgE к пищевым аллергенам с использованием тест-систем (компания «Алкор-Био», Россия) методом иммуноферментного анализа на полуавтоматическом анализаторе Thermo Scientific Multiskan FC. Расчет и анализ полученных данных проводили с помощью пакета программ Statistica 8.0.

Результаты и обсуждение. Концентрация общего иммуноглобулина Е в сыворотке крови больных ПС составила 57,9 [31,6; 135,1] МЕ/мл, больных АД – 210,4 [56,2; 1000,0] МЕ/мл, в контрольной группе – 45,1 [23,4; 144,0] МЕ/мл соответственно, $p_{1,2}$ = 0,005; $p_{2,3}$ = 0,001; $p_{1,3}$ = 0,4. Сенсибилизация к пищевым аллергенам статистически значимо чаще определялась в группе больных АД в сравнении с группой больных ПС: 95,0% (n = 19) против 37,2% (n = 19) соответственно, $p_{1,2}$ = 0,005. В группе больных АД сенсибилизация к куриному яйцу, томатам и арахису выявлена статистически значимо чаще в сравнении с группой больных ПС и группой контроля В группе больных ПС сенсибилизация к говядине, гречке и картофелю выявлена статистически значимо чаще в сравнении с контрольной группой.

Заключение. Таким образом, выявлена сенсибилизация на основе изучения концентрации аллерген-специфических IgE (sIgE) к пищевым аллергенам в сыворотке крови не только больных АД, но и больных ПС, причем спектр сенсибилизации к изучаемым аллергенам имеет свои особенности в зависимости от нозологии.

Ключевые слова: псориаз, атопический дерматит, пищевая аллергия, сенсибилизация, аллерген-специфические IgE

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

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INTRODUCTION

Despite multiple theories of psoriasis (PS), its etiology and pathogenesis remain elusive [1, 2]. Ineffectiveness of current curative and pathogen-specific therapies for PS makes its study relevant and determines the need to search for new approaches to the study of the disease, including its etiology and pathogenesis [3–5].

Psoriasis and atopic dermatitis (AD) are skin diseases both characterized by the simultaneous presence of systemic inflammation and skin damage. Canonically, PS is considered as a Th1 / Th17-mediated skin disease, while AD is characterized by a predominant type 2 immune response [1, 6]. Earlier, it was believed that the presence of AD excluded simultaneous development of PS in patients due to fundamentally different immune responses. Recently, researchers have described coexisting AD and PS [7–11]. Therefore, studying coexisting AD and PS may lead to discovery of new therapeutic targets and facilitate the development of strategies for personalized care [8–10].

AD is the earliest manifestation of allergic march characterized by progression of a systemic allergic reaction from eczema to allergic rhinitis and bronchial asthma [11]. In recent years, the term "psoriatic march" has been coined, which reflects the polysystemic nature of inflammation in PS [12].

Modern researchers have described that AD and PS are characterized by similar histologic changes in skin lesions. Thus, in patients with AD and PS, histology revealed neutrophil infiltration in foci of skin lesions [13, 14]. The hypothesis according to which chronic inflammation in PS contributes to an increase in the number of B lymphocytes in skin lesions followed by overproduction of total IgE in the blood serum and predominant Th2 response in these patients is of great interest [15]. The role of Th17- and Th22 lymphocytes in the pathogenesis of both AD and PS was discussed [14–18]. Studies on the concentration of specific IgE antibodies to various groups of allergens in PS are few, which determines the relevance of this research [19, 20].

According to some authors, patients with PS are characterized by an increase in specific IgE antibodies to birch pollen, timothy, rye, potato, and carrot [19]. According to the dual allergen exposure hypothesis, food allergen sensitization may develop not only via oral exposure but also following allergen penetration through impaired skin barrier [19, 21]. This fact determines the relevance of studying food allergen sensitization in patients with coexisting PS and AD, while a comparative analysis of food allergen sensitization patterns can help identify new factors and mechanisms leading to skin damage.

The aim of the study was to investigate and conduct a comparative analysis of food allergen sensitization patterns in patients with PS and AD.

MATERIALS AND METHODS

The study included patients with PS (group 1, $n = 51$) and AD (group 2, comparison group, $n = 20$) aged 18–57 years. The average age of patients in group 1 was 40.0 ± 1.8 years, in group 2 – 25.0 ± 2.0 years. In the gender profile of both groups, women prevailed: 52.9% ($n = 27$) in group 1, 55% ($n = 11$) in group 2. A control group (group 3, $n = 19$) encompassed apparently healthy sex- and age-matched individuals. The severity and extent of PS were measured by the Psoriasis Area and Severity Index (PASI). The average PASI score in the PS group was 10.0 [6.0; 14.4].

All patients underwent specific allergy testing, including allergy history and determination of sensitization patterns by analyzing serum concentrations of total immunoglobulin E (IgE) and allergen-specific IgE (sIgE) to food allergens using ELISA test systems (Alkor-Bio, Russia) on the Thermo Scientific Multiskan FC microplate photometer. We used reagents for determining sIgE to the following food allergens: cow milk, beef, chicken egg (whole), chicken meat, gluten, wheat, oat, rice, buckwheat, potato, carrot, tomato, apple, peanut.

According to the manufacturer's instruction (Alkor-Bio, Russia), the level of sIgE ≥ 0.35 kIU / l indicated a positive response. Sensitization to the studied allergens in the control group was not detected. The calculation and analysis of the obtained data were carried out using the Statistica 8.0 software package. Statistical processing of the results was performed by calculating the mean and the error of the mean

($M \pm m$). The data were presented as the median and the interquartile range Me [$Q_{25\%}$; $Q_{75\%}$] and as the absolute and relative number of sensitized patients n (%). The differences were considered statistically significant at $p < 0.05$.

RESULTS

In the group of PS patients, prior history of allergy (allergy and / or allergic diseases) was established in 41.2% (n = 21) of cases, seasonal allergy – in 7.8% (n = 4) of patients, family history of allergy (allergy and / or allergic diseases among close relatives) – in 27.5 % (n = 14) of cases, and family history of PS – in 39.2% (n = 20) of cases.

The average age at PS onset was 24.0 ± 2.1 years, the total disease duration was 11.0 ± 1.6 years. In 76.5% (n = 39) of cases, skin rash was accompanied by intense itching. The main clinical manifestations of PS were a monomorphic rash with flat papules of various sizes and large pink and red plaques, characterized by hyperproliferation and aberrant differentiation of the epidermis. In PS patients, relapsing – remitting skin disease was observed in 56.7% (n = 29) of cases, exacerbations in autumn and winter occurred in 33.3% (n = 17) of cases, and exacerbations in spring and summer were noted in 9.8% (n = 5) of cases, mainly in patients with coexisting pathology (seasonal allergic rhinoconjunctivitis).

According to allergy history, urticaria, allergic rhinitis, and insect and drug allergies were noted in 95% (n = 19) of AD patients. Family history of allergy in AD patients was detected in 55% (n = 11) of patients and seasonal allergic manifestations – in 60% (n = 12) of cases. The following clinical manifestations of AD were detected: erythema, dry skin, excoriations, peeling. Itching was observed in 95% (n = 19) of AD patients. Skin damage in most AD patients was as follows: limited – 60% (n = 12) of cases, generalized – 30% (n = 6) of patients, diffuse – 10% (n = 2) of cases. Moderate AD was found in 90% (n = 18) of cases, whereas a severe course of AD was recorded in 10% of patients.

The serum level of total IgE for PS patients was 57.9 [31.6; 135.1] IU / ml, for AD patients – 210.4 [56.2; 1,000.0] IU / ml, for the control group – 45.1 [23.4; 144.0] IU / ml, respectively, $p_{1,2} = 0.005$; $p_{2,3} = 0.001$; $p_{1,3} = 0.4$. Literature data on the concentration of total IgE in PS are conflicting. Increased serum levels of total IgE have been reported in some studies [15], whereas others did not demonstrate statistically significant differences in total IgE concentrations between PS patients and controls [22]. Higher concentrations of total IgE were shown to be associated with the duration of skin lesion in PS and correlated with the severity of the clinical course of the disease [19].

Food allergen sensitization was detected significantly more often in the group of AD patients compared to the PS group: 95.0 (n = 19) vs. 37.2% (n = 19), respectively, $p_{1,2} = 0.005$ (Table).

Table

Comparative characteristics of food allergy sensitization patterns in patients with psoriasis and atopic dermatitis, n (%)

Parameter	PS patients, n = 51	AD patients, n = 20	p
Cow milk	2 (10.5)	5 (26.3)	$p_{1,2} = 0.2$ $p_{1,3} = 0.1$ $p_{2,3} = 0.02$
Beef	5 (26.3)	2 (10.5)	$p_{1,2} = 0.2$ $p_{1,3} = 0.02$ $p_{2,3} = 0.1$
Chicken egg (whole)	0	4 (21.1)	$p_{1,2} = 0.04$ $p_{2,3} = 0.04$
Chicken meat	0	1 (5.3)	$p_{1,2} = 0.3$ $p_{2,3} = 0.3$
Gluten	0	1 (5.3)	$p_{1,2} = 0.3$ $p_{2,3} = 0.3$
Wheat	1 (5.3)	4 (21.1)	$p_{1,2} = 0.1$ $p_{1,3} = 0.3$ $p_{2,3} = 0.04$
Oat	3 (15.8)	7 (36.8)	$p_{1,2} = 0.1$ $p_{1,3} = 0.08$ $p_{2,3} = 0.004$
Rice	1 (5.3)	2 (10.5)	$p_{1,2} = 0.5$ $p_{1,3} = 0.3$ $p_{2,3} = 0.15$
Buckwheat	6 (31.6)	2 (10.5)	$p_{1,2} = 0.1$ $p_{1,3} = 0.01$ $p_{2,3} = 0.15$
Potato	4 (21.1)	0	$p_{1,2} = 0.04$ $p_{1,3} = 0.04$
Carrot	3 (15.8)	2 (10.5)	$p_{1,2} = 0.6$ $p_{1,3} = 0.07$ $p_{2,3} = 0.1$
Tomatoe	1 (5.3)	8 (42.1)	$p_{1,2} = 0.008$ $p_{1,3} = 0.3$ $p_{2,3} = 0.002$
Apple	3 (15.8)	6 (31.6)	$p_{1,2} = 0.3$ $p_{1,3} = 0.07$ $p_{2,3} = 0.008$
Peanut	2 (10.5)	12 (63.1)	$p_{1,2} < 0.001$ $p_{1,3} = 0.1$ $p_{2,3} < 0.001$

Note. In the control group, sensitization to the studied allergens was not detected.

Sensitization to chicken eggs, tomatoes, and peanuts was found significantly more frequently in the group of AD patients than in the group of PS patients. Chicken egg allergy is known to be one of the most common in the world [23]. According to the literature, chicken egg allergy is observed in one third of adult American population [23]. Consequently, high incidence of sensitization to chicken eggs in patients with AD identified in this study does not contradict the literature data [24]. Sensitization of AD patients to tomatoes and peanuts is likely associated with cross-reactivity with pollen allergens [25]. In the group of AD patients, the frequency of sensitization to cow milk protein, chicken eggs, wheat, oat, tomatoes, apples, and peanuts was significantly higher compared to the control group (Table).

An interesting aspect of the study is the presence of food allergen sensitization in patients with PS. The study showed that sensitization to beef, buckwheat, and potatoes in the group of PS patients was significantly more common than in the controls. According to the literature, sensitization to beef may be associated with cross-reactivity with cow milk proteins [26]. Literature data indicate the presence of antigenic determinants common for some foods and pollen allergens [27, 28]. Sensitization to potatoes and buckwheat in PS patients is most likely associated with cross-reactivity with pollen allergens, since this group of patients showed higher sensitization to birch pollen, sage pollen, timothy, and rye [19].

DISCUSSION

Literature data on the influence of food allergens on the development and course of PS are scarce, which determines the need for further study of the role of food allergy in the etiology and pathogenesis of PS. In the present study, sensitization was determined by studying the level of sIgE to food allergens in the blood serum of not only patients with AD, but also patients with PS. The study revealed features of sensitization patterns to food allergens depending on the type of skin lesion. Thus, AD was characterized by a wider range of sensitization to food allergens compared to PS: chicken eggs, tomatoes, and peanuts. Since AD is a classic example of an IgE-mediated disease, high incidence of sensitization to food allergens in this category of patients should have been expected.

The study revealed high frequency of sensitization to food allergens in patients with PS. Sensitization to beef, potatoes, and buckwheat was more often detected, which may indicate the influence of these allergens on the development of skin lesions in PS.

A steady increase in the incidence of allergies in all diseases is reported all over the world. Assessing the role of food allergies in PS development is of great interest. Recently, an increase in the incidence of coexisting AD and PS has been reported [8, 19]. The presence of food allergies can facilitate damage to digestive organs, leading to impairment of their barrier function, thus increasing the permeability and absorption of various allergens and endotoxins [28]. Moreover, the involvement of the gastrointestinal tract in the systemic allergic reactions to food allergy in PS patients is a characteristic sign of dermatologic manifestations of gastrointestinal diseases [29].

It is known that food allergen sensitization is marked by aggravation of allergy symptoms after consuming foods that are causal allergens [28]. This fact should be considered in clinical practice and requires prescription of an elimination diet for AD and PS patients with account of their individual sensitization patterns to food allergens.

CONCLUSION

Skin lesions and chronic inflammation in PS and AD result in impaired epidermal barrier and, therefore, facilitate more intense penetration of various allergens through the skin, which contributes to extension of the sensitization range and progression of the pathology [30, 31]. Food allergen sensitization in patients with PS can be both a trigger of the disease and a risk factor for its development and progression.

The preliminary results of the study determine the need for prescribing a personalized elimination diet therapy and other methods of allergy diagnosis, such as skin prick test, elimination and provocation tests.

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Analyzing serological screening of the functional state of gastric mucosa in clinical practice

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ABSTRACT

Aim. To analyze the results of the GastroPanel and GastroScreen-3 tests over a 15-year follow-up and determine the incidence of autoimmune gastritis (AIG) in clinical practice and in a random sample of Novosibirsk residents.

Materials and methods. Biomarkers were analyzed in two groups: 1,742 people, average age of 50.0 ± 13.53 years (GastroPanel test, Biohit Oy, Finland), and 170 people, average age of 53.8 ± 12.89 years (GastroScreen-3 test, Vector-Best, Russia), from 2007 to 2022. The AIG incidence was calculated in current clinical practice and in a random sample of Novosibirsk residents aged 45–69 years. The PGI level of 160 μg / l was taken as the upper limit of normal, PGI of 31–50 μg / l indicated moderate atrophy, PGI < 30 μg / l and the PGI / PGII ratio ≤ 3 indicated severe gastric fundus atrophy. AIG was considered at PGI ≤ 10.1 μg / l, the PGI / PGI ratio ≤ 1.3, and gastrin-17 ≥ 42.4 pmol / l (GastroPanel) and at PGI ≤ 16.8 μg / l and the PGI / PGII ratio ≤ 1.5 (GastroScreen-3). The *H. pylori* IgG level > 42 EIU was considered to be positive. Antibodies to CagA protein were determined using the Helico-Best Antibody test (Vector-Best, Novosibirsk).

Results. Serological signs of severe and moderate gastric fundus atrophy were detected in 10 and 9.4% (GastroPanel test) and in 13.3 and 7% (GastroScreen-3 test) of those examined, respectively. Signs of multifocal atrophy were found in 0.7% of cases. Antibodies to *H. pylori* were detected in 57.7%, CagA+ strain – in 56.1% of cases. Peptic ulcer disease (PGI ≥160 μg / l) was found in 15.3% (GastroPanel test) and 10% (GastroScreen-3 test) of the examined. According to the GastroPanel and GastroScreen-3 tests, the incidence of AIG was 1.6% in a random sample and 2.6 and 3.5% in current clinical practice, respectively.

Conclusion. Twenty percent of the examined persons were at risk of developing gastric cancer and 10–15% had peptic ulcer disease, which requires further examination. The incidence of AIG in different study groups based on serological screening was 1.6–3.5%.

Keywords: pepsinogens, GastroPanel, GastroScreen-3, *Helicobacter pylori*, fundus atrophy, autoimmune gastritis

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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Conformity with the principles of ethics. All patients signed an informed consent to participate in the study. The study was approved by the Bioethics Committee at Research Institute of Internal and Preventive Medicine, Branch of the Institute of Cytology and Genetics, SB RAS (Protocol No. 11 of 02.03.2021).

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Анализ серологической диагностики функционального состояния слизистой желудка в клинической практике

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РЕЗЮМЕ

Цель. Проанализировать результаты тест-систем «ГастроПанель» и «ГастроСкрин-3» за 15 лет наблюдения и определить частоту аутоиммунного гастрита (АИГ) в клинической практике и в случайной выборке жителей г. Новосибирска.

Материалы и методы. Показатели биомаркеров были проанализированы в двух группах: 1 742 человека, средний возраст 50,0 ± 13,53 лет (тест-система «ГастроПанель», компания «Биохит», Финляндия), и 170 человек, средний возраст 53,8 ± 12,89 лет (тест-система «ГастроСкрин-3», АО «Вектор-Бест», Россия), с 2007 по 2022 г. Расчет частоты АИГ проводился в текущей клинической практике и в случайной выборке жителей г. Новосибирска 45–69 лет. Верхней границей нормы считали показатель пепсиноген I (ПГI) – 160 мкг/л, умеренной атрофии соответствовал диапазон ПГI 31–50 мкг/л, а ПГI ≤ 30 мкг/л и соотношения ПГI/ПГII ≤ 3 – выраженной фундальной атрофии. Аутоиммунный гастрит рассматривали при показателях ПГI ≤ 10,1 мкг/л, ПГI/ПГI ≤ 1,3; гастрина-17 ≥ 42,4 пмоль/л («ГастроПанель») и ПГI ≤ 16,8 мкг/л, ПГI/ПГII ≤ 1,5 тест-система («ГастроСкрин-3», АО «Вектор-Бест», Россия). Положительным считали уровень иммуноглобулина класса (Ig) G *H. pylori* более 42 EIU. Антитела к CagA-белку определяли с помощью тест-системы «Хелико-Бест антитела» (АО «Вектор-Бест», г. Новосибирск).

Результаты. Серологические признаки выраженной и умеренной фундальной атрофии выявлены: 10 и 9,4% («ГастроПанель»), 13,3 и 7% («ГастроСкрин-3») соответственно. Признаки мультифокальной атрофии обнаружены в 0,7%. Иммуноглобулины класса G *H. pylori* определялись в 57,7%, CagA+ штамм – в 56,1% случаев. Язвенный фенотип гастрита был обнаружен у 15,3% («ГастроПанель») и у 10% («ГастроСкрин-3»). Частота АИГ по данным тест-систем «ГастроПанель» и «ГастроСкрин-3» в случайной выборке составила 1,6%, в текущей клинической практике – 2,6 и 3,5% соответственно.

Заключение. В группу риска развития рака желудка попали 20% обследованных, у 10–15% обнаружен язвенный фенотип, что требует дообследования. Частота АИГ в исследуемых группах на основании серологического скрининга составила 1,6–3,5%.

Ключевые слова: пепсиногены, «ГастроПанель», «ГастроСкрин-3», *Helicobacter pylori*, фундальная атрофия, аутоиммунный гастрит

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

Источник финансирования. Работа выполнена по государственному заданию в рамках бюджетных тем «Изучение молекулярно-генетических и молекулярно-биологических механизмов развития распространенных терапевтических заболеваний в Сибири для совершенствования подходов к их ранней диагностике и профилактике», 2024–2028 гг. (FWNR-2024-0004); «Совершенствование методов диагностики, профилак-

тики и лечения больных распространенными заболеваниями гепатобилиарной системы и желудочно-кишечного тракта в Сибири», 2023–2025, FWNR-2023-0003.

Соответствие принципам этики. Все пациенты подписали информированное согласие на участие в исследовании. Исследование одобрено комитетом биомедицинской этики НИИТПМ – филиала ИЦиГ СО РАН (протокол № 11 от 2.03.2021).

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INTRODUCTION

The function of the stomach and its mucosal structure are closely associated with each other. Normal levels of such biomarkers as pepsinogen I, pepsinogen II and their ratio (PG I, PG II, PG I/PG II), gastrin-17, as well as the absence of IgG antibodies to *H. pylori* and CagA cytotoxic protein are surrogate markers of healthy gastric mucosa, with the exception of nonspecific inflammation or microerosions that do not affect their profile [1, 2]. Test panels include biomarkers that reflect the morphological changes and the function of the gastric mucosa. In non-atrophic gastritis associated with *H. pylori* infection, the levels of pepsinogens, especially those of PG II, increase [1, 2].

Changes in the levels of biomarkers can also show the localization of the process. PG I is an indicator of damage to gastric glands in the stomach body, PG II is synthesized in every part of the stomach, and PG I / PG II ratio is correlated with the progression of fundus atrophy in the mucosa [1, 3]. Gastrin-17 is the principal hormone that regulates the secretion of hydrochloric acid by parietal cells of the stomach. Its basal level is decreased in persons with hyperacidity (hypersecretion). The development of atrophy in the antral part also leads to decreased levels of gastrin-17, including its postprandial fraction [4, 5].

According to the last Maastricht Consensus, atrophy determinates the risk of non-hereditary gastric cancer and can be found with the use of invasive (biopsy) and non-invasive methods [6]. A decrease in PG I and / or PGI / PG II ratio with high levels of gastrin-17 indicates the presence of gastric fundus atrophy and is characteristic of autoimmune gastritis as well as high levels of anti-parietal cell antibodies (APCAs) and / or anti-intrinsic factor antibodies (AIFAs) [2, 4, 7]. Thus, all these parameters provide important information about the functional state of the gastric mucosa [6].

Test kits that include a panel of atrophy biomarkers have proven to be effective in non-invasive diagnosis both in individual patients and in population screening [1, 2, 4, 8]. GastroPanel test (Finland) is one of the most used kits that includes PG I, PG II, PG I / PG II ratio, gastrin-17, and IgG antibodies to *H.pylori*. Its sensitivity is 83%, and its specificity ranges from 95 to 98% [9]. The Russian test system GastroScreen-3 has been introduced into clinical practice recently. It includes PG I, PG II, PG I / PG II ratio, and antibodies to CagA protein.

The aim of the study was to analyze the results of serological screening of the functional state of gastric mucosa using two test systems (GastroPanel and Gastroscreen-3) and to determine the frequency of autoimmune gastritis (AIG) in clinical practice and in a random sample of Novosibirsk residents.

MATERIALS AND METHODS

Biomarker data obtained using the GastroPanel test (Biohit Oy, Finland) were analyzed in 1,742 people with an average age of 50.0 ± 13.53 years during a 15-year follow-up from 2007 to 2022. Women made up a larger proportion of individuals in the group (1,210 people, which is 69.5 %) than men (532 men – 30.5 %, $p < 0.001$). Using the GastroScreen-3 biomarker panel (Vector Best, Russia), the analysis was carried out in 170 people with an average age of 53.8 ± 12.89 years over a 4-year follow-up from 2018 to 2022. The proportion of women in this group was also larger than that of men (79.4 and 20.6%, respectively; $p < 0.001$). All patients went to the clinic at the Research Institute of Internal and Preventive Medicine, Branch of the Institute of Cytology and Genetics, independently or following a doctor referral.

A random sample of Novosibirsk residents was examined using the GastroPanel test to study the incidence of AIG. The group consisted of 246 people (117 men and 129 women) with an average age of 59.4 ± 7.0 years, selected by simple random sampling

from 9,360 people aged 45–69 years based on the data of the cross-sectional study which was part of the HAPIEE project conducted at the Research Institute of Internal and Preventive Medicine in 2003–2005.

Serum samples were tested using the GastroPanel (Biohit Oy, Finland) and GastroScreen-3 (Vector-Best, Russia) tests for enzyme-linked immunosorbent assay, according to the manufacturer's instructions [10]. The PGI value of 160 μg / l was considered the upper limit of normal, and the PGI level ≤ 30 μg / l and / or the PGI / PGII ratio of ≤ 3 indicated severe gastric fundus atrophy. The PGI range of 31–50 μg / l indicated moderate gastric fundus atrophy. Multifocal (pangastritis) atrophic gastritis was determined when the level of PGI decreased to ≤ 30 and gastrin-17 was less than 1 pmol / l [10]. The level of IgG antibodies to *H. pylori* was considered significant in terms of diagnosis when the level was more than 42 EIU. *H. pylori* CagA antibodies were assessed using the Helico-Best Antibody test system (Vector-Best, Russia).

In our previous study, we determined cutoff values for autoimmune atrophic gastritis in patients with verified AIG. For the GastroPanel test, the values were the following: PG I ≤ 10.1 μg / l, the PG I / PG II ratio ≤ 1.3, and gastrin-17 ≥ 42.4 pmol / l. For the GastroScreen-3 test, the values were as follows: PG I ≤ 16.8 μg / l and the PG I / PG II ratio ≤ 1.5 [11].

Statistical analysis of the obtained results was performed using the SPSS statistics (16.0 version). The distribution of quantitative variables was assessed using the Kolmogorov – Smirnov test. We calculated mean values ($M \pm \sigma$) for a normal distribution and the median (*Me*) and the interquartile range [Q_{25}; Q_{75}] for a non-normal distribution. The Student's *t*-test and the Mann – Whitney *U* test were used to determine the statistical significance of the differences. The Pearson's chi-squared test was used to compare proportions. The critical value of the null hypothesis was considered at $p \leq 0.05$.

The study was conducted in accordance with the Declaration of Helsinki and approved by Biomedical Ethics Committee at the Research Institute of Internal and Preventive Medicine – a Branch of the Institute of Cytology and Genetics (Protocol No. 11 of 02.03.2021). All patients signed an informed consent to participate in the study.

RESULTS AND DISCUSSION

The mean and median values of biomarkers measured in all participants using two test systems during the follow-up are shown in Tables 1 and 2. In men, the levels of PGI and the PGI / PGII ratio (GastroPanel) were higher, and the level of gastrin-17 was lower than in women (Table 1).

Table 1

Parameters of the GastroPanel test over a 15-year follow-up in men and women, *Me* [$Q_{25\%}$; $Q_{75\%}$]

Parameter	Men, *n* = 532	Women, *n* = 1,210	Total, *n* = 1,742	p_{m-f}
PG I, μg / l	97.4 [65.2; 139.7]	83.9 [54.4; 125.0]	87.7 [57.9; 128.7]	<0.0001
PG II, μg / l	10.6 [6.5; 19.7]	10.1 [6.1; 19.0]	10.2 [6.2; 19.4]	0.168
PG I / PG II	8.5 [5.6; 11.8]	7.9 [4.9; 11.7]	8.2 [5.1; 11.7]	0.036
Gastrin-17, pmol / l	4.3 [1.4; 11.6]	4.9 [1.9; 15.2]	4.7 [1.7; 14.0]	<0.0001
IgG to *H. pylori*, EIU	62.0 [17.7; 105.1]	56.3 [16.9; 108.2]	57.8 [17.3; 107.4]	0.996

Table 2

Parameters of the GastroScreen-3 test over a 4-year follow-up in men and women, *Me* [$Q_{25\%}$; $Q_{75\%}$]

Parameter	Men, *n* = 35	Women, *n* = 135	Total, *n* = 170	p_{m-f}
PG I, μg / l	98.6 [53.5; 139.5]	89.8 [59.4; 122.7]	91.5 [58.8; 129.9]	0.458
PG II, μg / l	10.6 [6.5; 19.7]	10.1 [6.1; 18.9]	9.1 [5.7; 16.7]	0.906
PG I / PG II	8.6 [6.3; 12.0]	8.7 [5.6; 13.2]	8.6 [5.7; 13.0]	0.882

H. pylori infection is recognized as the main cause of atrophic gastritis and a class one carcinogen [4, 12]. In this study, IgG to *H. pylori* was detected in 57.7 % of the participants (out of 1,742 people) with high prevalence of cytotoxic CagA+ strain (56.1%). In the GastroScreen-3 group, a more carcinogenic CagA+ strain of *H. pylori* [6, 13] was found in 42% of the participants (out of 170 people). It is possible that the percentage of those infected was higher because IgG to *H. pylori* can be negative due to elimination of bacteria in individuals with severe atrophy or after successful treatment. The literature describes cases of spontaneous disappearance of *H. pylori* in patients with severe atrophic gastritis, while the

probability of developing gastric cancer may increase [14, 15].

Serological signs of severe and moderate gastric mucosal atrophy were detected in 10 and 9.4% of 1,742 people examined using the GastroPanel test, respectively. Severe and moderate atrophy was detected in 10.6 and 7.1% of individuals in the GastroScreen-3 group (170 persons), respectively (Table 2). In total, over the follow-ups, serological signs of gastric fundus atrophy of varying severity were identified in 19.4% (GastroPanel) and 17.6% of cases (GastroScreen-3). According to several studies, the PGI / PGII ratio may be a more reliable marker of gastric fundus atrophy than PGI alone [7, 16]. The PGI / PG II ratio was found to be low in 11% of individuals in the GastroPanel group, and a combination of low PGI levels and low PG I / PG II ratio was detected in 7.3% of cases. The PG I / PG II ratio ≤ 3 was found in 8.2 % of the participants in the GastroScreen-3 group, while the combination of PG I ≤ 30 μg / l and the PG I / PG II ratio ≤ 3 was detected in 7.1% of patients in this group. Serological signs of multifocal atrophic gastritis with a high risk of developing gastric cancer were detected in 13 of 1,742 individuals (0.7%) (Table 2).

The detection of low biomarker values corresponding to the serological criteria of atrophy requires further endoscopic examination with multifocal biopsy and gastric atrophy grading according to the OLGA integrated system [17]. Thus, according to the latest consensus, both international and Russian, serological tests are useful for assessing individual risk of gastric cancer [6, 18, 19].

Peptic ulcer disease, elevated levels of hydrochloric acid, and PGI higher than 160 μg / l were found in 15.3 % of cases in the GastroPanel group and in 10% of cases in the GastroScreen-3 group (Table 3).

Table 3

Frequency of GastroPanel and GastroScreen-3 parameters with interpretation of possible risks, %, *Me* [$Q_{25\%}$; $Q_{75\%}$]

Parameter	GastroPanel, *n* = 1,742	GastroScreen-3, *n* = 170	Interpretation
PG I (51–160 μg / l)	64.5 [62.3; 66.7]	71.8 [65; 78.5]	No signs of atrophy
PG I (≤ 30 μg / l)	10 [8.6; 11.4]	10.6 [6.0; 15.2]	Severe gastric fundus atrophy. Risk of gastric cancer
PG I / PG II ≤ 3	11 [9.5; 12.5]	8.2 [4.1; 12.3]	
PG I ≤ 30 μg / l + PG I / PG II ≤ 3	7.3 [6.1; 8.5]	7.1 [3.2; 11.0]	
PG I (31–50 μg / l)	9.4 [8.0; 10.8]	7.1 [3.2; 11.0]	Signs of moderate gastric fundus atrophy. Risk of gastric cancer
PG I ≤ 10.1 μg / l + Gastrin-17 ≥ 42.4 pmol / l	2.6 [1.9; 3.3]	–	Autoimmune gastritis. High risk of iron deficiency, vitamin B12 deficiency, anemia, and gastric cancer
PG I ≤ 16.8 μg / l + PG I / PG II < 1.5	–	3.5 [0.7; 6.3]	
PG I (≥ 160 μg / l)	15.3 [13.6; 17.0]	10 [5.5; 14.5]	Hypersecretory state. High risk of erosive and ulcerative damage to gastric mucosa
PG I ≤ 30 μg / l + Gastrin-17 <1 pmol / l	0.7 [0.3; 1.1]	–	Pangastritis. Multifocal atrophy (body + antrum). High risk of developing gastric cancer

Thus, over 15-year (GastroPanel) and 4-year (GastroScreen-3) follow-up, 20% of the participants were included in the gastric cancer risk group, and 10–15% of the participants were included in the risk group for erosive and ulcerative damage to the gastric mucosa which requires a further detailed examination.

According to the literature, the levels of pepsinogens, especially those of PGII, increase in *H. pylori*-associated gastritis [3, 20]. In this study, average values of PGI and PGII were also significantly higher in the *H. pylori*-positive individuals compared to the *H. pylori*-negative persons (111.6 ± 63.4 vs. 83.6 ± 56.7 μg / l and 18.4 ± 13.9 vs. 9.9 ± 9.2 μg / l, $p < 0.0001$, respectively), and PGII was also higher in the CagA-positive individuals (14.9 ± 10.5 vs. 10.6 ± 7.8 μg / l, $p = 0.004$).

In addition to *H. pylori* infection, AIG can also be the cause of atrophic changes in the mucosa [21]. Based on previously obtained cut-offs for atrophy biomarkers, the incidence of AIG in current clinical practice was 2.6% (GastroPanel, 1,742 participants) and 3.5% (GastroScreen-3, 170 participants) (Table 2). In the random sample (45–69 years, 246 participants), the incidence of AIG was 1.6% (GastroPanel). These values do not contradict the literature data [22, 23]. In addition to the risk of developing hematologic disorders, AIG poses a risk of developing neuroendocrine tumors and adenocarcinomas. However, it should be noted that stage III–IV atrophic gastritis (according to OLGA) associated with *H. pylori* infection determines a greater risk of developing gastric cancer [6].

CONCLUSION

The conducted analysis of the results of serological screening in clinical practice in people from a wide range of age groups over a long follow-up period showed high frequency of gastric fundus atrophy of varying severity with coexisting *H. pylori* infection with high prevalence of cytotoxic CagA+ strain or AIG, which requires a more detailed examination. The frequency of AIG was 1.6–3.5% in different study groups based on serological tests.

Therefore, serological screening of gastritis types using diagnostic panels, such as GastroPanel or GastroScreen-3 tests, is an effective tool to determine the functional state of the gastric mucosa.

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Authors' contribution

Belkovets A.V., Ozhiganova N.V. – conception and design, analysis and interpretation of the data, justification of the manuscript. Kruchinina M.V. – critical revision of important intellectual content, final approval of the manuscript for publication. Polonskaya Ya.V. – analysis and interpretation of the data. Shcherbakova L.V. – statistical processing of the data.

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Effect of pro- and metabiotic *Lactobacillus delbrueckii D5* strain on myocardial resistance to ischemia – reperfusion injury in the rat model of systemic inflammatory response

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ABSTRACT

Aim. To study the effect of lyophilized *L. delbrueckii D5*, as well as its inactivated culture, during intragastric administration on myocardial resistance to ischemia – reperfusion injury (IRI), markers of inflammation, and intestinal epithelial permeability.

Materials and methods. The experiments were performed on male Wistar rats with a model of systemic inflammatory response syndrome (SIRS). Myocardial IRI was reproduced on an isolated Langendorff heart.

Results. A significant increase in the levels of tumor necrosis factor (TNF)α, interleukin (IL)-1β, IL-6, and lactoferrin in SIRS was revealed. The introduction of both inactivated and lyophilized culture of *L. delbrueckii D5* resulted in normalization of these changes. Normalization of the increased blood level of lipopolysaccharide in SIRS was also noted with the introduction of both inactivated and lyophilized *L. delbrueckii D5*. However, the inactivated culture had no effect on the myocardial infarct size, which was increased in the SIRS group compared to the controls, whereas the introduction of the lyophilized strain led to a significant decrease in this parameter.

Conclusion. The inactivated culture of *Lactobacillus delbrueckii D5* has a pronounced anti-inflammatory effect, but does not impact myocardial resistance to IRI, unlike the lyophilized strain, which requires further research.

Keywords: myocardium, ischemia – reperfusion injury, infarct size, *Lactobacillus delbrueckii D5*, systemic inflammatory response syndrome, leukocytes, cytokines, probiotics

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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Влияние про- и метабиотической формы штамма *Lactobacillus delbrueckii D5* на устойчивость миокарда к ишемии-реперфузии в условиях системного воспалительного ответа у крыс

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РЕЗЮМЕ

Цель. Изучение эффекта лиофилизированного штамма *L. delbrueckii D5*, а также его инактивированной формы при внутрижелудочном введении на устойчивость миокарда к ишемическому-реперфузионному повреждению (ИРП), маркеры воспаления и проницаемость эпителиального барьера кишки.

Материалы и методы. Эксперименты выполнены на самцах крыс стока Вистар с улучшенным конвенциональным статусом на модели синдрома системного воспалительного ответа (ССВО). Ишемически-реперфузионное повреждение миокарда воспроизводили на изолированном сердце, перфузируемом по Лангендорфу.

Результаты. Отмечено значимое повышение уровня фактора некроза опухоли альфа, интерлейкина (ИЛ) 1β, ИЛ-6 и лактоферрина при ССВО. Введение как инактивированного, так и лиофилизированного штамма *L. delbrueckii D5* приводило к нормализации указанных изменений. Также отмечена нормализация повышенного при ССВО уровня липополисахарида в крови при введении как инактивированного, так и лиофилизированного штамма *L. delbrueckii D5.* Однако инактивированный штамм не оказывал влияния на размер инфаркта миокарда, который был увеличен при ССВО по сравнению с контролем, тогда как при введении лиофилизированной формы имелось значимое снижение размера инфаркта.

Заключение. Инактивированная культура *Lactobacillus delbrueckii D5* обладает выраженным противовоспалительным свойством, но не влияет на устойчивость миокарда к ИРП, в отличие от лиофилизированного штамма, что требует дальнейших исследований.

Ключевые слова: миокард, ишемическое-реперфузионное повреждение, размер инфаркта, *Lactobacillus delbrueckii D5*, синдром системного воспалительного ответа, лейкоциты, цитокины, пробиотики

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

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INTRODUCTION

The search for signaling molecules that increase myocardial resistance to ischemia – reperfusion injury (IRI) is of undoubted interest for experimental and clinical medicine due to systemic inflammatory response syndrome (SIRS). Recently, data on myocardial IRI reduction induced by various changes in the composition of gut microbiota (GMB) have appeared in the literature. Attempts to identify a single molecular mediator linking the GMB with immune and cardiovascular systems of the superorganism are most likely doomed to failure [1]. Given the infinite diversity of GMB composition and its involvement in all physiological and pathological processes of the macroorganism, the hypothesis of exclusive properties of the bacterium determining the health of the host organism and its microbiota also sounds unlikely. The most probable condition for the formation and maintenance of health seems to be a harmonious combination of key signaling factors of immunity and metabolic parameters to provide an adequate and balanced relationship between the organism, consumed food, and GMB composition.

In accordance with our previous results obtained on the rodent model of SIRS developed within the concept of probiotic-induced cardioprotection, the following intermediate conclusions can be made. In SIRS, which occurs with a combination of primary visceral obesity (PVO), antibiotic-induced dysbiosis (AID), and chemically induced colitis, there is a decrease in myocardial resistance to IRI [2], and administration of some probiotic strains is accompanied by a decrease in myocardial IRI [3].

In addition to the generally recognized and established cardiotropic markers of systemic inflammation (tumor necrosis factor alpha (TNFα), transforming growth factor beta (TGFβ), interleukin (IL)-1, IL-2, IL-6, IL-8, IL-10, monocyte chemoattractant protein 1 (MCP-1), etc.), metabolic products (short-chain fatty acids (SCFAs), bile acids (BA)), and leukocyte population related to changes in the composition of GMB [4], changes in endothelial and epithelial permeability and acute phase reactants, in particular haptoglobin (Hp) and lactoferrin (Lf), are of interest. The elucidation of physiological and molecular mechanisms regulating the effect of GMB and its metabolites on inflammation and myocardial resistance to IRI is of particular interest. In order to identify promising targets and mechanisms, it is necessary to experimentally substantiate the general and specific features of the effect of live and inactivated strains, called metabiotic, on the macroorganism [5].

In the present work, we studied the effect of *Lactobacillus delbrueckii D5* and its inactivated culture on changes in animal body weight, feed and water consumption, hematological and immunological parameters, hemodynamic characteristics, and myocardial resistance to IRI using an isolated Langendorff heart on the previously developed rat model of SIRS in the vivarium (Wistar rats). The working hypothesis was the assumption on differences in the efficacy of inactivated culture compared to live bacteria.

MATERIALS AND METHODS

The experiments were performed on male Wistar rats weighing 320–370 g in accordance with the European Council Directive (86/609/EEC) on the protection of animals used for scientific purposes and the protocol approved by the Commission for the control over care and use of laboratory animals at

Almazov National Medical Center. Modeling of SIRS was described in detail earlier [2].

The animals were randomly distributed into one of five groups (n = 10 in each group): 1) controls: rats received standard feed and drinking water *ad libitum*; 2) SIRS: in addition to the standard diet, rats received 2 g of polyunsaturated fat and 1 g of sucrose daily per os for 28 days, followed by modeling of chemically induced colitis. For this purpose, the animals were rectally injected once with 1 ml of a 3% acetic acid solution + ethanol. Starting from this day, the animals were intragastrically administered a mixture of antimicrobial drugs (AMD) – amoxicillin, metronidazole, and clarithromycin: 1 ml of antimicrobial solution at a daily dose of 15 mg of each AMD per rat for three days and 1 ml of normal saline (NS) for 8 days; 3) SIRS + *L. delbrueckii D5* – all manipulations corresponded to those described for the SIRS group, but instead of 1 ml of NS, the animals were administrated 1 ml of *L.delbrueckii D5* suspension at a concentration of 10*8 CFU per animal; 4) SIRS + inactivated culture – rats of this group were administered 1 ml of probiotic *L.delbrueckii D5* suspension after its pasteurization at 85–90 °C for 1 minute.

Under combined anesthesia, the heart of the rats was removed from the chest by thoracotomy after the end of drug administration (zoletil 20 mg / kg, IM, isoflurane 1.5–2%) and mounted on the Langendorf apparatus. Retrograde perfusion was performed through the aorta with the oxygenated Krebs – Henseleit buffer at a constant pressure of 80 mm Hg. The temperature of the heart and solutions was maintained at 37 °C throughout the experiment. A polyethylene balloon was inserted into the left ventricle for recording isovolumetric pressure. Diastolic pressure and pulse pressure in the left ventricle were recorded on the personal computer using the PhysExp 3.0 program. Heart rate (bpm) was estimated from the left ventricular pressure curve using the software-based method. Coronary flow (CF) in ml / min was also determined by measuring perfusate flow velocity from the pulmonary artery.

The study protocol included the following steps: 1) baseline monitoring of functional indices after a 10 min stabilization period; 2) induction of global ischemia for 30 min; 3) reperfusion through the aorta with the oxygenated Krebs – Henseleit buffer for 60 min with readings every 15 min. After completing 60-minute reperfusion, we performed planimetry of infarct size by staining heart slices with triphenyltetrazolium chloride (TTC) [6].

Complete blood count was performed on the automated veterinary hematology analyzer (URIT-3000 Vet Plus, URIT Medical Electronic, China). The analysis of the leukocyte count in the blood was performed on three leukocyte populations: LYM (lymphocytes), MID (total number of monocytes, eosinophils, basophils, and blasts), and GRAN (granulocytes). The levels of lipopolysaccharide (LPS), TGFβ1, TNFα, IL-1β, IL-6, Hp, and Lf were assessed by the enzyme-linked immunosorbent assay (MR-96A, Mindray, China). Throughout the experiment, the clinical status of the animals, feed and water consumption, and body weight were assessed daily from 9:00 to 10:00 AM.

Statistical processing of the experimental data was carried out using the STATISTICA 12.0 software package. The statistical analysis of discrete values was performed using the nonparametric Kruskal – Wallis H test to detect statistically significant differences, followed by post hoc comparisons using the Mann – Whitney U test. The tables presented the median (Me) and the lower and upper quartiles ($Q_{25\%}$;$Q_{75\%}$). The differences were considered statistically significant at $p < 0.05$.

RESULTS

Body weight, feed and water consumption

The body weight of the rats in the control group throughout the follow-up increased on average by 1.25 ± 0.33 g / day, while in the SIRS, SIRS + *L. delbrueckii D5*, and SIRS + inactivated culture groups, we noted a decrease in the animal body weight by 3.63 ± 1.21, 3.18 ± 0.95, and 3.25 ± 0.97 g / day, respectively ($p < 0.05$ compared to the controls). Water consumption over the same period per 100 g of body weight was 8.8 ± 1.1 ml / day in the control group and 7.3 ± 0.1; 10.1 ± 1.5, and 9.3 ± 0.3 ml / day for the SIRS, SIRS + *L. delbrueckii D5*, and SIRS + inactivated culture groups, respectively. Feed consumption in these groups was 1.2 ± 0.2; 1.4 ± 0.3, and 1.5 ± 0.1 g / day, respectively, which was 2.8, 2.4, and 2.2 times lower ($p < 0.05$) than in the control group (3.3 ± 0.4 g / day).

Complete blood count

The leukocyte count ($\times 10^9$ / l for all populations) in the control group was 5.3 (5.2; 5.4); in the SIRS, SIRS + *L. delbrueckii D5*, and SIRS + inactivated culture groups, it was 7.9 (6.6; 10.1) ($p < 0.05$), 7.5 (5.3; 10.4) ($p < 0.05$), and 6.2 (5.8; 7.3), respectively. The granulocyte population (GRAN) made the main contribution to the increase in the leukocyte count

compared to the control group (2.6 (2.3; 2.8)) and was 5.9 (4.2; 6.8) ($p < 0$ .05); 5.5 (4.8; 6.2) ($p < 0.05$), and 4.6 (4.3; 5.5) ($p < 0.05$) in the experimental groups, respectively. The MID values were 0.2 (0.2; 0.28), 0.4 (0.2; 0.5), 0.30 (0.2; 0.49), and 0.20 (0.2; 0.4) in the control, SIRS, SIRS + *L. delbrueckii D5*, and SIRS + inactivated culture groups, respectively. The LYM population accounted for 1.2 (1.1; 1.3), 1.6 (1.4; 2.0), 1.3 (0.9; 1.9), and 1.2 (1.1; 1.6) in the control, SIRS, SIRS + *L. delbrueckii D5*, and SIRS + inactivated culture groups, respectively.

Immunological parameters

The study revealed a significant increase in the blood LPS concentration by more than 3 times ($p < 0.05$) in the SIRS group compared to the controls, with no increase in other groups. The levels of inflammatory markers and hormones are presented in Table 1.

Table 1

Results of assessing plasma levels of cytokines, haptoglobin, lactoferrin, and lipopolysaccharide, pg / ml, *Me* ($Q_{25\%}$; $Q_{75\%}$)

Parameter	Controls	SIRS	SIRS + *L. delbrueckii D5*	SIRS + inactivated culture
TNFα	10.4 (8.7; 11.4)	19.0 (15.0; 21.5)*	11.2 (10.0; 13.3)#	14.8 (12.8; 14.6)
IL-1β	4.6 (2.2; 4.9)	9.6 (7.4; 10.2)*	4.9 (4.3; 5.2) #	6.6 (6.0; 7.6)
IL-6	1.5 (1.4; 1.8)	2.2 (2.0; 2.5)*	1.3 (1.2; 2.5)	1.4 (1.0; 2.1)
TTGFβ	3.2 (2.5; 4.4)	6.2 (5.1; 11.1)*	4.5 (3.1; 7.3)	3.8 (3.2; 4.9)#
Lactoferrin	86 (58; 121)	107 (90; 109)	133 (119; 145)*	55 (37; 63)#
Haptoglobin	31 (24; 37)	76 (64; 102)*	24 (22; 30)#	35 (26; 40)#
LPS	52 (42; 65)	163 (91; 168)*	60 (54; 71)#	63 (50; 69)#

* $p < 0.05$ compared to the control group, # $p < 0.05$ compared to the SIRS group.

In the SIRS group, the level of TNFα, IL-1β, IL-6, TGFβ, Hp, and LPS increased by 83, 108, 47, 94, 146, and 214%, respectively ($p < 0.05$), compared to the control group. In the SIRS + inactivated culture group, there was a decrease by 63, 94, and 117% in TGFβ, Lf, and Hp, respectively ($p < 0.05$), compared to the SIRS group. In the SIRS + *L. delbrueckii D5* group, the values of all parameters were close to the control ones, except for a significant increase in Lf by 133% ($p < 0.05$).

Histologic examination of the colon

The histologic examination of the corresponding section of the colon in the rats of the control group revealed normal histologic design of the tissue. In the SIRS group, large areas with ulcers and erosions with purulent exudate were identified on the colonic mucosa at the site of inflammation (5–7 cm from the anus). The mucosa and submucosa were replaced with granulation tissue. We detected edema and severe inflammatory infiltrate with prevalence of lymphocytes; however, macrophages, polymorphonuclear leukocytes, and eosinophils were also present in fairly large numbers. Plethora of microcirculatory and lymphatic vessels was noted. The muscular layer was relaxed, inflammatory infiltrate of varying severity was determined. The bottom of the ulcerative lesion had characteristic changes in the form of alternating layers of fibrinoid necrosis, granulation tissue, and fibrous tissue. The mucosa around extensive ulcerative lesions was ulcerated, with impaired histologic design, pronounced inflammatory infiltrate, and necrosis of crypts by more than 2/3.

In the SIRS + *L. delbrueckii D5* and SIRS + inactivated culture groups, extensive ulcerative lesions were also identified, but there were more pronounced signs of tissue regeneration, re-epithelialization at the borders of ulcerative lesions of up to 40%, predominance of the fibrinoid component, restoration of histologic design in the muscular layer and submucosa, and a lack of mucopurulent discharge. In the SIRS + inactivated culture group, the superficial layers of ulcerative lesions were characterized by formation of immature connective tissue in place of granulation tissue as well as by decreased inflammatory infiltrate. When the mucosa and submucosa were replaced with granulation tissue, signs of regeneration were noted. In the SIRS + *L. delbrueckii D5* group with similar changes in some samples, re-epithelialization by single-layered intestinal epithelium was detected at the borders of ulcerative lesions, indicating accelerated regeneration.

Morphofunctional characteristics of the isolated heart

The infarct size in the control group was 41% (38; 45), and in the SIRS group – 54% (52; 57), which was significantly greater ($p < 0.05$) compared to the controls. In the SIRS + *L. delbrueckii D5* group, the infarct size was 42% (36; 48), which was significantly smaller than in the SIRS group. In the SIRS + inactivated culture group, only a trend toward a decrease in infarct size was revealed compared to the SIRS group (47% (44; 58), $p = 0.6061$).

Table 2 presents a correlation between the values of the studied parameters and myocardial infarct size.

The figure shows significant differences between infarct size, TNF**α**, IL-1β, TGFβ, and Hp values in the groups.

Table 2

Spearman's correlation between plasma parameters (infarct size, TNFα, IL-1β, TGFβ, haptoglobin, and lactoferrin) in all the rats in the experiment, $p < 0.05$

Parameter	TNFα	IL-1β	TGFβ	Lactoferrin	Haptoglobin	Infarct size
TNF**α**, pg / ml	1.0000	0.4570*	0.2209	–0.0269	0.3077	0.4654*
IL-1β, pg / ml	0.4570*	1.0000	0.3944*	–0.3455*	0.4159*	0.5063*
T TGFβ, pg / ml	0.2209	0.3944*	1.0000	–0.0477	0.2638	0.3657*
Lactoferrin, pg / ml	–0.0269	–0.3455*	–0.0477	1.0000	–0.2164	–0.2102
Haptoglobin, pg / ml	0.3077	0.4159*	0.2638	–0.2164	1.0000	0.4244
Infarct size, %	0.4654*	0.5063*	0.3657*	–0.2102	0.4244*	1.0000

* $p < 0.05$ compared to the control group.

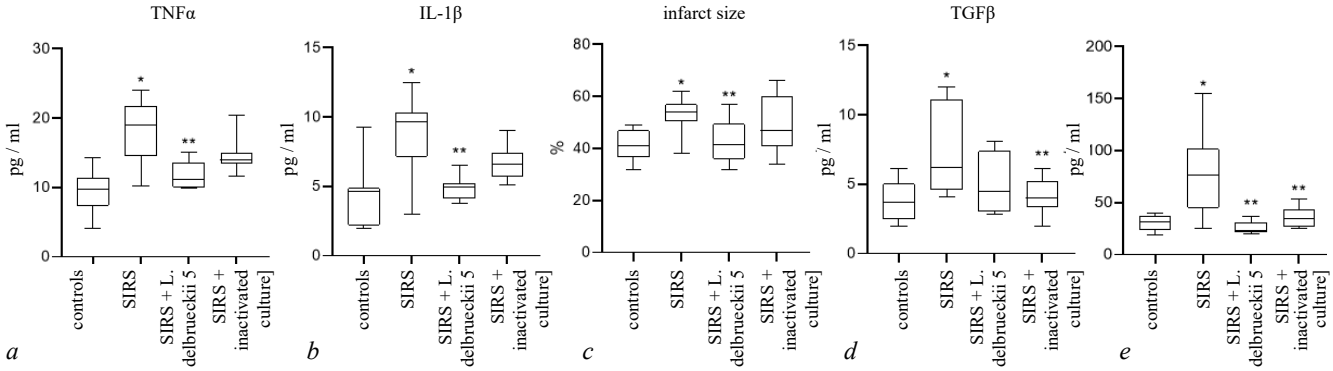

Figure. TNFα (*a*), IL-1β (*b*), infarct size (*c*), TGFβ (*d*), Hp (*e*) in the blood plasma: * $p < 0.05$ compared to the control group, ** $p < 0.05$ compared to the SIRS group

Due to limited possibilities of leukocyte differentiation into subpopulations and by maturity, it can be indirectly stated that neutrophils played the main role in the increase in the GRAN population in modeling SIRS. In the SIRS group, there was a decrease in the lymphocyte count and the MID population, which included monocytes, eosinophils, basophils, and blasts. In the SIRS + inactivated culture group, minimal MID and LYM values were noted. Administration of live and inactivated culture to the rats in the SIRS group resulted in normalization of the total leukocyte count and a decrease in the LYM, GRAN, and MID populations compared to SIRS.

In the SIRS group with a rise in TNFα, IL-1β, IL-6, and Hp in the blood and an increase in the GRAN population, a decrease in the LYM and MID populations was observed, indicating a causal role of SIRS in the production and migration of leukocytes. Regulation of lymphocyte proliferation and differentiation was affected by cytokines, intensively formed during exposure to various infectious and non-infectious antigens by lymphocytes and monocytes, in particular IL-2, IL-4, IL-6, and IL-7. In the SIRS group, degenerative and destructive changes in the intestinal wall with impairment of barrier function were noted, with a significant increase in the level of LPS in the blood, which triggered systemic inflammation. The main, but probably not the only reason for the decrease in LPS levels in the groups receiving probiotic and metabiotic therapy for SIRS may be competitive reduction of signs of opportunistic microbiota and / or a decrease in the permeability of

the intestinal wall to LPS. Each mechanism, either individually or in combination, may make a decisive contribution to endotoxinemia, since suppression of phagocytic and immunocompetent host cells is found only in individual strains.

Accumulating evidence in recent years suggests an important role of GMB in maintaining selective permeability of intestinal epithelium and preventing cellular barrier dysfunction leading to increased intestinal permeability. It provides nonspecific transport of proinflammatory antigens, microbes, and metabolites from the intestinal lumen to the mucosa and bloodstream, which in turn may be responsible for a variety of diseases, including inflammatory bowel disease and tumors, obesity, non-alcoholic fatty liver disease, depression, neurodegeneration, cardiovascular diseases, and type 1 diabetes mellitus [7]. Members of normal GMB apparently play a specialized role in maintaining the integrity of physical intestinal barrier, with their generation of microbial metabolites and bacterial components being the main way to achieve this goal [8].

For example, butyrate produced through bacterial fermentation of dietary fiber enhances the expression of tight junction (TJ) proteins – crucial multiprotein complexes that regulate the permeability of the intestinal barrier [9]. Microbial components, such as lipopeptides, enhance TJ function by stabilizing the levels of occludin-1 (ZO-1) and claudin by activating protein kinase C through interaction with Toll-like receptors (TLRs) on intestinal epithelial cells [10].

In this regard, a number of *in vitro* [11–15] and *in vivo* studies, including clinical trials [16, 17] on the effect of probiotic bacterial strains of the *Lactobacillus, Lactiplantibacillus, Limosilactobacillus, Bifidobacterium*, and *Streptococcus* genera on intestinal barrier function have been conducted, with promising results. Probiotic supplementation was found to markedly improve intestinal barrier function, as measured by transepithelial electrical resistance (TEER) and serum zonulin and LPS levels. Besides, it normalizes increased intestinal permeability and reduces the content of proinflammatory cytokines, including C-reactive protein (CRP), TNFα, and IL-6 in unfavorable conditions (bacterial infections, oxidative stress, high-fat diet, alcohol, chronic allergens, dysbiosis), in particular by enhancing the function of TJ in intestinal epithelial cells associated with increased synthesis of occludins [18, 19].

It should be noted that complex probiotics demonstrate higher efficacy in maintaining intestinal barrier function and preventing increased intestinal permeability compared to single strains. Thus, probiotics may play a crucial role in the treatment of autoimmune and metabolic diseases by improving intestinal barrier function. However, an exhaustive evaluation of probiotics regulating intestinal barrier function in various diseases is still lacking.

A significant inverse relationship of Lf values and a direct relationship of Hp values with IL-1β were found in the SIRS + *L. delbrueckii D5* group at high Lf concentration. Normal values of IL-1β and Hp with normal infarct size were observed, which may indicate that the live strain had specific properties compared the inactivated culture.

In this study, the administration of live and inactivated culture equally affected most of the studied parameters in the blood, indicating molecular sufficiency of pro- and metabiotic in this case. It is obvious that when the animals received live bacteria, the production of Lf increased significantly, while the production of Hp and proinflammatory cytokines decreased to control values. These trends were confirmed by significant differences between infarct size in the group of animals receiving live bacteria. It is likely that live lyophilized lactobacilli that became metabolically active had time to modulate immune responses, bringing them to control values. The obtained data indicate the expediency of probiotic therapy with live microorganisms in acute cardiac pathologies.

CONCLUSION

Lf and Hp, as acute phase proteins, showed different responses during pro- and metabiotic modulation of SIRS. Blood Hp levels were directly associated with LPS values, while Lf values were inversely correlated with the levels of the studied cytokines and myocardial infarct size.

In this study, synergistic changes in TNFα, IL-1β, IL-6, and Lf values across the groups are worth noting, with the maximum increase in the SIRS group, the intermediate increase in the SIRS + inactivated culture group, and the minimum increase in the SIRS + *L. delbrueckii D5* group. Taking into account the association of myocardial infarct size with an increase in the values of the studied parameters in the SIRS and SIRS + inactivated culture groups and a decrease in these parameters in the control and SIRS + *L. delbrueckii D5* groups, we can assume the presence of specific properties of the live probiotic culture and the corresponding mechanism.

The question remains open whether a combination of increased TNFα, IL-1β, IL-6, and Lf confirms decreased myocardial resistance to IRI. If the correlation between these markers and myocardial morphofunctional indices is established, targeted exclusion of one of the factors in the safest way is possible. Inactivated culture of *Lactobacillus delbrueckii D5* has a pronounced anti-inflammatory effect without increasing resistance to IRI, in contrast to the lyophilized strain, which requires further studies.

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Authors' contribution

Borshchev Yu.Yu., Galagudza M.M., Suvorov A.N. – conception and design. Borshchev Yu.Yu., Burovenko I.Yu., Minasean S.M., Borshcheva O.V., Semenova N.Yu., Gritsenko E.Yu., Sheptitsky V.A. – collection and processing of the material, statistical processing. Borshchev Yu.Yu., Sheptitsky V.A. – drafting of the article. Borshchev Yu.Yu., Galagudza M.M., Burovenko I.Yu. – editing of the manuscript.

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The state and vascularization of the bone marrow transplanted in the diffusion chamber to the rat neurovascular bundle

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ABSTRACT

Background. The diffusion chamber method helps solve the problem of delivering a biomaterial with minimal losses, while creating an isolated environment in the recipient's body. The issue of vascularization of diffusion chambers to preserve the functional capacity of the biomaterial remains relevant. A bioengineered diffusion chamber model, together with the vascular adventitia, promotes vascularization of the biomaterial placed in the chamber.

The **aim** of the study was to assess the state of the bone marrow placed in the diffusion chamber and transplanted to the femoral neurovascular bundle of a rat.

Materials and methods. The experimental part of the study was carried out on mature male Wistar rats. The animals were divided into two groups. Group 1 was experimental (n = 4), in which a polycaprolactone diffusion chamber filled with bone marrow was implanted in the femoral neurovascular bundle. Group 2 was control (n = 3), in which the diffusion chamber without bone marrow was implanted in a similar bundle.

Results. The histologic examination of the structure of the compact capsule in the bioengineered model in the experimental group revealed areas of woven bone tissue in 25% of the rats. An increase in the vascularization coefficient by 96% and a rise in the Kernohan index by 7% in the experimental group compared to the control group indicated that sufficient conditions were formed to develop the microvasculature while maintaining the bone marrow differentiation path.

Conclusion. The reliability of these results is confirmed by immunohistochemical markers of vascularization VEGF and CD34.

Keywords: diffusion chambers, biodegradable polymer, cell technologies, bone marrow transplant, microfluidic technologies, laboratory rats

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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Состояние и васкуляризация костного мозга при имплантации в диффузионной камере на сосудисто-нервный пучок

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РЕЗЮМЕ

Введение. Метод диффузионных камер способствует решению задачи по доставке биологического трансплантата с минимальными потерями, создавая при этом условия изоляции в организме реципиента. Актуальным остается вопрос васкуляризации диффузионных камер для сохранения функциональности биоматериала. Модель биоинженерной конструкции диффузионной камеры в совокупности с адвентицией сосуда способствует васкуляризации биоматериала, помещенного в камеру.

Целью исследования явилась оценка состояния сингенного костного мозга, имплантированного в диффузионной камере на бедренный сосудисто-нервный пучок крысы.

Материалы и методы. Дизайн исследования включал в себя экспериментальную часть, проводимую на половозрелых самцах крыс линии Вистар. Животные были разделены на две группы: 1-я – экспериментальная (n = 4), имплантация диффузионной камеры из поликапролактона на бедренный сосудисто-нервный пучок с костным мозгом; 2-я – контрольная (n = 3), на аналогичный пучок имплантировалась камера без содержимого.

Результаты. При гистологическом исследовании в структуре компактной капсулы биоинженерной конструкции в экспериментальной группе выявлены участки грубоволокнистой костной ткани у 25% крыс. Повышение коэффициента васкуляризации на 96% и индекса Керногана на 7% в экспериментальной группе по сравнению с контрольной свидетельствует о формировании достаточных условий для развития микроциркуляторного русла при сохранении направления дифференцировки костного мозга.

Заключение. Достоверность приведенных результатов подтверждается иммуногистохимическими маркерами васкуляризации VEGF и CD34.

Ключевые слова: диффузионные камеры, биодеградируемый полимер, клеточные технологии, имплантация костного мозга, микрофлюидные технологии, лабораторные крысы

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

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INTRODUCTION

The use of the diffusion chamber (DC) is associated with a loss of the volume and biological properties of the cellular material as well as with the possibility of expanding the range of possible implantation. The search for methods for isolating cellular biomaterial is relevant due to high percentage of cellular biomaterial loss despite a high number of mesenchymal stem cells (MSC) in the bone marrow. The complexity of bone marrow transplant is associated with the necessity to imitate bone marrow hematopoietic niche (vascular, endosteal) as a physiological microenvironment [1]. That is why understanding the difficulties of the implantation process contributed to the progress in the development of 3D constructs with the possibility of immobilizing proliferation and differentiation factors [2] and programming hypoxic gradient [3].

Bone marrow vascularization associated with the expansion of the vascular niche is considered to

be the key problem in its functionality. Studies have noted the association between changes in bone marrow microcirculation and the progression of hematologic tumors as well as solid cancer [4]. The features of bone marrow vascularization present an opportunity for *in vitro* microfluidic technologies to generate separate compartments of cell and molecular signals in one biomaterial [5]. In addition, maintenance of bone marrow stem cells is regulated by different types of blood vessels with different permeability properties [6]

Researchers who attempted to consider the listed parameters in the *in vitro* system succeeded in creating the first vascularized bone-marrow-on-a-chip models [7]. It is known that new capillaries can grow not just in the process of neoangiogenesis, but also via the vascularization mechanism. The adventitia of major vessels is a depo for progenitor cells that participate in the microvasculature regeneration in response to a wide range of pathological stimuli. In addition, *vasa vasorum* has a range of molecular cell factors that initiate vasculogenesis [8]. The listed above allows to consider the bioengineered DC and vascular adventitia as an element of an experimental model of *in situ* vascularization of a syngeneic bone marrow transplant.

The **aim** of the study was to assess the state of the bone marrow placed in the 3D DC and transplanted to the femoral neurovascular bundle (FNB) of a rat.

MATERIALS AND METHODS

In this study, a bioengineered DC was designed in the form of a closed polymer capsule with a removable lid, latches, and the possibility to fill the cavity with cellular material (Fig. 1). The 3D-model of the DC (Fig. 1, *b*) was designed in the open source software environment Blender. The experimental samples (Fig. 1, *a*) were obtained by fused filament fabrication (FFF) on the CreatBot Duo 3D printer (CreatBot 3D Printer, China). End-walls of the DC have recesses to fix the construct to the vessel. The DC was made of polycaprolactone (Natural Works Ingeo 40–43d NatureWorks LLC) that is a biodegradable polyester approved for medical use with a low melting point (59–64 °) [9].

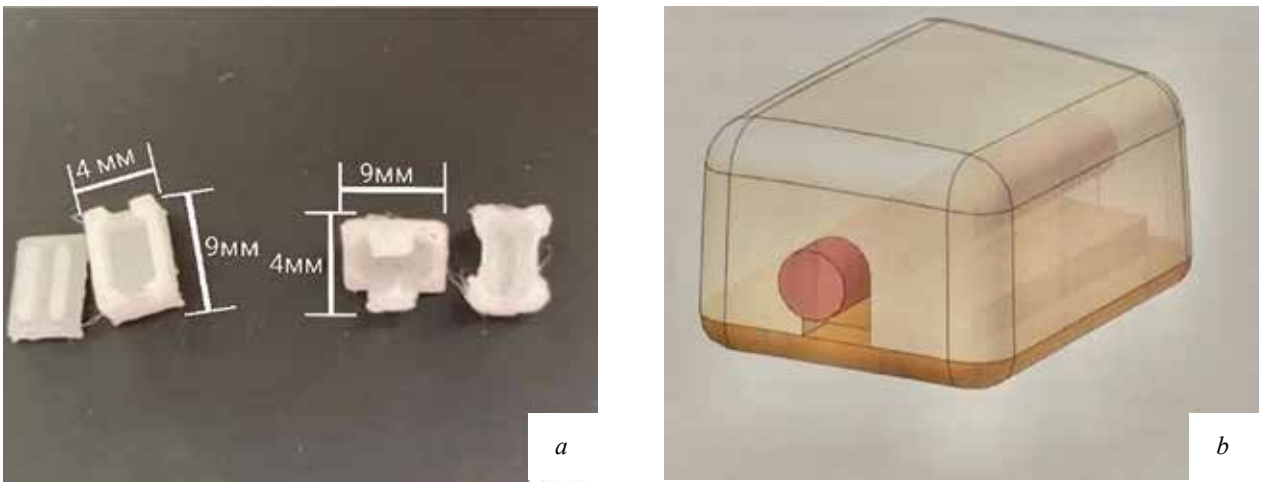

Fig. 1. 3D-printed construct (*a*) based on the 3D diffusion chamber model (*b*)

Sterilization of DC was done in 100% ethylene oxide vapor at 37 ˚C for 9 hours in the gas sterilizer 3M Steri-Vac Sterilizer/Aerator (3M, USA) according to the recommendations of GOST ISO 11135-2017 [10]. The study was conducted on adult male Wistar rats weighing 280–300 g. The animals were held in standard vivarium conditions in the Laboratory of Biological Models of Siberian State Medical University (Tomsk). The animals were divided into two groups. Group 1 was experimental (n = 4), in which a DC filled with bone marrow was implanted in FNB. Group 2 was control (n = 3) in which a DC without bone marrow was implanted in a similar bundle.

Two rats comparable in weight and age to the study groups became bone marrow donors. Bone marrow obtained under aseptic conditions in a laminar flow hood by washing the diaphysis of the femurs with a sterile culture medium, was placed *ex vivo* in an implantation chamber. Implantation of

DCs in experimental animals was carried out under isoflurane anesthesia. The volume of bone marrow placed in the DC was 100 μl. 15 minutes before surgery, atropine was administered intramuscularly at a dosage of 0.2 mg / kg to prevent hypersecretion of mucus in the bronchi. Surgical access was provided through a 2–3 cm incision deep in the inguinal fold, inward from the pulsation of the femoral artery [11]. All manipulations with the animals were carried out in accordance with the Directive of the European Parliament No. 2010/63eu of 22.09.2010 "On the protection of animals used for scientific purposes".

Six hours after the surgery, the rats were considered stable. On day 40 after the end of the implantation period, the animals were euthanized by CO_2 inhalation in compliance with the rules and norms of the European Council (86/609 EEC), the Declaration of Helsinki, and orders of the Ministry of Healthcare of the USSR (No. 742 of 13.11.1984 and No. 48 of 23.01.1985).

A macroscopic (visual) evaluation of the implantation site was carried out at 40 days of the experiment during necropsy. The implantation site was assessed by the following criteria: the degree of blood supply in the vessels, encapsulation and visual signs of an inflammatory reaction (the presence of hyperemia, edema, and infiltration) according to the scoring system, where 0 points was the absence of a sign, 1 point – weak degree, 2 points – moderate degree.

Histologic samples were prepared after necropsy according to standard methods [12]. Microscopy was performed on the Carl Zeiss Observer D1 light microscope (Germany). Rabbit polyclonal VEGF antibodies (Anti-VEGFA antibody, ab46154 antibodies from Dako (Mouse monoclonal [EIC] to VEGF receptor 2), CD34 (Anti-CD34 antibody, ab185732 antibodies from Dako to CD34 (Clone QBEnd 10)), and CD45 (CD45-APC-Cy7, Biolegend, USA)) were used for immunohistochemistry staining. Staining was carried out according to the manufacturer's instructions. To analyze the results of immunohistochemical reactions, a method for assessing dye expression on a point scale was used, where 3 points (+++) was strong staining, 2 points (++) – moderate staining, and 1 point (+) – weak staining [13]. Microscopy was performed on the Carl Zeiss Observer D1 light microscope (Germany). Morphometry with an assessment of the vascularization coefficient was carried out using images obtained by the Zeiss AxioCam ICc5 digital camera for light microscopy (Germany).

To quantify the degree of bone marrow vascularization in the DC implanted in rat FNB, the following coefficients were used: the vascularization coefficient (CV) was estimated using the formula: $CV = S_v/S_p \times 100\%$, where Sv was the area of all microvessels, Sp was the area of the photograph [14], excluding the femoral artery and vein, the Kernohan index (KI) was calculated as: $KI = (2 \times L_{\text{artery wall}}) / D_{\text{artery}}$, where L was the thickness of the tunica media of the artery), D was the diameter of the artery lumen [15].

Ten fields of view were assessed in each group to calculate the parameters. Statistical processing was carried out using the Statistica 10.0 program, IBM (USA). Statistical hypothesis testing to determine the nature of trait distribution was carried out using the Shapiro – Wilk test for small ($n < 30$) samples. Descriptive and nonparametric statistical methods were used to process the results obtained. The studied parameters were described as the median (Me) and the interquartile range (Q_1; Q_3). When comparing independent samples, the Kruskal – Wallis test with the median test was used; and the Wilcoxon test was used for paired comparisons. The differences were considered statistically significant at $p < 0.05$.

RESULTS

Intraoperatively, camera fixation did not disrupt general blood circulation and innervation (Fig. 2). There were no visible postoperative inflammatory reactions at the implantation sites during the follow-up period (40 days) (Fig. 3).

Fig. 2. The diffusion chamber implantation bed

a *b* *A*

Fig. 3. The diffusion chamber implantation site after the surgery: *a* – experimental group, *b* – control group

The results of the macroscopic (visual) assessment of the implantation site according to the degree of blood supply in the vessels, encapsulation and visual signs of an inflammatory reaction (presence of hyperemia, edema, and infiltration) showed (Table 1) that in the experimental group, implantation was accompanied by mild (according to GOST ISO 10993-6-2021) changes in the form of hyperemia and formation of a connective tissue capsule.

Table 1

Macroscopic changes in the implantation bed of the diffusion chamber filled with bone marrow implanted in FNB on day 40 of the experiment, points, *Me* (Q_1; Q_3)

Group	Inflammation	Hyperemia	Chamber encapsulation
Experimental group, $n = 4$	0 (0; 0)	1 (1; 1.5)	1.5 (1; 1.5)
Control group, $n = 3$	0 (0; 0)	0 (0; 1)	–

Note. The number of animals in each group – *n*.

The histologic examination revealed that the implanted bioengineered structures were covered with a compact capsule more than 50 microns thick, made of mature connective tissue (Fig. 4). This tissue was classified as loose fibrous irregular connective tissue with developed microcirculation. These compact capsule structures could not be detected in the control group.

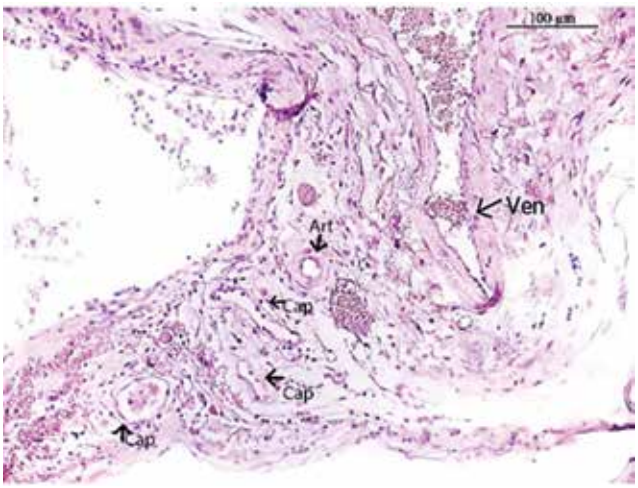

Fig. 4. Microscopic images of the distribution of microvasculature in the connective tissue capsule around the diffusion chamber on day 40 after the implantation. Light microscopy, × 40

Connective tissue was also found in the cavity of the chamber adjacent to the polymer wall (Fig. 5, *a*), similar in its structure and components to the connective tissue capsule of the DC (Fig. 5).

Microscopy of the chamber contents showed that all animals in the experimental and control groups had areas of loose fibrous connective tissue with a large number of microvessels (Fig. 5). Microscopy of a transverse section of the FNB, as an implantation site of DC filled with bone marrow, did not reveal a narrowing of the vessel lumen compared to a similar parameter in animals of the control group. The vessel walls showed homogeneity of endothelial cells and an increase in the layer of muscle cells. An increase in the number of small vessels in the muscle layer and adventitia (*vasa vasorum*) with an increase in the lumen of the latter was noted (Fig. 6).

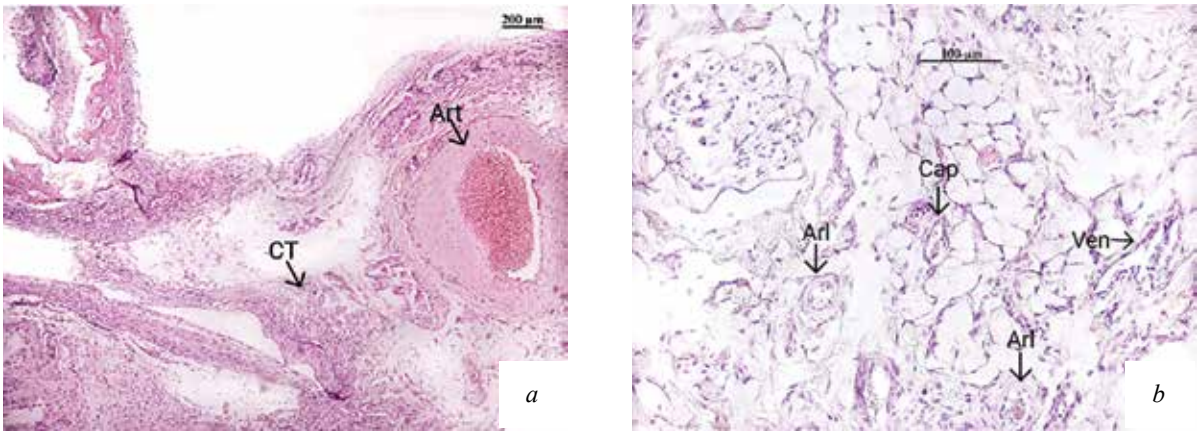

Fig. 5. Microscopic image of the connective tissue content of the diffusion chamber (*a*) and its microcirculation (*b*) on day 40 after implantation: CT – connective tissue, Cap – capillaries; Art – arterioles; Ven – venules. H&E stain. Light microscopy, ×10 (*a*); × 40 (*b*)

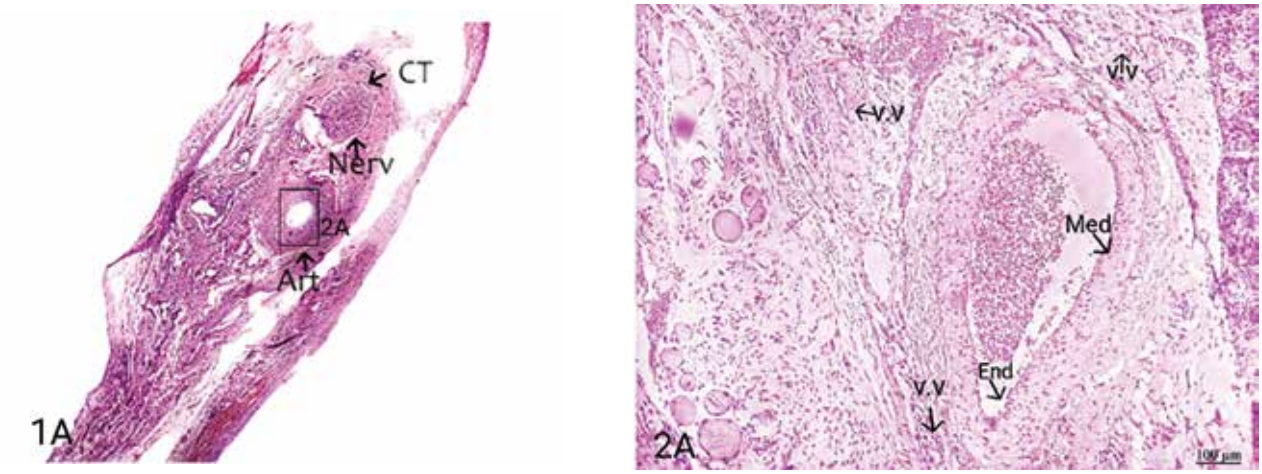

Fig. 6. 1A – microscopic image of the rat FNB with the DC: Art – artery, Nerv – nerve, CT – connective tissue content in the diffusion chamber. H&E stain. Light microscopy, × 10. *2A* – Microscopic image of an artery in the neurovascular bundle. Med – media, *v.v.*– *vasa vasorum*, End – endothelium. H&E stain. Light microscopy, × 40

The results of a quantitative assessment of the angiogenesis of DC with bone marrow implanted in rat FNB, according to the vascularization coefficient and the Kernohan index, are presented in Table 2. According to Table 2, during the DC implantation in FNB, sufficient conditions are formed along the femoral artery with a bioengineered construct that promote the growth of microvasculature in the zone of regenerative metaplasia of the bone marrow into connective tissue and its derivatives (adipose and bone tissue).

According to the immunohistochemical analysis, expression of VEGF, CD34, and CD45 was observed in the sections in all animals of the experimental and control groups, but in the experimental group with bone marrow metaplasia, it was more pronounced for CD34 (Fig. 7, *c, d*) and VEGF (Fig. 8, *a, b*). In both groups, the presence of the hematolymphoid marker CD45 was shown before day 40 of the study (Fig. 7, *e, f*).

Table 2

Parameters of vascularization of the diffusion chamber filled with bone marrow implanted in the FNB, *Me* (Q_1; Q_3)

Parameter	Group	
	Experimental group, $n = 4$	Control group, $n = 3$
Vascularization coefficient, %	1.28* (0.93; 1.60)	0.65 (0.37; 0.71)
Kernohan index, pxl	0.72* (0.69; 0.73)	0.67 (0.66; 0.68)

* – statistically significant differences in the parameters of the experimental group compared to the corresponding control value at $p < 0.05$.

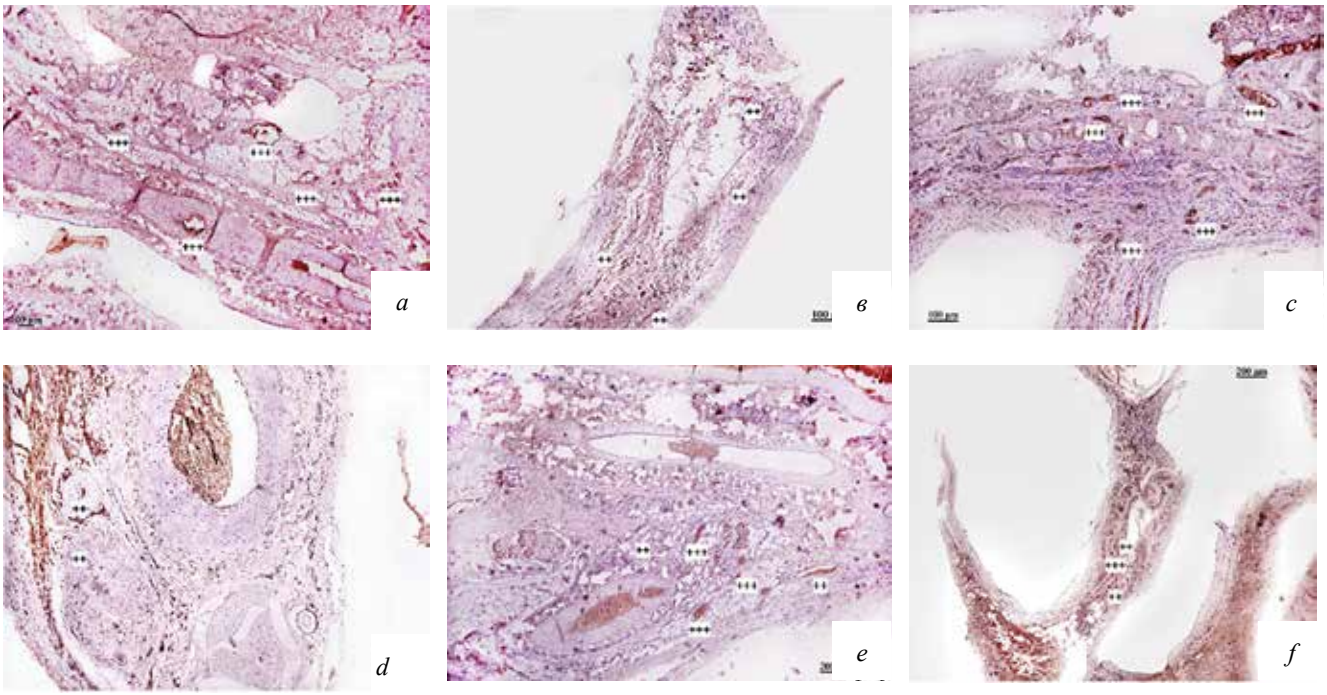

Fig. 7. Expression of VEGF, CD34, and CD45 in the experimental and control groups, × 10: +++ – strong staining, ++ – moderate staining. Expression of VEGF in DC contents in the experimental group. The expression degree is 3 points (+++) (*a*); VEGF expression in DC contents in the control group. The expression degree is 2 points (++) (*b*); CD34 expression in DC contents in the experimental group. The expression degree is 3 points (+) (*c*); CD34 expression in DC contents in the control group. The expression degree is 2 points (+) (*d*); CD45 expression in DC contents in the experimental group. The expression degree is 3 points (+) (*e*); CD45 expression in DC contents in the control group. The expression degree is 2 points (++) (*f*)

DISCUSSION

The study demonstrated the main morphological features of vascularization during bone marrow transplant to the FNB in DC. The material used for DC is polycaprolactone that is a biocompatible and biodegradable polymer. The degradation products of polycaprolactone are water, carbon dioxide, and caproic acid that are safe for animals [16].

The above literature data are confirmed by the conducted study which showed the absence of the damaging effect of polycaprolactone degradation products in the form of inflammatory reactions at the implantation site. The assessment of postoperative tissue state in the animals revealed no signs of inflammatory reactions. The revealed increase in the number of microvessels and the vascularization coefficient in the DC filled with bone marrow showed that bone marrow promotes the formation of vascularized stroma around major vessels. Immunohistochemical vascularization markers VEGF and CD34 confirmed the histology results. VEGF is a signaling protein produced for the induction of vasculogenesis and angiogenesis; it is responsible for restoring oxygen flow to tissues [17]. According to the manufacturer's instructions, CD34 expression in the present study was interpreted as a

marker of endothelial cells in blood and lymphatic vessels [18], and it increased during implantation of the DC filled with bone marrow. CD45 is a member of the tyrosine phosphatase family. The gene encoding this protein is specifically expressed in hematopoietic cells. The protein plays a role in signal transmission from cellular antigen receptors [19]. Expression of CD45 in histology sections at the implantation site of DC with bone marrow and without it (control) suggests migration of blood cells to the damaged area with subsequent active participation in regenerative processes [20].

Polycaprolactone has adsorption properties toward mesenchymal stem cells (MSCs) and low cellular toxicity [21]. Both local (vascular and BM) and circulating MSCs and pericytes can induce neoangiogenesis [22]. However, the presence of bone marrow in the DC significantly enhances the vascularization of the implant. At the same time, the literature also indicates the anti-inflammatory / regenerative effect of bone marrow MSCs at the implantation site.

The cytokine profile of MSCs may affect the absence of an inflammatory reaction at the implantation site [23]. Thus, vasculogenesis inducers (for example, VEGF, interleukin (IL)-10) secreted by MSCs are cytokines that also regulate tissue regeneration [24]. From the point of view of physiology, bone marrow MSCs can be differentiated based on the formation of well-vascularized loose irregular connective tissue during subcutaneous implantation [24], which was preserved under conditions of bone marrow implantation to the FNB in DC.

CONCLUSION

Diffusion chamber made of polycaprolactone and implanted in the femoral neurovascular bundle does not cause mechanical damage, inflammation, and post-implantation complications. Bone marrow placed in diffusion chambers undergoes regenerative metaplasia with differentiation of mesenchymal stem cells into fibroblasts and, possibly, endothelial cells. Increased vascularization in the zone of ectopically regenerating bone marrow creates conditions for *in situ* engineering of parenchymal organs that require preserved blood supply (liver, etc.). In general, the formation of a functional "DC – bone marrow – macrocirculation" system can be useful for the development of experimental tissue engineering.

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Authors' contribution

Marzol E.A., Dvornichnko M.V., Khlusov I.A. – conception and design. Marzol E.A., Zinovyev E.A., Mitryaikin N.S. – analysis and interpretation of the data. Marzol E.A., Zinovyev E.A., Dvornichenko M.V. – justification of the manuscript or critical revision of the manuscript for important intellectual content. Dvornichnko M.V., Khlusov I.A. – final approval of the manuscript for publication.

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Clinical and immunological characteristics of post-COVID syndrome

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ABSTRACT

Aim. To evaluate changes in clinical manifestations and the cytokine profile of blood serum in patients with post-COVID syndrome.

Materials and methods. The study involved 46 patients (37 women and 9 men) with signs of post-COVID syndrome 1–12 months after COVID-19 infection. COVID-19 infection was laboratory-confirmed (patients were tested positive for SARS-Cov-2 RNA using polymerase chain reaction (PCR), or they were tested positive for SARS-Cov-2 immunoglobulin (Ig)G antibodies after the end of the acute phase and in asymptomatic infection). Along with mandatory tests included in the regular health checkup of medical staff, the levels of interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-17, tumor necrosis factor alpha (TNFα), interferon gamma (INFγ), and total IgE were determined in the blood serum of patients.

Results. The results showed that the development of post-COVID syndrome did not depend on the age and gender of patients and the severity of the acute phase of infection. Patients were more likely to develop post-COVID syndrome in the absence of antiviral therapy or in case of its ineffectiveness. A high level and imbalance of pro- and anti-inflammatory cytokines without laboratory signs of inflammation underlie the development of clinical manifestations at early stages of post-COVID syndrome (up to 3 months). The clinical presentation was characterized by symptoms of asthenia and functional disorders in the nervous, cardiovascular, and respiratory systems and gastrointestinal tract. After 3 months, the content of most cytokines returned to normal levels, whereas only the concentration of IL-17 remained elevated. Allergic and autoallergic mechanisms of damage to the skin, respiratory organs, and joints, as well as progression of cardiovascular pathology determined the clinical symptoms of post-COVID syndrome for 3–12 months.

Conclusion. The changes in the cytokine profile over 12 months reflect different damage mechanisms at different periods of the post-COVID syndrome, which determines the range of its clinical manifestations.

Keywords: post-COVID syndrome, asthenic syndrome, cytokines

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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Conformity with the principles of ethics. All persons signed an informed consent to participate in the study. The study was approved by the Ethics Committee at the National Ilizarov Medical Research Center for Traumatology and Orthopedics (Protocol No. 2 (72) of 07.10.2022).

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Клинико-иммунологическая характеристика постковидного синдрома

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РЕЗЮМЕ

Цель – оценить динамику клинических проявлений и цитокиновый профиль сыворотки крови у пациентов с постковидным синдромом.

Материалы и методы. Обследовано 46 пациентов (37 женщин и 9 мужчин) с признаками постковидного синдрома спустя 1–12 мес после перенесенной инфекции COVID-19. Факт перенесенной инфекции COVID-19 был лабораторно подтвержден (положительный результат полимеразной цепной реакции РНК SARS-Cov-2 в анамнезе или положительный титр антител иммуноглобулина (Ig) класса G к SARS-Cov-2 после купирования острого периода и при бессимптомном течении инфекции). Наряду с обязательным перечнем исследований, предусмотренных порядком проведения обязательных периодических осмотров медицинских работников, в сыворотке крови пациентов определяли содержание цитокинов интерлейкина (IL) 1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-17, фактора некроза опухоли альфа (TNFα), интерферона гамма (IFNγ) и уровень общего IgE.

Результаты. Формирование постковидного синдрома не зависит от возраста, пола пациентов и тяжести течения острого периода перенесенной инфекции. При отсутствии противовирусной терапии или ее неполноценности вероятность развития постковидного синдрома повышается. В основе формирования клинических проявлений в ранние сроки – до 3 мес – постковидного синдрома лежит высокий уровень и дисбаланс про- и противовоспалительных цитокинов при отсутствии лабораторных признаков воспаления. Клиническая картина характеризуется симптомами астенизации и функциональными нарушениями нервной, сердечно-сосудистой, дыхательной систем и желудочно-кишечного тракта. Спустя 3 мес уровень большинства цитокинов нормализуется, но остается высокой только концентрация IL-17. Аллергические и аутоаллергические механизмы повреждения кожи, органов дыхания, суставов, а также прогрессирование сердечно-сосудистой патологии определяют клиническую симптоматику постковидного синдрома на протяжении 3–12 мес.

Заключение. Динамика цитокинового фона в течение 12 мес отражает различные механизмы повреждения в разные сроки постковидного синдрома, что и определяет спектр его клинических проявлений.

Ключевые слова: постковидный синдром, астенический синдром, цитокины

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 независимым этическим комитетом «Национальный медицинский исследовательский центр травматологии и ортопедии имени академика Г.А. Илизарова» (протокол № 2 (72) от 07.10.2022).

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INTRODUCTION

The pandemic caused by SARS-CoV-2, with its undulating course due to new mutations of the virus, led to the development of a chronic condition called "long COVID" [1]. About 20% of people who went through the acute phase of COVID-19 with completed replication of SARS-CoV-2 experience a combination of various clinical symptoms in the post-COVID period. They make up post-COVID syndrome [1–3] which affects up to 5 million people worldwide [4, 5].

Post-COVID syndrome (PCS) is included in ICD-10 (U09.9) as a condition after COVID-19 (Post COVID-19 condition, unspecified) with a time criterion of at least 12 weeks [6, 7]. Most patients in the post-COVID period tend to have normal laboratory and radiological parameters, which indicates that virological recovery has been achieved. Despite this, some patients do not return to their initial physical activity and do not notice a full recovery [8–10]. Particular attention should be paid to the fact that the development of PCS does not depend on age, gender differences, severity of the acute phase, and prior hospitalization, and symptoms can appear at different times after the disease.

Currently, in Russia, guidelines for rehabilitation measures after COVID-19 have been developed and introduced into clinical practice [6]. The proposed scale for individual rehabilitation routing of people who had COVID-19 determines the possibility of rehabilitation measures at various stages of medical care, in particular in the outpatient setting. The priority in this program is the recovery period of the first 3 months after the acute phase of coronavirus infection. The early start of rehabilitation is aimed at preventing complications and speeding up full recovery and return to the previous lifestyle.

However, some patients do not achieve full recovery even after 12 months, which significantly reduces their quality of life. In addition, in clinical practice, both the onset and progression of many chronic diseases are increasingly recorded not only in a period of up to 12 weeks but also after 3–8 months after the infection. In this regard, identifying the mechanisms of PCS formation is relevant and can serve as a basis for predicting the development of complications and justifying the prevention and correction of its manifestations.

The aim of the study was to evaluate changes in clinical manifestations and the cytokine profile of blood serum in patients with PCS.

MATERIALS AND METHODS

The analysis of the obtained data was carried out in the outpatient department of one of Tyumen healthcare facilities from January to May 2022. The study involved employees of this healthcare facility who underwent a regular health checkup (a total of 302 people, of which 204 were women). The mandatory list of examinations included an examination by an internal medicine physician, complete blood count and blood biochemistry (glucose, total cholesterol, HDL, LDL, triglycerides); ECG, chest fluoroscopy in accordance with the procedure approved by the Ministry of Healthcare of the Russian Federation for mandatory health checkups of medical workers who work with industrial health and safety hazards (Order of the Ministry of Healthcare of the Russian Federation No. 29N of 28.01.2021, as amended on 01.02.2022).

The study involved 46 patients (37 women and 9 men) with signs of asthenia 1–12 months after COVID-19 infection. They did not have somatic symptom disorders that could provoke or aggravate asthenia in the post-COVID period, which means that they represented health status group I–II. All of them had a laboratory-confirmed history of COVID-19 infection (a positive SARS Cov-2 RNA PCR or positive SARS Cov-2 IgG after the end of the acute phase and in asymptomatic infection).

The severity of COVID-19 infection in the acute phase was assessed according to the Temporary Guidelines for the Prevention, Diagnosis and Treatment of Novel Coronavirus Infection (COVID-19) (Ministry of Healthcare of Russia. Edition 17 (09.12.2022)). According to their medical history, 5 people had no symptoms in the acute phase of COVID-19; 26 individuals had mild symptoms, 10 – moderate and 5 – severe. The last acute phase of infection occurred 1–3 months ago (n = 12); 3–6 months ago (n = 18); 6–12 months ago (n = 16). 49% of those surveyed had COVID-19 more than twice over the past 2 years.

The content of interleukin (IL) -1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-17, tumor necrosis factor alpha (TNFα), and interferon gamma (INFγ) in the blood serum of patients with PCS was determined by enzyme immunoassay using a standard reagent kit (Protein Contour LLC, Russia). The analysis was carried out according to the manufacturer's instructions. The results were recorded on the Multiskan photometer (Labsystems, Finland). The parameters obtained from the study of serum from healthy blood donors (n = 25) were used as control values. The level of total IgE in blood serum was determined using the enzyme-

linked immunosorbent assay (ELISA) with the result recorded on the Multiskan SkyHigh microplate reader (Thermo FS, Finland).

The results were statistically processed using the Statistica 9 software package (StatSoft, USA). In order to choose the method for the statistical analysis, the Shapiro – Wilk test was used to check data for normality of distribution. Only one of the studied parameters had normal distribution. The data were presented as the median and the interquartile range Me [Q_{25}; Q_{75}]. The differences were analyzed using the nonparametric Mann – Whitney *U*-test. The differences between the groups were considered statistically significant at $p < 0.05$.

RESULTS

Asthenia as the main manifestation of PCS was more common in women (n = 37) than in men (n = 9). The average age of women was 49.43 [18.0; 73.0] years. The average age of men was higher – 57. 62 [31.0; 73.0] years (Table 1).

Table 1

Gender and age profile of patients with PCS

Parameter	Number of cases, *n* (%)	Age distribution
Men	9 (20%)	18–30 years old : 0 31–40 years old: 1 41–50 years old: 0 51–60 years old: 3 61–70 years old: 3 71 years and older: 2
Women	37 (80%)	18–30 years old: 5 31–40 years old: 6 41–50 years old: 10 51–60 years old: 9 61–70 years old: 3 71 years and older: 4

Basically, both men and women had mild symptoms in the acute phase of infection (57%, n = 26), receiving treatment in the outpatient setting. More than half of them (52%) did not receive antiviral therapy to the full extent, that is, they did not complete the course of antiviral therapy and/or did not always take antiviral drugs at a therapeutic dose or independently reduced the dose and/or volume of prescribed medications. Ten patients, most of them women, had a moderate course of the disease with a complication of interstitial pneumonia.

Five employees, three of whom were men, had severe COVID-19 infection. All patients with moderate and severe infection received treatment in hospital followed by an outpatient rehabilitation course. They completed a full course of etiotropic antiviral therapy. Five patients, four of whom were women, had no symptoms in the acute phase and learned about the history of COVID-19 only from a positive SARS-Cov-2 IgG.

During the first three months after the acute phase of COVID-19, younger people more often needed medical care, including 5 women aged 32.53 [31.0; 48.0] years (Fig. 1). On the contrary, after 3–6 months, men over 50 years of age complained more often about the deterioration of their general condition. During 6–12 months, the gender profile of patients was the same, but older people (52.3 [45.0; 73.0] years) noted deterioration of the condition more often (Fig. 2).

During the second or third month after acute infection, asthenia was the main sign in all patients (Fig. 3). Its main manifestations included severe unmotivated general weakness, rapid fatigue, and a decreased ability to work.

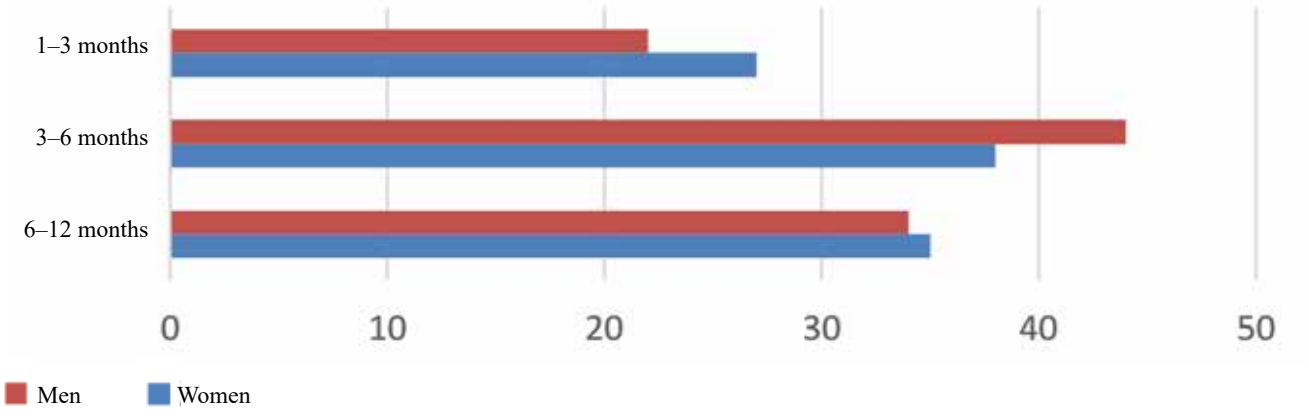

Fig. 1. Gender profile of patients with PCS depending on the duration of the acute phase of infection

Seven out of twelve patients complained of damage to the central nervous system in the form of persistent diffuse headaches, cognitive and mental disorders (sleep disturbance: insomnia at night and drowsiness during the day, vivid dreams, nightmares, short-term memory impairment, and inability to concentrate). In five patients, asthenia was accompanied by symptoms of cardiovascular system dysfunction, such as decompensation or the onset of cardiovascular diseases in the form of inappropriate tachycardia, increased or fluctuating blood pressure, and increased shortness of breath during habitual physical activity. Further examination (transthoracic echocardiography, 24-hour ECG) did not detect significant organic damage to the heart. Four patients complained of visual impairments in the form of a decrease in visual acuity, the appearance of blurry vision, and gritty and dry eyes. Signs of respiratory dysfunction persisted with the same frequency. There were single patient complains of skin rash in the form of polymorphic spots or pustular rash, dyspeptic disorders, and polyarthralgia.

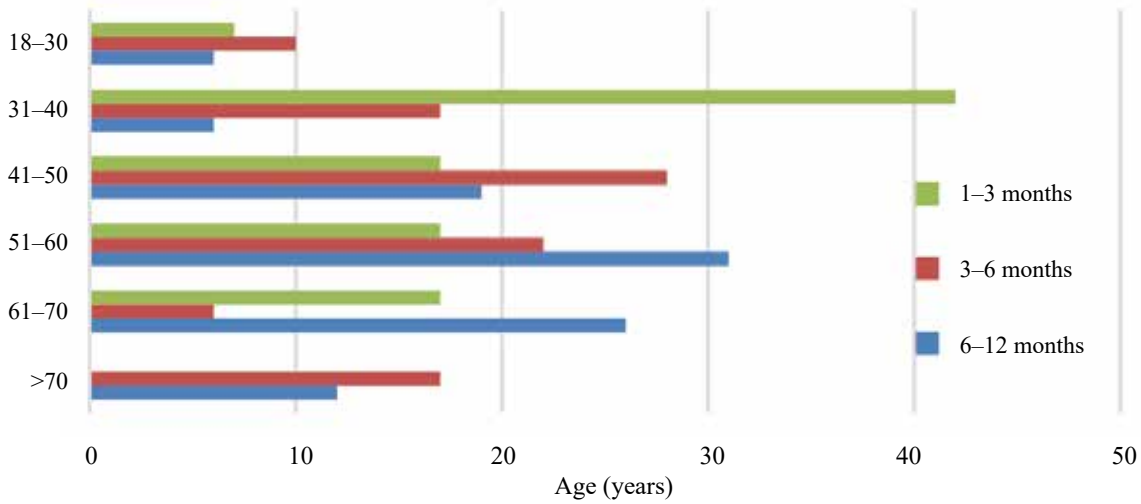

Fig. 2. Age profile of patients with PCS depending on the duration of the acute phase of infection

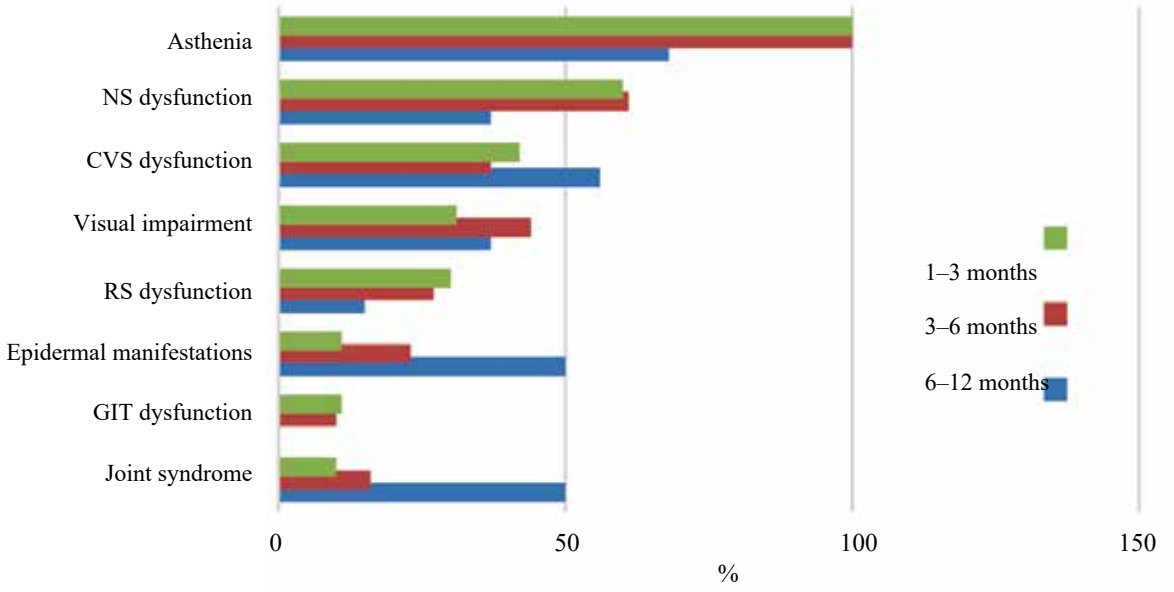

Fig. 3. Complaints of patients during various periods of PCS. NS – nervous system, CVS – cardiovascular system, RS – respiratory system, GIT – gastrointestinal tract

The analysis of the serum cytokine profile revealed high levels of all the studied cytokines in patients with PCS during a period of 2–3 months (Table 2). The median levels of IL-1ß and IL-2 were 1.3 and 1.4 times higher than those of healthy blood donors; IL-8 and IL-10 were 2 times higher, respectively. The content of IL-17 exceeded the control values by 2.8 times, IL-4 and IL-6 – by 3 and 4.5 times, respectively. The highest values were reached by TNFα and INFγ, whose concentrations exceeded the normal values by 5.5 and 70 times, respectively.

Table 2

Variability of serum cytokine levels in clinical groups depending on the duration of post-COVID syndrome, pg / ml, *Me* [Q_{25}; Q_{75}]

Parameter	Group 1, 1–3 months of PCS, *n* = 12	Group 2, 3–6 months of PCS, *n* = 18	Group 3, 6–12 months of PCS, *n* = 16	Group 4, healthy blood donors, *n* = 25	*p*		
					Groups 1–4	Groups 2–4	Groups 3–4
IL-1β	3.85 [3.45; 5.35]	2.62 [2.45; 3.05]	2.98 [2.45; 3.37]	2.88 [2.46; 3.12]	0.001	0.249	0.965
IL-2	0.18 [0.13; 0.26]	0.12 [0.09; 0.15]	0.13 [0.12; 0.14]	0.13 [0.09; 0.15]	0.002	0.484	0.655
IL-4	1.28 [0.98; 1.6]	0.37 [0.34; 0.52]	0.43 [0.38; 0.64]	0.42 [0.31; 0.55]	0.000	0.531	0.403
IL-6	3.77 [2.51; 4.64]	1.23 [1.12; 1.30]	0.98 [0.89; 1.17]	1.09 [0.97; 1.29]	0.000	0.363	0.129
IL-8	3.65 [2.85; 5.1]	1.66 [1.34; 2.67]	1.57 [1.36; 2.04]	1.6 [1.25; 2.42]	0.000	1.000	0.633
IL-10	2.48 [1.46; 3.22]	1.15 [1.08; 1.25]	1.2 [1.00; 1.27]	1.14 [1; 1.24]	0.001	0.686	0.720
IL-17	13.3 [11.16; 14.31]	5.74 [5.36; 6.99]	5.49 [4.88; 9.22]	4.75 [3; 5.61]	0.000	0.018	0.030
TNFα	1.7 [0.97; 2.65]	0.32 [0.25; 0.42]	0.25 [0.13; 0.46]	0.31 [0.17; 0.44]	0.000	0.919	0.467
INFγ	9.20 [0.67; 1.13]	0.14 [0.11; 0.15]	0.14 [0.10; 0.19]	0.13 [0.08; 0.08]	0.000	0.879	0.550

Despite the high levels of cytokines, there were no laboratory signs of inflammation in the complete blood count of patients with PCS: the values of the leukocyte content in peripheral blood were 6.12 [5.29; 7.29] × 10^9 / l; ESR 9.7 [4; 17] mm / h; C-reactive protein 6.9 [5.2; 11.3] mg / l. At the same time, an increase in the level of total IgE was recorded in 3 out of 12 people, who did not have a history of allergies and any clinical manifestations of allergies.

In the period of 3–6 months after the acute phase of COVID-19 ended, asthenia remained the main sign in all patients of the study group (100%). In 10 patients out of 18, manifestations of damage to the nervous system and gastrointestinal tract (nausea, increased and decreased appetite, disrupted taste preferences) were recorded. Symptoms of respiratory and cardiovascular dysfunction were present in 6 patients and, as a rule, were less severe and much easier to tolerate than in the early stages of PCS.

However, during this period, an increase in the number of patients (8 out of 18) with vision impairments was recorded. When examined by an ophthalmologist, 4 patients had changes in the fundus in the form of hypertensive and dystonic retinal angiopathy, and in half of the patients, these changes were recorded for the first time.

The number of patients with complaints of skin itching, petechiae, urticaria, and pustular rash increased to 4. Three patients had arthralgia and polyarthralgia, which debuted in two patients while in one patient cartilage destruction progressed.

The total IgE in the study group was 80.8 [25.0; 112.0] IU / ml and was significantly higher than in the control group (p = 0.009). An increase in total IgE was detected in 9 patients, which in 5 cases was accompanied by clinical symptoms of allergy in the form of atopic rash and bronchial obstructive syndrome.

The concentration of all the studied cytokines in patients at this stage of PCS decreased and often reached the values of healthy donors, but the IL-17 content remained significantly high at the level of 5.74 [5.36; 6.99] pg / ml versus 4.75 [3.0; 5.61] pg / ml in the controls (p = 0.018) (Table 2).

During 6–12 months after infection, clinical symptoms of PCS changed. Asthenia was diagnosed in 11 out of 16 patients, and signs of nervous system dysfunction were noted only in 6 cases. In 10 patients, persistent cardiac rhythm disturbances of various types and/or disturbances in myocardial repolarization (mainly in women) were recorded. Stabilization of arterial hypertension and formation of retinal angiopathy in 7 patients can be considered as signs of organic damage to the cardiovascular system.

Gastrointestinal disorders were not detected at this stage of PCS. Only 2 patients had shortness of breath when walking and mild cough as symptoms of impaired pulmonary function. However, two patients developed bronchial obstructive syndrome with persistent cough for the first time. In one case,

atopic asthma was diagnosed for the first time with a change in the spirogram manifested by a decrease in forced expiratory volume in 1 second (FEV1) / forced vital capacity (FVC) < 70%, FEV1 < 80% and a positive bronchodilator test (the increase while taking salbutamol was +12%).

Half of the patients (8 out of 16 cases) had changes in skin color in the form of pink and red spots, one patient had purple spots, and none of them sought medical help for this. These epidermal manifestations were identified during regular health checkup by a doctor. In one case, papulae were identified. Two patients had urticaria-like rash. One woman in the post-COVID period was newly diagnosed with recurrent urticaria in the form of pale pink and light red blisters, rising above the level of the skin and accompanied by itching. The patient had no history of allergy before COVID-19 infection. Antihistamine therapy did not have any clear effect. Subsequently, monoclonal gammopathy (Schnitzler syndrome) was diagnosed.

Over a period of 6–12 months, 8 patients had musculoskeletal impairments, the appearance or progression of which the subjects associated with COVID-19 infection. In 3 cases, it was isolated arthralgia or polyarthralgia without signs of joint damage according to the results of an ultrasound examination. In 3 patients, cartilage deformations and mixed arthritis with signs of synovitis were detected. In two patients, while receiving a full course of NSAIDs, chondroprotectors, intra-articular administration of corticosteroids, and physiotherapy, the destruction of the knee cartilage tissue progressed, which resulted in cartilage destruction and the development of aseptic necrosis and was an indication for knee arthroplasty.

The increase in allergic and autoallergic manifestations during 6–12 months of PCS was accompanied by an increase in the average IgE level in the blood to 98.98 [40.1; 172] IU, as well as in the relative and absolute content of eosinophils in peripheral blood (5.75 [3.2; 7.2]% and 0.54 [0.19; 0.76] $\times 10^9$ / l, respectively). In the meantime, acute-phase blood serum parameters did not exceed the established clinical norms. The blood cytokine profile was characterized only by a high level of IL-17, which was higher than that of donors (5.49 [4.88; 9.22] and 4.75 [3; 5.61] pkg / ml, respectively ($p = 0.03$)). The content of this cytokine depended on the clinical manifestations of PCS. In patients with isolated atopic epidermal manifestations, the concentration of IL-17 was 9.46 [6.20; 13.66] pkg / ml, in patients with musculoskeletal impairments, it was 12.56 [8.10; 14.56] pkg / ml, and in coexisting joint and epidermal syndromes, the parameter reached 13.95 [13.05; 14.05] pkg / ml.

DISCUSSION

Consequently, the clinical manifestations of PCS change dynamically during the year after acute infection, which is accompanied by natural changes in the plasma cytokine profile. The clinical symptoms of the first 3 months of PCS in the form of asthenia and CNS and cardiovascular dysfunctions are caused by an increased level and imbalance of both pro- and anti-inflammatory cytokines.

Cytokine levels gradually reach the normal range, which is accompanied by a decrease in the frequency of asthenia and CNS and respiratory dysfunction. However, after 3 months and further, only high levels of IL-17 are noted in the blood. Mast cells can intensely produce IL-17 [11], the activity of which increases in PCS [12, 13]. Moderate synthesis of IL-17 by T lymphocytes promotes the production of antimicrobial peptides. However, prolonged production of the IL-17 family can lead to chronic inflammation [14]. Perhaps a constant and long-term increase in IL-17 levels is associated with long-term post-COVID inflammation.

It is also likely that one of the factors activating mast cells is an increase in IgE. If its increase in the early stages of PCS was asymptomatic, then the increase in cases of hyperimmunoglobulin E syndrome and a rise in its values in the long term are already accompanied by clinical manifestations of allergies in the form of epidermal lesions and bronchial obstructive syndrome.

IL-17 is an important mediator of the formation of allergic and autoallergic damage, which is confirmed by significant differences in the degree of its increase depending on the localization of clinical manifestations and especially on their coexistence. It is known that IL-17 usually induces proinflammatory reactions, often associated with allergies, and also promotes the production of many other cytokines, chemokines, and prostaglandins [15]. IL-17 is assumed to play an important role in autoimmune diseases [16].

Most likely, an increase in the concentration of IL-17 also contributes to an increase in the incidence of visual impairments in the long term of PCS. High concentrations of IL-17 and increased levels of Th17 producing it have been found in a number of

ocular diseases associated with neovascularization [17]. The pathogenic role of IL-17 in the occurrence of joint syndrome has also been proven, which may be due to its participation in the formation of synovial inflammation with subsequent cartilage destruction [18].

Considering the ability of IL-17 to cause endothelial dysfunction and stimulate the activity of the renin – angiotensin – aldosterone system and arteriolar remodeling [19, 20], it is possible to explain the formation of stable hypertension and retinal angiopathy during 3–12 months of PCS. It is likely that the gradual recovery of gastrointestinal function is associated with the restoration of the gut microbiota and stimulation of the protection against bacterial and fungal pathogens by IL-17 [21].

CONCLUSION

Thus, the development of PCS does not depend on age and gender of patients and the severity of the acute phase of infection. However, patients are more likely to develop PCS in the absence of antiviral therapy or its inadequacy. The range of clinical manifestations of PCS changes over 12 months, which is determined by natural changes in the cytokine levels.

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Restoring antibiotic sensitivity to lincomycin in compositions with nanosilver and humic substances

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ABSTRACT

Aim. To study the effect of compositions with nanosilver and humic substances on restoration of sensitivity of methicillin-resistant *Staphylococcus aureus* to lincomycin.

Materials and methods. Compositions of humic substances with silver nanoparticles were synthesized from commercial sodium humate Powhumus and silver nitrate in the presence of NaOH (1 M) to modulate alkaline pH. To synthesize one of the two compositions, sodium humate was modified with hydroquinone. To describe the characteristics of the resulting compositions, surface plasmon resonance spectra of silver nanoparticles and their images obtained by transmission electron microscopy were recorded. Sensitivity of a clinical strain of methicillin-resistant *Staphylococcus aureus* was determined by measuring the minimum inhibitory concentration (MIC) with the addition of lincomycin and tetracycline to the compositions.

Results. 100% conversion of ionic silver into metallic silver with a characteristic nanoparticle size of 6 nm was shown. The effects of tetracycline and lincomycin on the studied strain of *Staphylococcus aureus* were compared, and high sensitivity to tetracycline (MIC < 10 μg / ml) and resistance to lincomycin (MIC > 200 μg / ml) were shown. Studying the effect of the composition containing sodium humates with nanosilver with the introduction of lincomycin into it showed that this approach can significantly reduce MIC of lincomycin to 0.1 μg/ml in the presence of compositions with hydroquinone-modified sodium humate at a concentration of 40 μg / ml and compositions with unmodified sodium humate at a concentration of 60 μg / ml.

Conclusion. The study demonstrated that the use of compositions with humic substances and nanosilver completely restores sensitivity of methicillin-resistant *Staphylococcus aureus* to lincomycin.

Keywords: silver nanoparticles, humic substances, lincomycin, synergistic effect, antibiotic resistance, *Staphylococcus aureus*

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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Восстановление антибиотикочувствительности к линкомицину в составе композиций с наносеребром и гуминовыми веществами

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РЕЗЮМЕ

Цель – исследование восстановления чувствительности метициллин-резистентного штамма *Staphylococcus aureus* к линкомицину при добавлении гуминовых веществ с наночастицами серебра.

Материалы и методы. Композиции гуминовых веществ с наночастицами серебра синтезировали из коммерческого гумата натрия Powhumus и нитрата серебра в присутствии NaOH (1 моль) для создания щелочной среды. Для синтеза одной из двух композиций гумат натрия модифицировали гидрохиноном. Для описания характеристик полученных составов снимали спектры поверхностного плазмонного резонанса наночастиц серебра и их изображения, полученные методом просвечивающей электронной микроскопии. Чувствительность клинического штамма *Staphylococcus aureus*, устойчивого к метициллину, определяли, измеряя минимальную ингибирующую концентрацию (МИК), с введением в состав композиций линкомицина и тетрациклина.

Результаты. Показана 100%-я конверсия ионного серебра в металлическое с характерным размером наночастиц 6 нм. Проведено сравнение действия тетрациклина и линкомицина на исследуемый штамм *S. aureus*, показаны высокая чувствительность к тетрациклину (МИК < 10 мкг/мл) и отсутствие чувствительности к линкомицину (МИК > 200 мкг/мл). Исследование действия композиции гуматов натрия с наносеребром при введении в них линкомицина показало, что подобный подход позволяет существенно снизить МИК линкомицина до 0,1 мкг/мл в присутствии композиций с модифицированным гидрохиноном гумата натрия в концентрации 40 мкг/мл и композиций с немодифицированным гуматом натрия в концентрации 60 мкг/мл.

Заключение. В проведенном исследовании продемонстрировано, что при использовании композиций гуминовых веществ с наносеребром происходит полное восстановление чувствительности к линкомицину устойчивого к метициллину штамма *Staphylococcus aureus*.

Ключевые слова: наночастицы серебра, гуминовые вещества, линкомицин, синергетический эффект, антибиотикорезистентность, *Staphylococcus aureus*

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

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INTRODUCTION

After the discovery of the first antibiotic penicillin, already in 1942, drug-resistant strains of *Staphylococcus aureus* emerged [1]. An increase in the number of antibiotic-resistant bacterial strains over time led to the need to develop new antibiotics, which, in turn, brought about new multidrug-resistant microorganisms [2]. As a result, searching for ways to enhance the effects of existing antibiotics became a pressing issue [3]. Silver nanoparticles, which are known for their antibacterial properties, attracted particular attention [4]. This is because resistance to metal particles develops much more slowly than to antibiotics [5].

Numerous studies on antibacterial activity of compositions with silver nanoparticles and antibiotics showed that nanosilver is capable of not only enhancing the effect of antibiotics against bacteria [6], but also of restoring sensitivity of resistant bacterial strains to antibiotics [7, 8]. Previous studies showed that the use of silver nanoparticles together with a wide range of antibiotics provided a synergistic antibacterial effect of both components against various gram-positive bacteria. It was shown that sensitivity of *Bacillus cereus* to lincomycin increased four-fold in the presence of silver nanoparticles [9]; sensitivity of *Entorococcus faecium* to ampicillin rose by 16 times, and to amikacin – by 32 times [10]. The shape and size of synthesized nanoparticles had a significant impact on antibacterial activity [11].

The main problem with the use of silver nanoparticles (NPs) in clinical practice is a potential increase in cytotoxicity of this kind of compositions. The problem is complicated by a poor understanding of the mechanism of action of such compositions [12, 13]. It is assumed that they might increase the concentration of Ag+ near the bacterial cell wall, which exerts a higher cytotoxic effect [14], induces hydroxyl radical formation [15], and affects DNA transcription [16].

Studies on the biological activity of natural and modified humic substances (HS) show that they have a great potential for incorporation into nanoparticle-based compositions due to plentiful properties and functional groups in their molecular ensemble [17–19]. The use of HS as both reducing and stabilizing agents for the synthesis of silver NPs without application of additional reducers is of particular interest [20–23]. In our previous studies, we described a method for synthesizing nanosilver-based drugs with a strong safety profile, which resulted from high antioxidant activity and low cytotoxicity of HS [24]. Addition of antibiotics to compositions with silver NPs and HS might be of particular interest in terms of developing novel combination drugs to overcome antibiotic resistance. Various mechanisms regulating interaction of HS with a wide range of antibiotics were discussed in the review article [25]. We hypothesized that antibiotic binding to HS on the surface of silver NPs could facilitate its penetration into the cell together with silver NPs.

The choice of antibiotics for the experimental studies was guided by the aim of the study – to develop effective bionanomaterial for treatment of infected wounds with a possibility of application in semisolid dosage forms (ointments, gels, etc.). Hence, an antibiotic was to penetrate both into soft tissues and bones to treat chronic and sluggish purulent inflammations, such as pressure ulcers and diabetic foot. These requirements are met by penicillins, lincosamides, fluoroquinolones, aminoglycosides, tetracyclines, etc. An antibiotic should mix well with lipophilic ointments for external application in semisolid dosage forms. This requirement is met only by ointments with lincomycin and tetracycline, since penicillins are not used externally, levofloxacin (fluoroquinolones) is used in the form of an aqueous spray, and neomycin (aminoglycosides) is applied in the form of an aerosol. As a result, two antibiotics were chosen for the study – lincomycin and tetracycline, resistance to which evolved in the methicillin-resistant *Staphylococcus aureus* (MRSA).

The MRSA strain F-182 was chosen as one of the most abundant and dangerous forms of *Staphylococcus aureus* causing a broad spectrum of diseases, including bloodstream infections, pneumonia, and skin and bone infections. Moreover, MRSA strains are highly resistant to the majority of existing antibiotics, which makes it difficult to treat and control. The strain F-182 is very well studied and described in the literature, which allowed for more in-depth research. At the next stages of our research, we plan on looking into other strains in search of promising combinations.

The aim of the study was to investigate the effect of compositions with nanosilver and humic substances on restoration of sensitivity of MRSA to lincomycin and tetracycline.

MATERIALS AND METHODS

Chemicals and materials. Sodium hydroxide (Kemphasol), silver nitrate (Molychem), Powhumus sodium humate (Humintech, Germany), and *Staphylococcus aureus* Rosenbach 1884 (strain

F-182) were used for bioassays. The value of pH was measured using the pH meter 713 (Metrohm, Switzerland) equipped with a glass electrode.

Synthesis of the compositions was carried out according to the procedure described in [24]. In brief, an aqueous solution of sodium humate and hydroquinone (HQ) derivatives at a concentration of 11.8 g / l was prepared. The pH value of the solution was brought to 12 by adding 1 M NaOH. Then, 50 ml of the solution was placed in a 100 ml three-neck round-bottom flask equipped with a reflux condenser and heated to 80 °C with constant stirring. When the temperature reached 80 °C, 2 ml of $AgNO_3$ solution at a concentration of 110 g / l was added dropwise to the hot humate solution, while maintaining pH at 12 with 1 M NaOH. Then, the solution was maintained at 80 °C with constant stirring for 4 hours. The total concentration of silver in the resulting solution was 41 mM / l.

Analysis of the synthesized compositions containing nanosilver and HS. The synthesized Ag NPs stabilized with HS were analyzed by transmission electron microscopy (TEM). The experiment was carried out using the JEOL JEM-2100F analytical electron microscope (JEOL, Akishima, Japan). The data were processed using the ImageJ program. Morphology and size of the particles were analyzed for both compositions.

The total silver concentration was measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES) using the 720-ES ICP-OES spectrometer (Agilent Technologies, USA). Ultraviolet-visible absorption spectra were registered to confirm the presence of silver NPs, which were detected by a surface plasmon resonance (SPR) peak at 400–430 nm for silver NPs. Absorption spectra were measured using the UV / Vis spectrometer (Cary 50 Probe, USA). The results of UV-visible spectroscopy and ICP-AES were compared to determine the degree of conversion of ionic silver to metallic silver.

The minimum inhibitory concentration (MIC) of the synthesized compositions to MRSA was determined using 96-well plates with Mueller Hinton broth (MHB). To determine MIC, the required volume of 0.9% NaCl and 0.142 ml of MHB containing the appropriate concentrations of lincomycin as part of compositions of HS with silver NPs were added to each well. The range of resulting concentrations was 0.1–20 μg / ml for lincomycin and 0–150 μg / ml for compositions of silver NPs and HS. MRSA colonies were pre-incubated at 35 °C in MHB for 12 hours. 0.025 ml of a bacterial suspension adjusted to McFarland 2 standard was added to each well and analyzed on the ImmunoChem-2100 microplate reader (High Technology Inc., USA) at a wavelength of 630 nm for 24 hours. MHB without the addition of test substances, to which a bacterial suspension was also added, served as a control.

Statistical processing of the obtained results was carried out by measures of variability using the SPSS statistical package (version 15.0, SPSS Inc., Chicago, IL, USA) with determination of the mean and the error of the mean and the probability of differences p for small samples with the Bonferroni correction (significance of differences at $p < 0.05$).

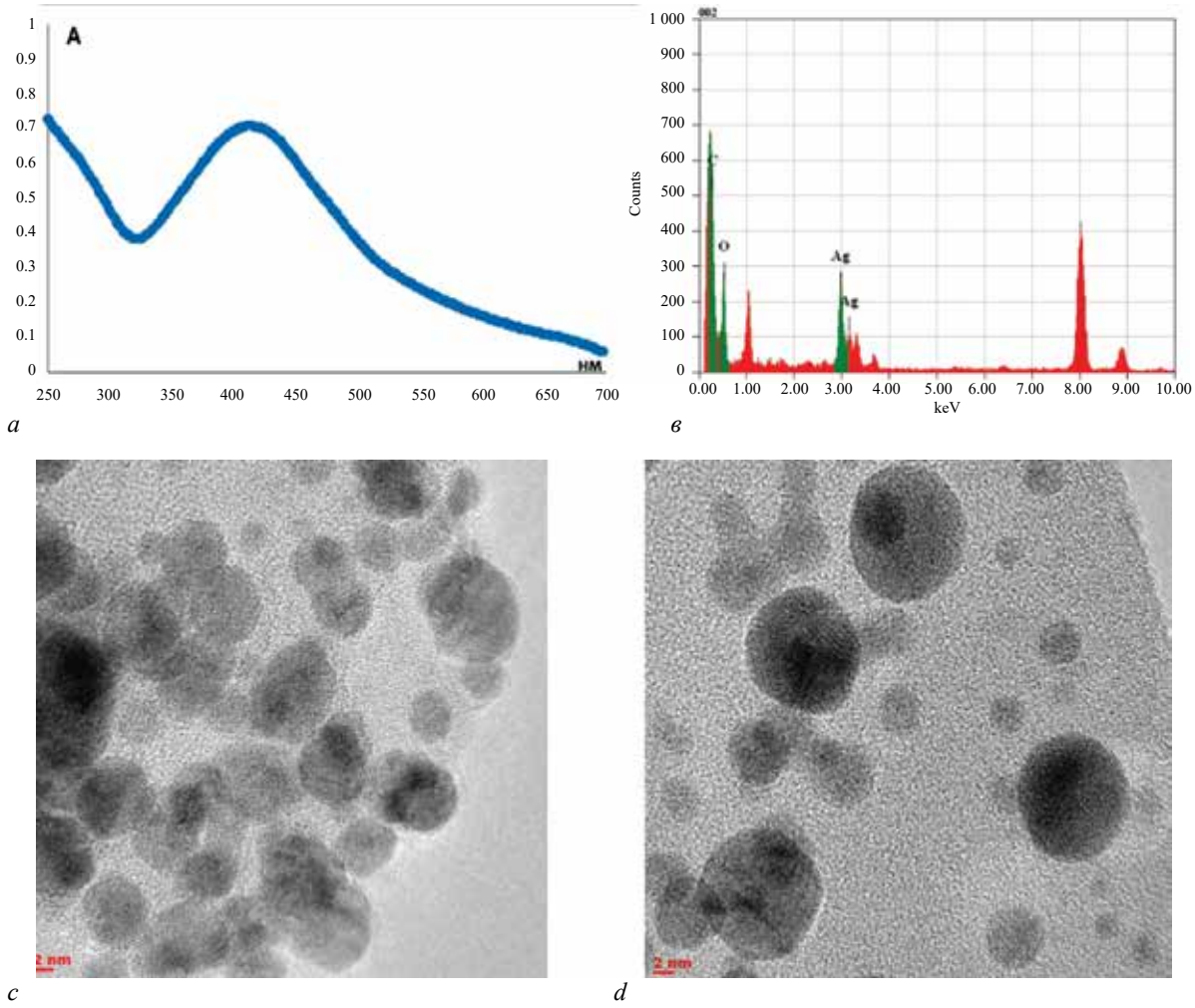
RESULTS

Synthesis of compositions of silver NPs with HS was accompanied by 100% conversion of ionic silver into metallic particles. The particles were spheric and around 6 nm in size. The resulting compositions contained 11.8 g / l of HS and 4.6 g / l (43 mM) of silver NPs. Figure 1 shows the surface plasmon resonance (SPR) spectra of silver NPs and their TEM images.

Figure 1*a* demonstrates a characteristic SPR peak of nanosilver at 414 nm, which indicates small size of synthesized nanoparticles. TEM images of AgNP – CHP (Fig. 1*b*) confirm that the average size of silver NPs is 6–7 nm. The size of nanoparticles is of particular importance for their biological activity. AgNPs with sizes less than 10 nm were reported to have maximum antimicrobial activity [13]. The size of the AgNPs used in this study satisfied this condition.

At the first stage of the experiment, we studied the effect of HS on the antibacterial activity of tetracycline and lincomycin. The studied strain was found to lack resistance to tetracycline: the antibiotic inhibited bacterial growth over the entire range of the studied concentrations. Lincomycin, on the contrary, did not suppress bacterial growth over the entire range of HS (0–1, 500 μg / ml) and lincomycin (10–200 μg / ml) concentrations.

Both combined compositions of AgNPs – CHP and AgNPs – CHP – HQ did not cause any changes in sensitivity of MRSA to tetracycline, but resulted in complete recovery of sensitivity to lincomycin (HS > 0 μg / ml for AgNPs – CHP, and HS 200 μg / ml for AgNPs – CHP – HQ). As a result, we decided to study the properties of the lincomycin-containing compositions where sensitivity to the antibiotic was restored.



a *в* *c* *d*

Fig. 1. Characteristics of silver nanoparticles (AgNPs) synthesized in the presence of coal humate (CHP): *a* – UV-visible absorption spectrum of the AgNP – CHP with a SPR peak at 414 nm; *b* – TEM elemental map; *c* and *d* – TEM images of AgNPs stabilized with HS

The antibacterial effect of AgNP synthesized in the presence of HS toward MRSA growth was studied in the presence or absence of lincomycin. We studied suppression of bacterial growth by AgNPs stabilized with CHP and CHP – HQ in the concentration range of 20–150 μg / ml and lincomycin in the concentration range of 0.1–20 μg / ml (Table 1, Fig. 2).

Table 1

Changes in the optical density of MRSA cells over 24 hours registered for MIC in the compositions with AgNPs, lincomycin, and modified sodium humate (CHP – HQ), mg / l

Lincomycin concentration	Concentration of AgNPs in the presence of CHP – HQ							Concentration of AgNPs in the presence of CHP						
	0	20	40	60	80	100	150	0	20	40	60	80	100	150
0.1	0.5	0.4	**0**	**0**	**0**	**0**	**0**	0.4	0.4	0.4	**0**	**0**	**0**	**0**
0.2	0.4	**0.2**	**0**	**0**	**0**	**0**	**0**	0.3	0.3	0.4	**0**	**0**	**0**	**0**
0.5	0.5	**0.2**	**0**	**0**	**0**	**0**	**0**	0.3	0.4	0.4	**0**	**0**	**0**	**0**
1	0.4	0.3	**0**	**0**	**0**	**0**	**0**	0.3	0.4	0.4	**0**	**0**	**0**	**0**
2	0.5	0.3	**0**	**0**	**0**	**0**	**0**	0.3	0.4	0.4	**0**	**0**	**0**	**0**
5	0.5	0.3	**0**	**0**	**0**	**0**	**0**	0.4	0.4	0.4	**0**	**0**	**0**	**0**
10	0.5	0.3	**0**	**0**	**0**	**0**	**0**	0.4	0.4	0.4	**0**	**0**	**0**	**0**
20	0.5	0.4	**0**	**0**	**0**	**0**	**0**	0.4	0.3	0.4	**0**	**0**	**0**	0

Note. Cases of statistically significant suppression of MRSA growth compared to the controls are presented in bold ($p < 0.05$).

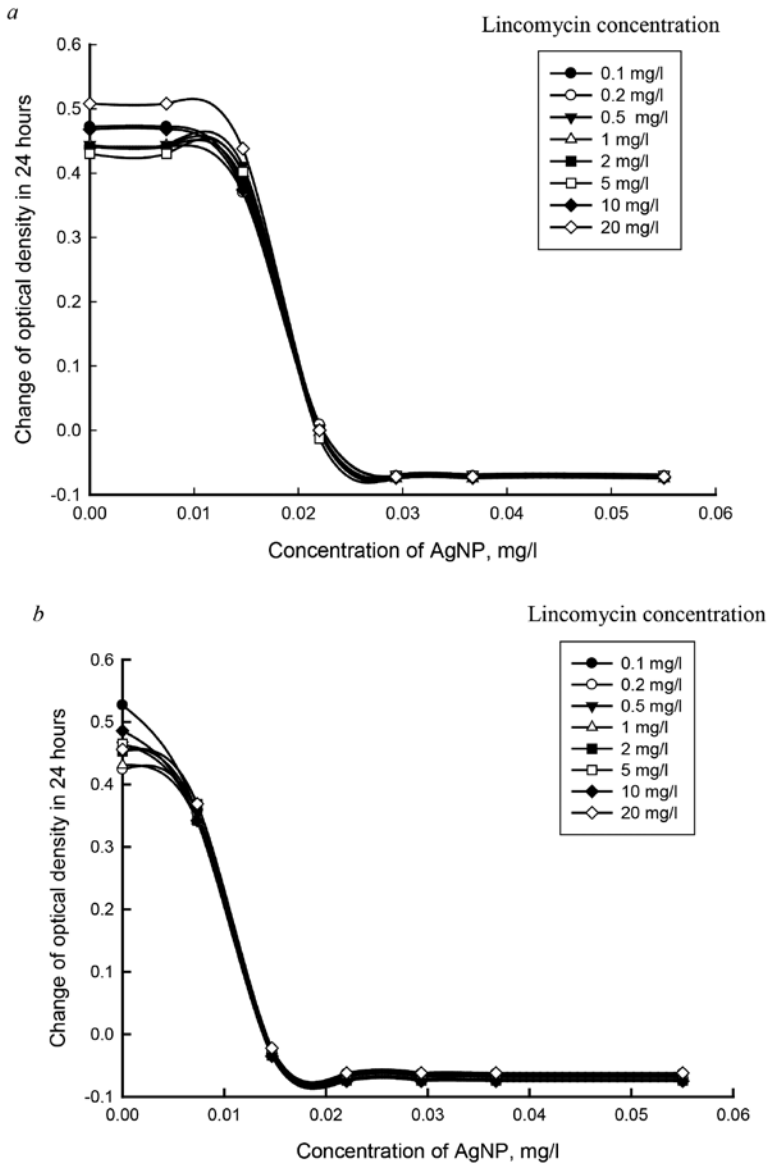


Fig. 2. A dependence of changes in the optical density of MRSA cells over 24 hours on the concentration of AgNPs at lincomycin concentrations in the range of 0.1–20 μg / ml in compositions with unmodified humic substances (*a*) and HQ-modified humic substances (*b*)

For lincomycin compositions in the absence of silver NPs, the MIC was > 200 μg / ml, whereas the presence of AgNPs in AgNP – CHP (40 μg / ml) and AgNP – CHP – HQ (20 μg / ml) compositions resulted in a drop in the MIC value down to 0.1 μg / ml (Fig. 2). The obtained data indicate significant suppression of bacterial growth compared to the use of the AgNP – HS – lincomycin composition.

DISCUSSION

The mechanism of lincomycin action is associated with the suppression of protein biosynthesis by binding to A-site in the 23S rRNA of the 50S subunit and, consequently, preventing the binding of tRNA and disrupting the translation process [26]. The mechanism of antibiotic resistance to lincomycin was described in detail in [27] and can be associated with inactivation of the antibiotic by enzymes [28], mutations in the MFS efflux pump [29], modification of the 23S rRNA binding site by methyltransferases [30] or mutation in the 50S ribosomal subunit. Although the mechanism of action of antibiotic compositions with AgNPs is not precisely known, it was shown that the use of

AgNPs could be of particular interest in the fight against such antibiotic-resistant microorganisms as MRSA [31, 32].

MRSA F-182 is a classical gram-positive bacterium used in numerous studies on a variety of antibacterial compositions [33]. The genome of this strain contains the *NorB* and *ErmY* genes, which are responsible for modification of the MFS efflux pump and methylation of the lincomycin binding site (23S rRNA), respectively. The above considerations allowed us to suggest that the combination of lincomycin with AgNP and HS made it possible to overcome the MLSB resistance to lincosamides, which is typical of this strain. The found MIC value for lincomycin among MRSA ATCC 43300 isolates was less than 0.1 μg / ml in the presence of 40 μg / ml of CHP and 20 μg / ml of CHP – HQ, respectively, while in the absence of AgNP – HS, the MIC for lincomycin exceeded 200 μg / ml.

The obtained MIC values for AgNPs obtained in this study in the presence of lincomycin and HS were shown in Table 2 and were 0.022 and 0.015 μg / ml for AgNP – CHP and AgNP – CHP – HQ compositions, respectively. Comparison of these values with those reported in the literature indicated higher activity of the obtained compositions compared to values for both engineered AgNPs and bio-AgNPs synthesized by various microorganisms (Table 2).

Table 2

Comparison of the MIC values for lincomycin and AgNPs with respect to MRSA found in this study and in the literature

Antibacterial agent	NP size, nm	Microorganism	MIC			Reference
			Lincomycin, μg / ml	AgNP, μg / ml	HS, μg / ml	
Lincomycin	–	MRSA ATCC 43300	>200	–	–	This study
Humate + lincomycin	–	MRSA ATCC 43300	>200	–	>1,500	This study
Humate + AgNP + lincomycin	6.0	MRSA ATCC 43300	<0.1	0.022	40	This study
Humate – HQ + AgNP + lincomycin	6.0	MRSA ATCC 43300	<0.1	0.015	60	This study
Lignin + AgNP	20.0	Multidrug resistant S. aureus	–	10	–	[34]
Dendrimer-encapsulated AgNP	3.3	S. aureus USA 300	–	128	–	[34]
Polystyrene sulfonate particles, with incorporated AgNP	5.0	S. aureus ATCC 29213	–	1.14	–	[34]
AgNP synthesized using the extract of Dracocephalum kotschyi	19.0	MRSA ATCC 43300	–	15.6	–	[34]
AgNP synthesized using the extract of Artemisia haussknechtii	10.7	MRSA ATCC 43300	–	10.0	–	[34]
AgNP synthesized using marine Streptomyces sp.	13.5	MRSA ATCC 43300	–	0.039	–	[34]
AgNP immobilized on copolymer	25.0	MRSA ATCC 43300	–	0.54	–	[34]

The discovered synergistic effects of AgNPs with regard to lincomycin support viability of the idea of applying silver NPs [35] and their compositions [36] on clinical isolates that pose a significant danger to human life. Moreover, they show a significant advantage of the proposed approach to overcoming antibiotic resistance of gram-positive bacteria through the use of ternary compositions of humic substances with nanoparticles and antibiotics. Although similar results were achieved for antibiotic compositions with silver NPs [37] and nanoparticles of other metals [38], the results presented in this study are new and of particular importance for improving safety profile of compositions based on nanosilver due to biogenic properties of HS [24]. Moreover, successful restoration of susceptibility to antibiotics using a novel composition of lincomycin with AgNPs and HS is a significant breakthrough in the fight against antibiotic resistance.

CONCLUSION

The study for the first time demonstrated the synergistic effect of restoring MRSA susceptibility to lincomycin in the presence of silver NPs stabilized with humic substances. Modification of coal humate with hydroquinone slightly enhanced the observed

synergistic effect. We suggested that the synergistic effect of lincomycin and nanosilver stabilized with humic substances could be explained by formation of weak surface complexes between humic substances and lincomycin, which dissociate and release the antibiotic after silver nanoparticles enter the cell. This opens new avenues in the fight against antibiotic resistance. The synthesized compositions of lincomycin with AgNPs stabilized with coal humates could be considered as candidates for further preclinical and clinical trials with the aim of their rapid implementation into clinical practice.

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Authors' contribution

Zykova M.V., Belousov M.V. – final approval of the manuscript for publication. Zhang Yun –development of the method for synthesizing silver nanoparticles in the presence of humic substances, determination of the characteristics of the synthesized compositions. Lysenko I.V., Mikhalev D.A. – carrying out of the experiment. Arutyunyan D.A. – processing and analysis of the obtained data, drafting of the article. Azarkina L.A. – drafting of the original manuscript. Perminova I.V. – supervision of the project, setting tasks, discussion of the results, drafting of the article.

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Results of *UGT1A1* gene sequencing in individuals with the Gilbert syndrome phenotype

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ABSTRACT

Aim. To evaluate the effectiveness of automated Sanger sequencing of the *UGT1A1* gene to search for pathogenic mutations in individuals with the Gilbert syndrome phenotype.

Materials and methods. Automated Sanger sequencing of exons and part of the promoter in the *UGT1A1* gene was carried out for 24 people with unconjugated hyperbilirubinemia, in whom all other causes except for genetic ones were excluded and DNA analysis was performed to determine the number of TA repeats in the promoter of the *UGT1A1* gene (rs3064744). Distribution of rs3064744 genotypes in the group was the following: 5 people – 7TA/7TA genotype, 5 people – 6TA/6TA genotype, 12 people – 6TA/7TA genotype, 1 person – 5TA/7TA genotype, 1 person – 6TA/8TA genotype. DNA was isolated using phenol – chloroform extraction or express methods. The sequencing was performed by capillary electrophoresis on the Hitachi 3500 Genetic Analyzer (Applied Biosystems, USA).

Results. Single nucleotide variants of uncertain significance were identified: rs3755319 (in 21 people) and rs28899472 (in three people with the 7TA/7TA genotype of rs3064744) in the promoter of the *UGT1A1* gene, rs2125984650 in the first exon of the *UGT1A1* gene (in one person with the 5TA/7TA genotype of rs3064744). In two individuals with the 6TA/7TA genotype of rs3064744, gene variants were identified that were pathogenic or likely pathogenic for the Gilbert syndrome according to some sources (rs4148323, rs1273237448).

Conclusion. According to the results of the study, automated Sanger sequencing of the *UGT1A1* gene may be the next stage of DNA analysis after determining the rs3064744 genotype for individuals with 6TA/6TA, 6TA/7TA rs3064744 genotypes and suspected Gilbert syndrome.

Keywords: Gilbert syndrome, *UGT1A1* gene, unconjugated hyperbilirubinemia, Sanger sequencing

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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Conformity with the principles of ethics. All patients signed an informed consent to molecular genetic testing. The study was approved by the local Ethics Committee at Research Institute of Internal and Preventive Medicine – Branch of the Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences (Protocol No. 4 of 14.02.2023).

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Результаты секвенирования гена *UGT1A1* у лиц с фенотипом синдрома Жильбера

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РЕЗЮМЕ

Цель. Оценка эффективности прямого автоматического секвенирования гена *UGT1A1* для поиска мутаций у пациентов с фенотипом синдрома Жильбера.

Материалы и методы. Проведено прямое автоматическое секвенирование по Сэнгеру экзонов и части промотора гена *UGT1A1* для 24 человек с непрямой гипербилирубинемией, у которых были исключены все другие ее причины, кроме генетических, и сделан ДНК-анализ на определение количества TA-повторов в промоторе гена *UGT1A1* (rs3064744). Распределение генотипов rs3064744 в группе: пять человек – генотип 7TA/7TA, пять человек – генотип 6TA/6TA, 12 человек – генотип 6TA/7TA, один человек – генотип 5TA/7TA, один человек – генотип 6TA/8TA. ДНК выделена методом фенолхлороформной экстракции или экспресс-методами. Секвенирование выполнено методом капиллярного электрофореза на аппарате Hitachi 3500 Genetic Analyzer (Applied Biosystems, США).

Результаты. Идентифицированы однонуклеотидные варианты неопределенной клинической значимости rs3755319 (у 21 человека) и rs28899472 (у трех человек с генотипом 7TA/7TA rs3064744) в промоторе гена *UGT1A1*, rs2125984650 в 1-м экзоне гена *UGT1A1* (у одного человека с генотипом 5TA/7TA rs3064744). У двух лиц с генотипами 6TA/7TA rs3064744 выявлены варианты гена, которые являются патогенными и вероятно патогенными для синдрома Жильбера по данным некоторых источников (rs4148323, rs1273237448).

Заключение. Прямое автоматическое секвенирование по Сэнгеру гена *UGT1A1* может быть следующим этапом ДНК-анализа после определения генотипа rs3064744 для лиц с генотипами 6TA/6TA, 6TA/7TA rs3064744 и подозрением на синдром Жильбера.

Ключевые слова: синдром Жильбера, ген *UGT1A1*, неконъюгированная гипербилирубинемия, секвенирование по Сэнгеру

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 добровольное информированное согласие на молекулярно-генетический анализ. Исследование одобрено локально-этическим комитетом НИИТПМ – филиала ИЦиГ СО РАН (протокол № 4 от 14.02.2023).

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INTRODUCTION

According to our study, in almost 35% of individuals with the Gilbert syndrome (GS) phenotype and excluded known causes of unconjugated hyperbilirubinemia, except for genetic ones, it is not possible to find a common variant rs3064744 of the *UGT1A1* gene (the number of TA repeats in the gene promoter) in the 7TA/7TA homozygous state, which would explain the cause of hyperbilirubinemia in these patients [1]. For individuals with 6TA/6TA and 6TA/7TA genotypes of rs3064744, with unconjugated hyperbilirubinemia and suspected GS, the next stage of molecular genetic diagnosis may be automated Sanger sequencing of the *UGT1A1* gene to search for gene variants that may be the cause of the GS development.

The aim of this work was to evaluate the effectiveness of automated Sanger sequencing of the *UGT1A1* gene to search for pathogenic mutations in individuals with the GS phenotype.

MATERIALS AND METHODS

Automated Sanger sequencing of exons and part of the promoter in the *UGT1A1* gene was carried out for 24 people with unconjugated hyperbilirubinemia, in whom all other causes except for genetic ones were excluded. The patients were examined and referred to DNA analysis by highly qualified gastroenterologists from 2012 to 2023. Distribution of rs3064744 genotypes in the group was the following: 5 people had 7TA/7TA genotype (the number of TA repeats in the promoter), 5 people – 6TA/6TA genotype, 12 people – 6TA/7TA genotype, 1 person – a rare 5TA/7TA genotype, 1 person – a rare 6TA/8TA genotype. The characteristics of the patients are presented in Table 1. The concentrations of total and unconjugated bilirubin shown in the table are random – they were recorded at the doctor's visit and could be higher during patient lifetime.

DNA was isolated by phenol–chloroform extraction or a rapid DNA extraction method (PREP-RAPID GENETICS, DNA Technology LLC, Moscow). The polymerase chain reaction (PCR) conditions are described in Table 2. The PCR temperature regime included 1 preheating cycle at 95 °C for 5 minutes and 1 final cycle at 72 °C for 7 minutes. To amplify the required DNA region, primers designed by us were used along with primers described by N. Abdellaoui et al. and E. Costa et al. [2, 3]. To amplify exon 1 in carriers of the heterozygous genotype of rs3064744 (6TA/7TA, 5TA/7TA, 6TA/8TA), primers were used that excluded the zone of rs3064744 to improve the quality of reading during automated sequencing.

Table 1

Patients included in the study

No.	Sex	Age, years	Genotype of rs3064744	Total bilirubin, umol/l	Unconjugated bilirubin, umol/l	Sequencing results				
						rs28899472 genotype	rs3755319 genotype	rs2125984650 genotype	rs1273237448 genotype	rs4148323 genotype
394	male	32	7TA/7TA	56.4	47.0	CT	CC	AA	CC	GG
386	female	50	7TA/7TA	48.0	42.0	CT	CC	AA	CC	GG
301	male	57	7TA/7TA	34.9	32.6	CT	CC	AA	CC	GG
405	male	18	7TA/7TA	54.8	46.4	CC	CC	AA	CC	GG
533	female	38	7TA/7TA	68.2	58.2	CC	CC	AA	CC	GG
240	female	61	6TA/8TA	51.0	46.2	CC	CC	AA	CC	GG
56	female	22	5TA/7TA	170.0	155.8	CC	CC	AT	CC	GG
404	male	18	6TA/7TA	55.4	47.8	CC	AC	AA	CC	GG
447	male	76	6TA/7TA	36.7	29.5	CC	AC	AA	CG	GA
12	male	18	6TA/7TA	58.0	53.4	CC	AC	AA	CC	GG
442	male	21	6TA/7TA	46.4	37.8	CC	AC	AA	CC	GG
475	male	38	6TA/7TA	41.7	27.2	CC	AC	AA	CC	GG
498	male	38	6TA/7TA	35.0	20.3	CC	AC	AA	CC	GG
495	male	17	6TA/7TA	32.0	26.0	CC	AC	AA	CC	GG
523	male	9	6TA/7TA	23.0	17.0	CC	AC	AA	CC	GG
535	female	17	6TA/7TA	25.2	21.2	CC	CC	AA	CC	GG
558	male	18	6TA/7TA	33.5	22.8	CC	CC	AA	CC	**GA**
587	male	15	6TA/7TA	36.3	33.1	CC	AC	AA	CC	GG
43	male	52	6TA/7TA	60.7	49.5	CC	AC	AA	CC	GG
11	male	56	6TA/6TA	54.2	45.5	CC	AC	AA	CC	GG
9	female	54	6TA/6TA	50.0	30.0	CC	AA	AA	CC	GG
206	male	40	6TA/6TA	40.2	28.6	CC	AA	AA	CC	GG
224	female	46	6TA/6TA	29.0	24.0	CC	AC	AA	CC	GG
104	male	28	6TA/6TA	33.7	29.6	CC	AA	AA	CC	GG

Table 2

Polymerase chain reaction conditions, *n* = 33

Gene region	Primer sequence	Temperature Duration	PCR mixture	Product length (bps)
Promoter	D: 5'-ctctaagcacatccccaagta-3' R: 5'-taagcaagtttccatccttca-3' [3]	95 °C 30 sec, 54 °C 30 sec, 72 °C 30 sec	10 µl BioMaster LR HS-PCR-Color reaction mixture (2×) (BIOLABMIX LLC, Novosibirsk), 0.4 mM each primer	525
Exon 1 (heterozygous for rs3064744)	1-1_D: 5'-gaacctctggcaggagcaa-3' 1_1_R: 5'-aaagctgctttctgccag-3'	95 °C 30 sec, 62 °C 30 sec, 72 °C 30 sec	75 mM Tris-HCI (pH 9.0), 20 mM $(NH_4)_2$ SO_4, Tween-20 0.01%, 2.5 mM $MgCl_2$, 0.4 mM each primer, 0.3 mM dNTP mixture, 2 µg DNA, 1 unit of Taq-DNA polymerase (SibEnzyme, Novosibirsk)	461
	1_2_D: 5'-acttactgcacaacaagga-3' 1_2_R: 5'-ggctagttaatcgatcca-3'	95 °C 30 sec, 56 °C 30 sec, 72 °C 30 sec	10 µl BioMaster LR HS-PCR-Color reaction mixture (2×) (BIOLABMIX LLC, Novosibirsk), 0.6 mM each primer	551
Exon 1 (homozygous for rs3064744)	1_1_D: 5'-aacttggtgtatcgattgg-3' 1_1_R: 5'-aaagctgctttctgccag-3'	95 °C 30 sec, 50 °C 30 sec, 72 °C 30 sec	110 µl BioMaster LR HS-PCR-Color reaction mixture (2×) (BIOLABMIX LLC, Novosibirsk), 0.24 mM each primer	516
	1_2_D: 5'-acttactgcacaacaagga-3' 1_2_R: 5'-ggctagttaatcgatcca-3'	95 °C 30 sec, 56 °C 30 sec, 72 °C 30 sec	10 µl BioMaster LR HS-PCR-Color reaction mixture (2×) (BIOLABMIX LLC, Novosibirsk), 0.6 mM each primer	551
Exon 2	D: 5'-tgtaagcaggaacccttcctcc-3' R: 5'-gaagctggaagtctgggattag-3' [2]	95 °C 30 sec, 60 °C 30 sec, 72 °C 30 sec	10 µl BioMaster LR HS-PCR-Color reaction mixture (2×) (BIOLABMIX LLC, Novosibirsk), 0.4 mM each primer	409
Exon 3	D: 5'-cctcccactctgttaaagactgttc-3' R: 5'-agtgttactcacatgcccttgc-3'[2]	95 °C 30 sec, 60 °C 30 sec, 72 °C 30 sec	10 µl BioMaster LR HS-PCR-Color reaction mixture (2×) (BIOLABMIX LLC, Novosibirsk), 0.4 mM each primer	402
Exon 4	D: 5'-tgcaagggcatgtgagtaacac-3' R: 5'-ttgaaacaacgctattaaatgctacg-3' [2]	95 °C 30 sec, 44 °C 30 sec, 72 °C 30 sec	10 µl BioMaster LR HS-PCR-Color reaction mixture (2×) (BIOLABMIX LLC, Novosibirsk), 0.4 mM each primer	434
Exon 5	D: 5'-gagaggattgttcataccacagg-3' R: 5'-cactgattctgttttcaagtttgg-3' [2]	95 °C 30 sec, 60 °C 30 sec, 72 °C 30 sec	10 µl BioMaster LR HS-PCR-Color reaction mixture (2×) (BIOLABMIX LLC, Novosibirsk), 0.8 mM each primer	429

Note. The number of cycles – *n*, base pair – bp.

The obtained amplification products were purified from salts, non-activated primers, and deoxynucleotide triphosphates using a CleanMag DNA suspension (Eurogen, Moscow). The samples were sequenced by capillary electrophoresis using the BigDye® Terminator v3.1 (Applied Biosystems, USA) and BrilliantDye™ Terminator (v3.1) Cycle Sequencing Kit (NimaGen, Netherlands) on the Hitachi 3500 Genetic Analyzer (Applied Biosystems, USA) using the POP-7 separation matrix. The sequencing results were analyzed using the SeqScape v.2.7 and Sequence Scanner software.

RESULTS AND DISCUSSION

The results of automated Sanger sequencing are shown in Table 1. A common single nucleotide variant rs3755319 was found in 21 people in a heterozygous or homozygous state (Fig. 1, 2). The rs3755319 variant (g.234667582A>C) is localized in the promoter of the *UGT1A1* gene and is common among the population [4]. According to gomAD data, the frequency of the rare C allele for Europeans is about 0.43. According to ClinVar, the variant is pathogenic for transient familial neonatal hyperbilirubinemia. However, in a study performed in Korea, its effect on the level of gene expression was not found (when evaluated as part of haplotypes): the haplotypes rs3755319C-rs2003569A-rs887829C-rs3064744(TA)6 and rs3755319A-rs2003569G-rs887829C-rs3064744(TA)7 were associated with lower gene expression compared to the haplotype rs3755319C-rs2003569G-rs887829T-rs3064744(TA)6 [5]. Studies on rs3755319 are found, devoted to its effect on the pharmacokinetics of moxifloxacin and irinotecan [6, 7]. The genetic variant was associated with the level of total bilirubin and cholelithiasis in patients with sickle cell anemia [8]. In order to make an unambiguous conclusion about the association of the variant with GS, additional research is required.

In 3 people with 7TA/7TA rs3064744 genotype (patients No. 301, 386, and 394), a common single

nucleotide variant rs28899472 in a heterozygous state was identified (Fig. 3). The single nucleotide variant rs28899472 (g.234667809C>T) is localized in the promoter of the *UGT1A1* gene [9]. According to gnomAD data, the frequency of the rare allele for Europeans is about 0.03. The variant is not described in ClinVar, and no scientific articles have been found devoted to it. According to the *in silico* predictive analysis, the variant is classified as benign or neutral (PolyPhen-2, PhD-SNP, SNPs&GO).

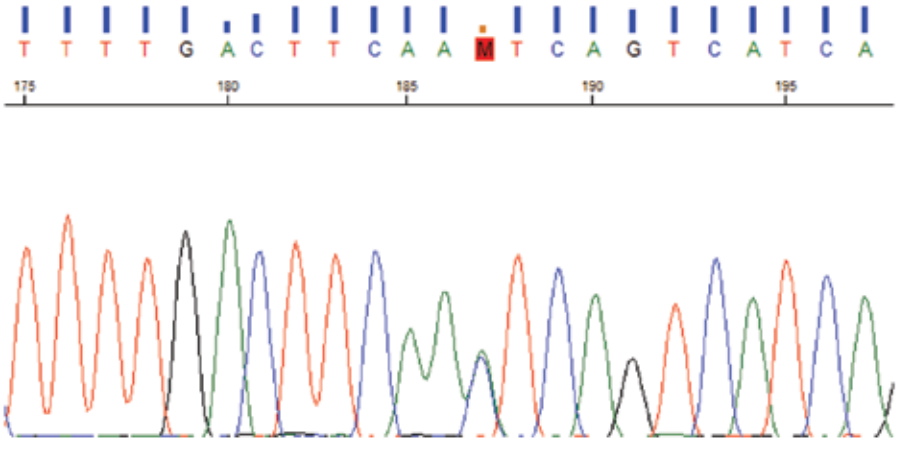

Fig. 1. Sequence of the sample (rs3755319 in a heterozygous state)

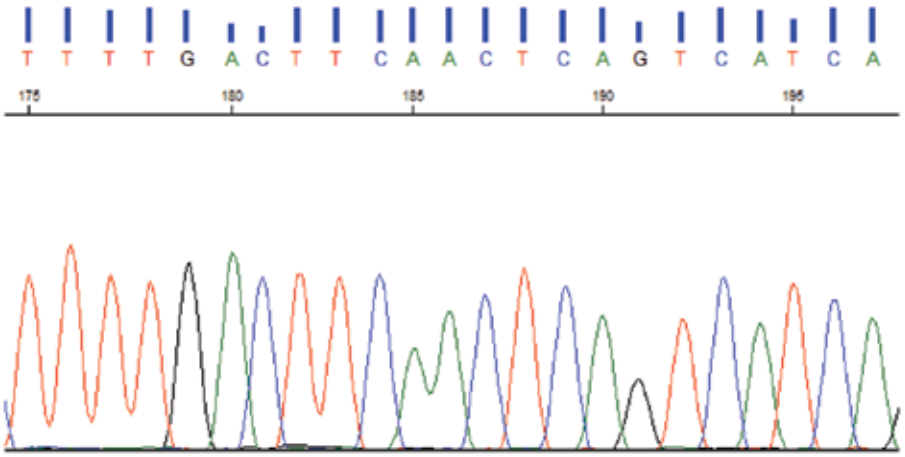

Fig. 2. Sequence of the sample (rs3755319 in a homozygous state)

The rs28899472 variant was searched for in the next of kin of patients No. 386 and 394. All the relatives of the patients included in the study did not show clinical symptoms of hyperbilirubinemia and never had an increase in the level of bilirubin and its fractions, according to the results of blood biochemistry. Both sons of patient No. 386 (a 50-year-old woman; the maximum concentration of bilirubin was recorded during pregnancy; during life, fluctuations in bilirubin levels from normal to elevated figures were observed) were heterozygous carriers of the rs3064744 variant (6TA/7TA). In one of the sons (28 years old), the rs28899472 variant in a heterozygous state was identified; the second son (24 years old) was not a carrier of the rare allele of rs28899472. The father of patient No. 394 (a 32-year-old man with newly diagnosed unconjugated hyperbilirubinemia during treatment of diffuse toxic goiter with thyrostatics) was also a heterozygous carrier of the rs3064744 variant (6TA/7TA) and was not a carrier of the rare allele of rs28899472.

Therefore, the rs28899472 variant was identified in a heterozygous state in a person without hyperbilirubinemia, a carrier of the 6TA/7TA genotype of rs3064744. Consequently, it is currently impossible to consider the variant as pathogenic in relation to GS; case-control studies are required to determine the frequency of the variant in the group of people with hyperbilirubinemia and in the control group.

In patient No. 56 (rare genotype 5TA/7TA rs3064744, female, 22 years old, without a history of liver and gallbladder diseases), a rare single nucleotide variant rs2125984650 was identified in a heterozygous state (Fig. 4). The rs2125984650 variant in exon 1 of the *UGT1A1* gene (c.188A>T) is a missense variant leading to the replacement of the aspartic amino acid with valine p.Asp63Val in position 63 of amino acid sequence [10]. The variant is not described in ClinVar. There are no data on the frequency of the variant in gnomAD. No scientific articles have been found devoted to the rs2125984650 variant. According to the *in silico* predictive analysis, the variant is classified as benign or neutral (PolyPhen-2, PhD-SNP, SNPs&GO). Thus, the rs2125984650 variant in the *UGT1A1* gene can be currently regarded as a variant of uncertain significance.

In patient No. 447, (genotype 6TA/7TA rs3064744, male, 76 years old, hyperbilirubinemia was detected accidentally at the age of 76, Tatar, history of gallstone disease, ultrasound examination revealed cysts in the left lobe of the liver), we identified a rare single nucleotide variant rs1273237448 in a heterozygous state (Fig. 5). The rs1273237448 variant localized in exon 1 of the *UGT1A1* gene (c.182C>G) is a missense variant leading to the replacement of alanine with glycine p.Ala61Gly in position 61 of amino acid sequence [11].

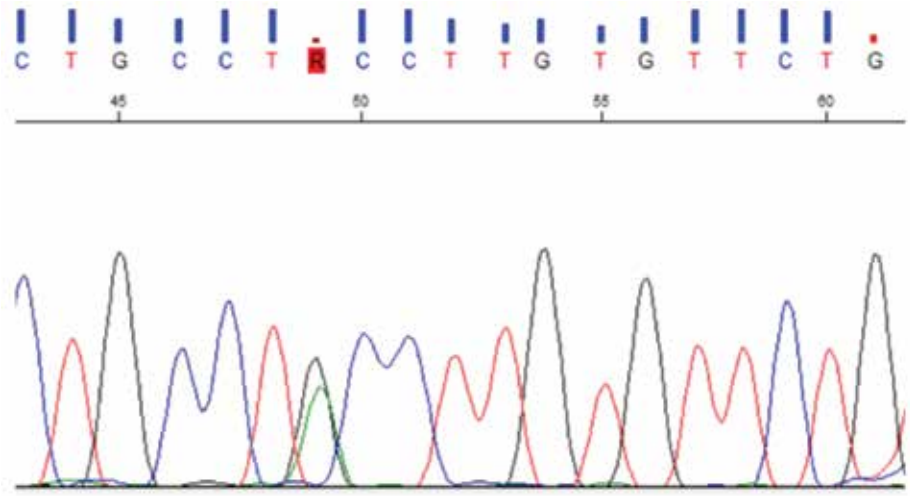

Fig. 3. Sequence of samples No. 301, 386, 394 (rs28899472 in a heterozygous state)

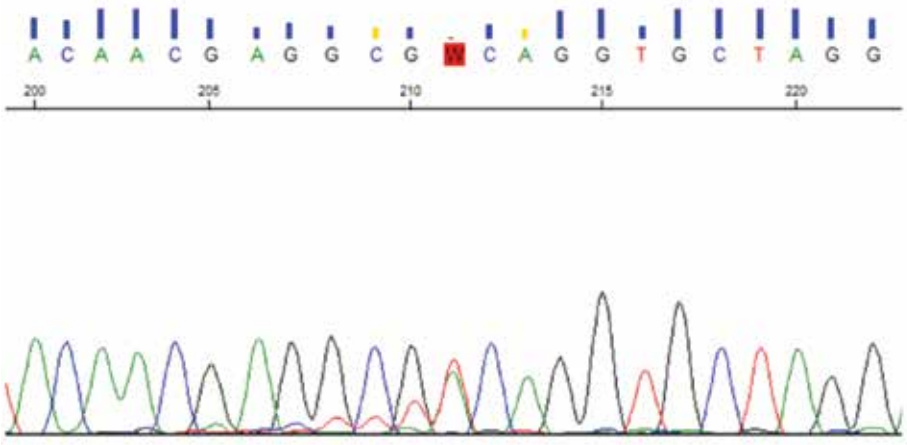

Fig. 4. Sequence of sample No. 56 (rs2125984650 in a heterozygous state)

According to ClinVar, the variant is likely pathogenic for GS [12]. The frequency of the rare allele, according to gnomAD, is very low – about 0.000009; no homozygotes were recorded. No scientific articles have been found that mention the rs1273237448 variant. Following the *in silico* predictive analysis, the variant is classified as benign or neutral (PolyPhen-2, PhD-SNP, SNPs&GO). Currently rs1273237448 can be regarded as a variant of uncertain significance; however, it may be related to the patient's phenotype.

Patient No. 558 (genotype 6TA/7TA rs3064744, male, 18 years old, without a history of liver and

gallbladder diseases) was a carrier of the rare variant rs4148323 (*UGT1A1*6*) in a heterozygous state (Fig. 6). The rs4148323 variant is localized in exon 1 of the *UGT1A1* gene (c.211G>A, p.Gly71Arg) [13]. The frequency of the rare allele, according to gnomAD, is low –about 0.002. The variant is most common in Asian countries (the frequency of the rare variant is about 0.15). The rs4148323 variant (*UGT1A1*6*) in the homozygous state was associated with the development of GS, neonatal hyperbilirubinemia, and a 70% decrease in the activity of the UDP-glucuronosyltransferase 1A1 enzyme compared to the wild type [14, 15].

Previously, we conducted a search for the variant in a group of people with GS (125 people). Patients No. 447 and 558 were not included in the group. In two individuals with GS in this group, the rs4148323 variant was also identified in a heterozygous state. Except for the rs4148323 variant, patients were heterozygous for rs3064744 (genotype 6TA/7TA) [1]. The main studies on rs4148323 were conducted in Asian countries (India, China). They showed that both homozygous carriers of rs4148323 and compound heterozygotes for rs4148323 and rs3064744 were found in GS, which was observed in patients No. 447 and 558 [16, 17].

Therefore, patient No. 447 was a heterozygous carrier of four variants: rs3064744 (a common variant in GS), rs1273237448 (a rare variant, likely pathogenic for GS, according to ClinVar data), and rs4148323 (a known variant for GS in Asian countries), which may explain unconjugated hyperbilirubinemia, and rs3755319 which is common in the population and the clinical significance of which is currently uncertain.

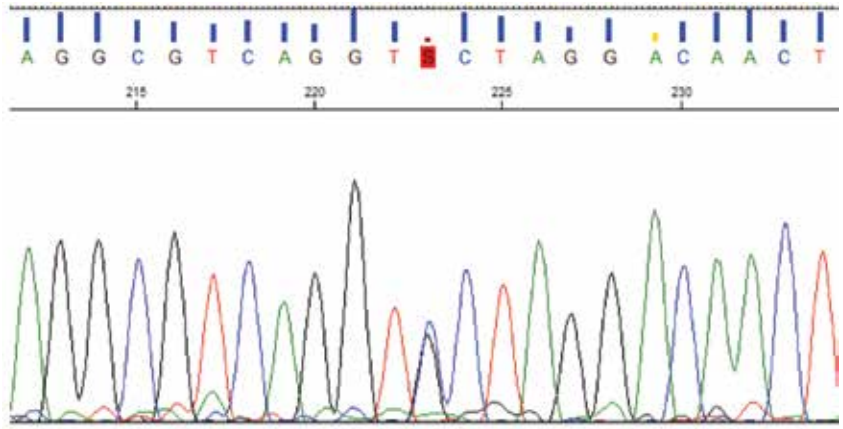

Fig. 5. Sequence of sample No. 447 (rs1273237448 in a heterozygous state)

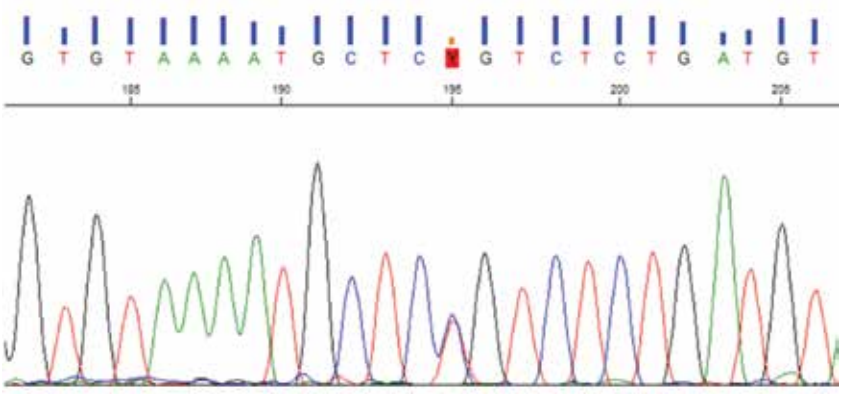

Fig. 6. Sequence of sample No. 447 (rs4148323 in a heterozygous state)

CONCLUSION

According to the results of the study, automated Sanger sequencing of the *UGT1A1* gene may be the next stage of DNA analysis after determining the rs3064744 genotype for individuals with genotypes 6TA/6TA and 6TA/7TA rs3064744 and suspected GS.

A common single nucleotide variant rs3755319 was identified in the gene promoter, whose significance in relation to unconjugated hyperbilirubinemia will have to be evaluated in future scientific studies. In three individuals with confirmed GS (7TA/7TA rs3064744), we identified the rs28899472 variant, the role of which

in the development of the GS phenotype is not yet clear and requires further study. A single nucleotide variant of uncertain significance rs2125984650 was identified in a patient with a rare genotype 5TA/7TA rs3064744. In two individuals with genotypes 6TA/7TA rs3064744, gene variants that were pathogenic and likely pathogenic for GS (according to some authors) were revealed (rs4148323, rs1273237448).

Therefore, none of the six people with unconjugated hyperbilirubinemia and 6TA/6TA rs3064744 genotype had any pathogenic variants for GS in the *UGT1A1* gene. Among twelve people with unconjugated hyperbilirubinemia and 6TA/7TA rs3064744 genotype, two had variants explaining their condition. Consequently, automated Sanger sequencing of the *UGT1A1* gene revealed causal variants of the gene only in 11% of people (2 out of 18 people with 6TA/6TA and 6TA/7TA rs3064744 genotypes). The results obtained may indicate either the presence of variants of other genes that are associated with GS or an insufficient examination of patients with 6TA/6TA and 6TA/7TA rs3064744 genotypes and a false clinical diagnosis of GS.

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Authors' contribution

Ivanova A.A. – conception and design, molecular genetic analysis, interpretation of the data. Apartseva N.E., Nemcova E.G., Kurilovich S.A., Kruchinina M.V. – recruitment of the GS group. Kashirina A.P., Ivanova Ju.V. – molecular genetic analysis. Maksimov V.N. – critical revision of the manuscript for important intellectual content, final approval of the manuscript for publication.

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External validation of a multivariate model for predicting the risk of death in patients with chronic heart failure and an implantable cardioverter – defibrillator

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ABSTRACT

Aim. To perform external validation of a multivariate model for predicting the risk of death in patients with an implantable cardioverter – defibrillator (ICD) in an independent sample.

Materials and methods. The group for model development included 260 patients from the Implantable Cardioverter – Defibrillator Patient Registry who had an ICD implanted between 2015 and 2019. External validation of the model was carried out in an independent, prospective, observational cohort study of patients from the same registry, in whom an ICD was implanted between 2020 and 2021, a total of 94 patients, median age 66 (52;73) years, 73 (77.6%) men, 21 (22.4%) women. In 89 (94.7%) patients, an ICD was implanted for primary prevention of sudden cardiac death. Following a telephone survey and examination of medical records from hospital and clinic databases, data on the vital status (alive / dead) and causes of death were obtained during a 2.5-year follow-up. The actual and predicted mortality from the estimated multivariate model were compared.

Results. During the follow-up, a total of 26 (27.7%) patients died in the external validation group, which was comparable to the development group ($p > 0.05$). In the group of deceased, 15 (57.7%) people developed acute decompensated heart failure, 4 (14.8%) had myocardial infarction, 6 (23.1%) had pneumonia caused by a new coronavirus infection, and one (3.8%) patient died due to an infectious complication.

The diagnostic accuracy of the multivariate model for predicting the risk of death in patients with ICD in an independent sample was sufficient (the area under the curve (AUC) of the created model was 0.8). The sensitivity of the model was 76.2%, specificity – 76.1%. Previously, in the development cohort, AUC of the created model was 0.8, the sensitivity of the model was 75.7%, and the specificity was 80%. Model significance did not differ significantly between the development and external validation groups ($p = 0.102$, McNeil test).

Conclusion. The multivariate prediction model has sufficient statistical power to predict the risk of long-term death after ICD implantation, which was externally validated.

Keywords: implantable cardioverter – defibrillator, heart failure, prognostic model, death, validation

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

Source of financing. The study was carried out within the basic research topic of Research Institute for Complex Problems of Cardiovascular Diseases "Developing innovative risk management models for cardiovascular diseases with account of comorbidity based on the study of fundamental, clinical, and epidemiological mechanisms and medical care organization technologies in conditions of the industrial region of Siberia" (state registration No. 122012000364-5 of 20.01.2022).

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Conformity with the principles of ethics. All patients signed an informed consent to participate in the study. The study was approved by the local Ethics Committee at Research Institute for Complex Problems of Cardiovascular Diseases (Protocol No. 1 of 26.01.2015).

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Внешняя валидация многофакторной модели прогнозирования риска смерти у пациентов с хронической сердечной недостаточностью и имплантированным кардиовертером-дефибриллятором

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РЕЗЮМЕ

Цель. Внешняя валидация многофакторной модели прогнозирования риска смерти у пациентов с имплантированным кардиовертером-дефибриллятором (ИКД) на независимой выборке.

Материалы и методы. Группа разработки модели была представлена 260 пациентами из Кузбасского регистра пациентов с имплантированным кардиовертером-дефибриллятором, которым ИКД был имплантирован в период с 2015 по 2019 г. Внешняя валидация модели проведена в когорте независимого проспективного наблюдения пациентов из этого же регистра, которым ИКД был имплантирован в период с 2020 по 2021 г., всего 94 пациента, медиана возраста 66 (52;73) лет, 73 (77,6%) мужчин, 21 (22,4%) женщина. У 89 (94,7%) пациентов ИКД был имплантирован с целью первичной профилактики внезапной сердечной смерти. Путем телефонного опроса, изучения медицинской документации баз данных стационаров и поликлиник были получены данные о статусе «жив/умер» и о причинах смерти в течение 2,5 лет наблюдения. Сравнивалась фактическая и прогнозируемая по оцениваемой многофакторной модели смертность.

Результаты. За период наблюдения в группе внешней валидации всего умерли 26 (27,7 %) пациентов, что было сопоставимо с группой разработки ($p > 0,05$). В группе умерших у 15 (57,7%) развилась острая декомпенсация сердечной недостаточности, у 4 (14,8 %) установлен инфаркт миокарда, у 6 (23,1%) – пневмония, вызванная новой коронавирусной инфекцией, 1 (3,8%) пациент умер из-за инфекционного осложнения.

Диагностическая точность многофакторной модели прогнозирования риска смерти у пациентов с ИКД на независимой выборке была достаточной (площадь под ROC-кривой (AUC) созданной модели составила 0,8). Чувствительность модели составила 76,2%, специфичность – 76,1%. Ранее на когорте разработки площадь под ROC-кривой (AUC) созданной модели составила 0,8; чувствительность модели – 75,7%; специфичность – 80%. Значимость модели в группах разработки и внешней валидации существенно не отличалась ($p = 0,102$, тест McNeil).

Заключение. Многофакторная модель прогнозирования обладает достаточной статистической мощностью для прогнозирования риска смерти в отдаленном периоде после имплантации ИКД, что подтверждено внешней валидацией.

Ключевые слова: имплантируемый кардиовертер-дефибриллятор, сердечная недостаточность, прогностическая модель, смерть, валидация

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

Источник финансирования. Работа выполнена в рамках фундаментальной темы НИИ КПССЗ «Разработка инновационных моделей управления риском развития болезней системы кровообращения с уче-

том коморбидности на основе изучения фундаментальных, клинических, эпидемиологических механизмов и организационных технологий медицинской помощи в условиях промышленного региона Сибири» (№ госрегистрации 122012000364-5 от 20.01.2022).

Соответствие принципам этики. Все лица подписали информированное согласие на участие в исследовании. Исследование одобрено локальным этическим комитетом КПССЗ (протокол № 1 от 26.01.2015).

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INTRODUCTION

According to recent data, the prevalence of severe chronic heart failure (CHF) in Russia has increased to 8.2% [1]. Low left ventricular ejection fraction (LVEF) is one of the main predictors of the development of life-threatening ventricular arrhythmias (VA) and the associated high risk of sudden cardiac death (SCD) [2]. Current clinical guidelines for the prevention of SCD consider LVEF lower than 35% as the main indication (class Ia evidence) for implantation of a cardioverter – defibrillator (ICD) for primary prevention of SCD [1, 3].

Therefore, the need for ICD for SCD prevention is very high. However, despite a steady increase in the number of ICDs implanted, Russia occupies last places in European ratings in the availability of interventional treatment for cardiac arrhythmia in the regions [4]. On the other hand, data from clinical practice indicate that patients with low LVEF are more likely to die from acute decompensated heart failure than from other causes, including SCD [5]. Thus, a prediction model that can assess mortality risk in patients with low LVEF before ICD implantation will help implement a patient-oriented approach to selecting patients for this type of medical care.

Clinical prognosis is based on available clinical data and the use of modern statistical methods and allows specialists to assess the risk of developing an event, making it an important area of research with a clear practical purpose. In this regard, in the medical field in general and in cardiology, in particular, an exponential growth in the number of prediction models has been seen. However, not all developed models undergo external validation. Thus, it was shown that out of 1,366 different models for predicting cardiovascular diseases, only 43.4% provided data on external validation [6]. Moreover, only single externally validated models have proven their clinical effectiveness by demonstrating that their use leads to improved results for patients and doctors.

The aim of this study was to perform external validation of a multivariate model for predicting the risk of death in patients with ICD in an independent sample.

MATERIALS AND METHODS

A multivariate prognostic model for determining the risk of nonarrhythmic death in patients with CHF and ICD was developed and internally validated as a result of a single-center, observational, prospective study based on data from the Kuzbass ICD Patient Registry. The registry consistently included all patients of the Kemerovo region who had ICD implanted from 2015 to 2019 and reached a total of 264 patients. The development of the registry and the informed consent form were approved by the local Ethics Committee and complied with the ethical principles of the Declaration of Helsinki. All study participants signed an informed consent upon admission to the hospital. When maintaining the registry, all the requirements of the Federal Law No. 152-FZ of 07.27.2006 “On Personal Data” were met.

The mean age of the patients included in the development group was 59 (53; 66) years, 214 (82.3%) were men, 28 (10.8%) were working. All patients were diagnosed with CHF. Median LVEF was 30 (25;36)%. A total of 158 (60.8%) patients received ICD for SCD prevention. Prior to ICD implantation, only 122 (46.9%) patients received triple combination therapy (renin – angiotensin – aldosterone system blocker (RAAS), mineralocorticoid receptor antagonist (MCRA), beta-adrenergic blocker (BAB)), according to the relevant clinical guidelines for the treatment of CHF.

To determine the most significant predictors of death during the follow-up period, a step-by-step logistic regression analysis with the inclusion of

the most important variables was performed (all variables can be assessed during patient screening), and prognostic models were developed for the risk of death, composite endpoint, and CHF progression. The regression equation was as follows: $y = a + b1 \times x1 + b2 \times x2 + ...bi \times xi$, where y is a dependent variable that can have two values: 0 – no event, 1 – event; a – constant; bi – regression coefficients; Xi – variables.

The probability of the event P was calculated according to the formula: $P = 1 / (1 + e^{-y})$, where P is predictive probability, e – exponent, whose approximate value equals to 2.718.

The verification of the null hypothesis regarding the validity of the model was carried out using the Hosmer – Lemeshow test; $p > 0.05$ indicated the validity of the model.

Following the development of the model, the qualitative assessment of the predictive probability of an event was conducted. When predicting death, the cut-off value was 0.2; for other events, it was 0.5.

Initially, the model included factors that had significant differences in comparison assessments. The parameters obtained during the model development using step-by-step regression are presented in Table 1.

Table 1

Regression coefficients used in the model for predicting the risk of low-term mortality (4 years) in patients with an implanted cardioverter – defibrillator

Parameter	Variables in the equation					
	B	Standard error	Wald	df	Significance	Exp (B)
P(PA) mmHg, X1	0.049	0.014	12.696	1	0.000	1.050
NYHA, X2	1.312	0.353	13.854	1	0,000	3.715
Type of prevention of SCD, X3	–1.396	0.370	14.203	1	0.000	0.248
Age, X4	0.054	0.017	9.596	1	0.002	1.055
RAAS + BAB+ MCRA, X5	1.244	0.380	10.737	1	0.001	3.470
BAB, X6	–1.626	0.681	5.701	1	0.017	0.197
Constant	–5.691	1.336	18.145	1	0.000	0.003

Note. P (PA) – systolic pressure in the pulmonary artery, NYHA – functional classification of heart failure proposed by New York Heart Association.

The probability of death was calculated as follows:

$$P = 1/(1 + 2.718^{-(-5.691 + 0.049 \times X1 + 1.312 \times X2 - 1.396 \times X3 + 0.054 \times X4 + 1.244 \times X5 - 1.626 \times X6)}) \times 100\%$$

P above 28% indicated a high risk of death.

Thus, the developed prediction model takes into account systolic pulmonary artery pressure above 45 mm Hg ($p = 0.000$), NYHA functional class ($p = 0.000$), type of SCD prevention ($p = 0.000$), triple combination therapy for CHF ($p = 0.001$), and therapy with BAB ($p = 0.017$). During internal validation, the Hosmer – Lemeshow test value for this model was $\chi 2 = 4.210$, $p = 0.838$, area under the curve (AUC) for the model was 0.8, sensitivity was 80%, and specificity was 75.7%, which indicated a high predictive ability. The model appears as a computer program for Microsoft Windows 9x / NT / 2000 / Vista, 7, 8 operating systems entitled "Calculator of mortality risks in patients with an implanted cardioverter – defibrillator" [7].

In order to externally validate this prediction model on an independent sample, 94 patients hospitalized at the Research Institute for Complex Issues of Cardiovascular Diseases for ICD implantation in 2020–2021 were included in a single-center, prospective study. The mean age of the patients was 66 (52; 73) years, 73 (77.6%) were men, 21 (22.4%) were women, 16 (17%) patients were still working. The comparative clinical characteristics of the groups and external validation of the model are presented in Table 2.

We assessed the risk of long-term mortality after ICD implantation in all patients using the developed model [7]. After that, we conducted a prospective follow-up with annual accumulation of data regarding the vital status of patients and causes of death. The follow-up period was 2.5 years.

Statistical processing of the results was carried out using the Statistica 10.0 (StatSoftInc., USA) and SPSS Statistics ver.23.0 (IBM, USA) software packages. The normality of data distribution was checked using the Shapiro – Wilk test. The Student's t-test was used to compare continuous variables with normal distribution; for the non-normally distributed data, the nonparametric Mann – Whitney U test was used. Discrete variables were compared using the $\chi 2$ test with the Yates correction. In case the number of variables was too small in one of the compared groups

(5 or less), the Fisher's exact test was applied. The data were presented as the median and the interquartile range *Me* (Q_{25}; Q_{75}) and as the absolute and relative values *n* (%). The differences were considered statistically significant at $p < 0.05$.

External validation was performed using the ROC analysis. By constructing curves, we analyzed the diagnostic accuracy of the model. Sensitivity and specificity were calculated for each diagnostic criterion. The diagnostic significance in different groups was compared by AUC values using the McNeil test. The classification and compliance assessment with actual events was performed using the Hosmer – Lemeshow test. The model was considered as adequate in the absence of significant differences ($p > 0.05$).

RESULTS

Comparative characteristics of the development and validation groups are presented in Table 2.

Table 2

Baseline clinical and anamnestic characteristics of the groups, *n* (%)

Parameter	Development, *N* = 260, 2015–2019	External validation group, *N* = 94, 2019–2020
Men	214 (82.3)	73 (77.6)
Age, years, *Me* (Q_{25}; Q_{75})	59 (53; 66)	66 (52; 73)
Still working	28 (10.8)	16 (17)*
CAD	194 (74.6)	76 (80.8)
PICS	156 (60)	58 (61.7)
Non-coronary diseases	66 (25.4)	18 (19.2)
LVEF, %, *Me* (Q_{25}; Q_{75})	30 (25; 36.5)	29.5 (24;37)
All types of AF	106 (40.8)	41 (43.6)
NYHA I–II	179 (68.8)	44 (46.8)*
NYHA III–IV	81 (31.2)	50 (53.2)*
Primary prevention of SCD	158 (60.8)	89 (94.7)*

Note. CAD – coronary artery disease, PICS – post-infarction cardiostenosis, AF – atrial fibrillation, *N* – number of patients. * $p < 0.01$.

The patients were comparable in gender, age, and etiology of CHF and LVEF. The external validation group had more severe cases of HF, and the majority of patients received ICD for SCD prevention (Table 2). Considering the fact that optimal drug therapy for CHF was an important prognostic factor, we carried out a comparative analysis of drug therapy in the development and external validation groups (Table 3)

Table 3

Frequency of prescription of drug therapy for heart failure prior to ICD implantation, *n* (%)

Drug	Development group, *N* = 260	External validation group, *N* = 94
ACEI	164 (57.3)	56 (59.5)
ARBs*	41 (14.3)	36 (38.2)
ARNI*	5 (1.7)	14 (14.9)
BAB	259 (90.6)	87 (92.5)
MCRA*	167 (58.4)	65 (69.1)
Amiodarone	144 (50.3)	54 (57.4)

Note. ACEI – angiotensin-converting enzyme inhibitor, ARB – angiotensin receptor blocker, ARNI– angiotensin receptor – neprilysin inhibitor, *N* – number of patients. * $p < 0.01$.

When comparing the drug therapy received before ICD implantation, it turned out that patients in the validation group were prescribed MCRA and RAAS inhibitors more often. However, only 122 (46.9%) patients in the development group and 49 (52.1%) patients in the validation group received triple combination CHF therapy ($p < 0.05$).

During the follow-up period, 54 patients died in the development group, and 4 patients were lost to follow-up and considered as dead; thus, the mortality rate in the group was 21.9%. Among these patients, 19 (35.2%) patients died in hospital, of which 3 (17.6%) had myocardial infarction, 1 (5.9%) had stroke, 13 (76.5%) died due to CHF and 2 (3.7%) died from pneumonia caused by novel coronavirus infection. Thirty-five (64.8%) patients died outside hospital, they suffered acute decompensated HF, and the cause of death was the underlying disease: 10 (27%) had dilated cardiomyopathy, 1 (2.8%) had rheumatic heart valve disease, and the remaining 24 (68.6%) had ischemic cardiomyopathy. The vast majority of deaths occurred in the first 1.5 years of the follow-up.

During the 2.5-year follow-up, 26 (27.7%) deaths were recorded in the external validation group, which is comparable to the development group ($p > 0.05$). Among the deceased patients, 15 (57.7%) developed CHF, 4 (14.8%) had myocardial infarction, 6 (23.1%) had pneumonia caused by novel coronavirus infection, and 1 (3.8%) patient died due to an infectious complication (sepsis).

The prognostic value of the developed model in the external validation group proved to be high (Figure).

The Hosmer – Lemeshow test for this predictive model was the following: $\chi 2 = 4.210$; $p = 0.838$. During the ROC analysis, AUC of the model was

0.8, indicating high predictive ability. Sensitivity of the model was 76.2%, and specificity was 76.1%. All these parameters confirmed the validity of the model.

The diagnostic value of the model in the development and external validation groups did not differ significantly ($p = 0.102$, McNeil test).

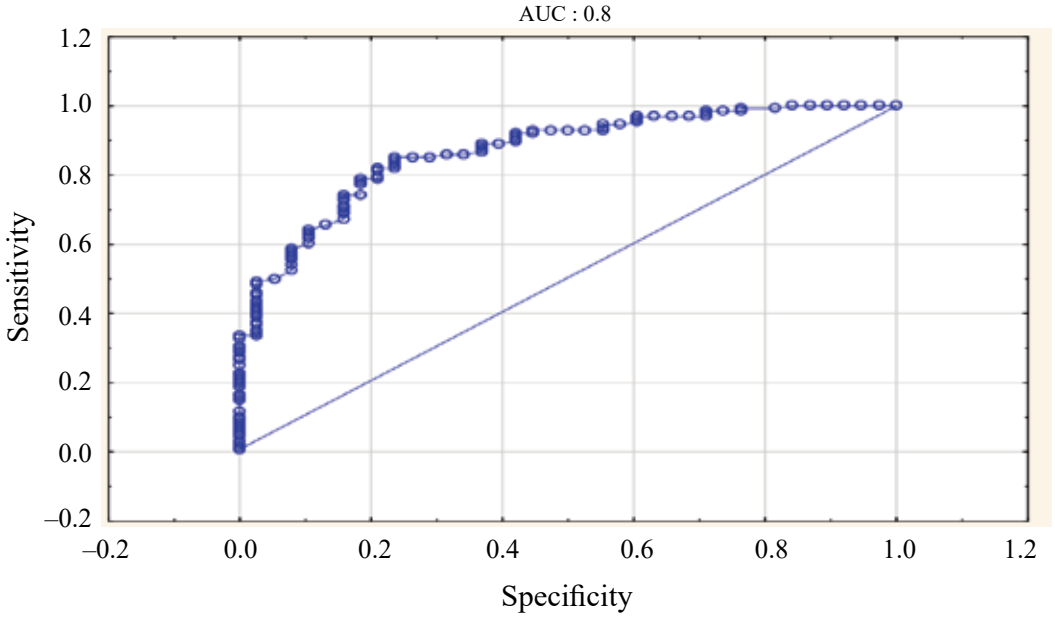

Figure. ROC curve for assessing mortality risk in patients with an implanted cardioverter – defibrillator in the external validation group

Table

Classification table

Patients		Predicted		
		Death		Percentage of correct predictions, %
		0	1	
Death	0	143	45	76,1
	1	15	48	76,2
Total percentage				76,1

Cut-off value was 0.280.

DISCUSSION

The data obtained in this study confirm that patients with low LVEF, including patients with ICD, die from CHF more frequently [8]. Currently, much attention is being paid to the issue of residual high mortality in patients with ICD and the search for predictors that would help identify ICD patients at high-risk of adverse outcomes [9–12]. In this regard, the possibility of predicting the risk of death becomes necessary when considering ICD implantation. In this context, the development of prediction models for application in clinical practice becomes relevant.

The MADIT-II risk score, intended for stratification of benefits of ICD implantation, includes eight predictors of the development of VA (male, age < 75 years, history of unstable ventricular tachycardia, heart rate >75 beats / min, systolic blood pressure < 140 mmHg, LVEF ≤ 25%, history of myocardial infarction and atrial arrhythmia) and seven predictors of nonarrhythmic death (age ≥ 75 years, diabetes mellitus, body mass index > 23 kg / m^2, LVEF ≤ 25%, NYHA class ≥ II, ICD instead of cardiac resynchronization therapy, atrial fibrillation, the level of the brain natriuretic peptide (BNP), and the duration of the QRS complex) [13]. Based on the combined analysis of these predictors, scientists developed a model for an individual assessment of a risk of developing VA compared to nonarrhythmic death. However, this scale was developed using data from studies conducted more than 20 years ago, its application is limited to patients with ischemic cardiomyopathy only, and the scale has not been validated in the Russian population.

Prior studies on the use of ICD are mainly aimed at determining the risk of developing VA and inappropriate ICD shocks in patients with CHF. Prospective Observational Study of Implantable Cardioverter – Defibrillators (PROSE-ICD) is one of the few studies to analyze predictors of mortality in patients with ICD. It included 1,189 patients with systolic HF who had ICD implanted for primary prevention of SCD. During the four-year follow-up, 343 (28.8%) patients died, and appropriate ICD shocks occurred in 137 (11.5%) patients. The study results showed that elevated levels of C-reactive protein, tumor necrosis factor alpha, BNP, troponin T, and iinterleukin-6 increased the risk of death ($p < 0.001$ for all parameters) [14]. To predict the risk of mortality, this study used biochemical markers that would not be routinely assessed in clinical practice. In addition, it has not been validated in the Russian population as well.

The well-known Seattle Heart Failure Model (SHFM), used to assess the life expectancy of patients with CHF at the outpatient stage, and the Meta-Analysis Global Group in Chronic (MAGGIC) Heart Failure scale are also based on the results of long-standing studies, do not take into account comorbid pathology, cannot be applied at the inpatient stage, and cannot be used to assess risks in patients with implanted devices, in particular, with ICD [15, 16].

In a study by T.E. Verstraelen et al. (2021) on the development and external validation of a model for predicting mortality in the ICD group (primary prevention of SCD) during a 2.7-year follow-up, 193 (13.4%) patients died in the development group and 223 (15.4%) patients died in the validation group, which is significantly different from the Russian population [10]. The predictors of all-cause mortality were age, diuretic intake, sodium and BNP levels, and intake of RAAS inhibitors. The C-statistic was 0.74 for both external and internal validation groups. Russian researchers are also actively working on the possibility of predicting outcomes in patients with CHF, however, almost all proposed methods include the need to determine either genetic markers or complex biochemical parameters, but do not include patients with ICD, thereby limiting practical application of these methods [17].

Therefore, currently there are no adequate ways to assess the risk of long-term nonarrhythmic mortality after ICD implantation in patients with CHF and low LVEF, suitable for use in the Russian clinical practice. The proposed and validated model for assessing the risk of nonarrhythmic death in patients with ICD differs from existing ones because it takes into account the presence of both factors (CHF with low LVEF and ICD), as well as comorbidity and adherence to optimal drug therapy (an important prognostic factor for the Russian population) to determine the prognosis.

The application of the prediction model involves the assessment of routine parameters included in a standard examination of a patient with CHF and does not require additional costs. It is important to note that this prediction model can and should be used before ICD implantation. It is supposed to identify the patients who would not significantly benefit from ICD implantation in the long term due to a high risk of SCD. In general, the predictive value of the studied model, estimated on the basis of an independent sample, is comparable with the results of the internal validation.

CONCLUSION

The presented multivariate model has sufficient prognostic power to predict the risk of death in patients with ICD in the long term, as confirmed by the external validation. However, risk stratification remains a difficult task, and based on the conducted research, identifying a group of patients who would not benefit from ICD implantation is still an issue. However, the proposed prediction model can provide clinical value by identifying cases in which ICD implantation could be delayed.

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Authors' contribution

Lebedeva N.B. – conception and design, analysis and interpretation of the data, approval of the draft version of the article. Parfenov P.G. – keeping the registry, acquisition of the data, analysis and interpretation of the data, search for literature. Egle A.P. – keeping the registry, statistical processing and interpretation of the data. GalintsevYu.V. – keeping the registry, carrying out of the prospective stage of external validation group monitoring. Ivanov V.I. – statistical processing of the data, development of a multivariate prediction model, calculation of risks. KashtalapV.V. – critical revision of the manuscript for important intellectual data. Barbarash O.L. – conception of the study, editing of the article, final approval of the article for publication.

All the authors gave their consent to submission of the manuscript and agreed to bear responsibility for all aspects related to it.

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Clinical and anamnestic characteristics of patients depending on left ventricular ejection fraction: results of a register study

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ABSTRACT

Aim. To study the clinical and anamnestic features of patients followed up in the Center for Chronic Heart Failure at the Regional Vascular Center according to the data of the corresponding register.

Materials and methods. The study included data of 802 patients included in the Kuzbass Register of Patients Followed up at the Center for Chronic Heart Failure from 2020 to 2022. The median follow-up was 1.6 ± 0.7 years.

Results. According to the present register, men dominated in the gender profile of patients with chronic heart failure – 612 (76.3%) participants ($p < 0.001$). The largest group of subjects was represented by patients with a low left ventricular ejection fraction (less than 40%) – 546 people. This category was also characterized by a more severe functional class of chronic heart failure (New York Heart Association); patients with functional class III–IV chronic heart failure prevailed ($p < 0.001$).

The most common comorbidities revealed were chronic kidney disease (glomerular filtration rate of less than 60 ml / min / 1.73 m² according to the CKD-EPI equation) – 614 (76.5%) patients and obesity (body mass index of more than 30 kg / m2) – 334 (41.6%) patients. Type 2 diabetes mellitus was reported in 193 (24%) patients. The analysis of the etiology of chronic heart failure showed that the main causes of heart failure in the groups with low and intermediate left ventricular ejection fraction were coronary heart disease and combined causes, whereas in the group with preserved left ventricular ejection fraction, the disease resulted from coronary heart disease and arrhythmogenic causes.

Conclusion. Assessing the clinical and anamnestic features of patients with heart failure, it can be said that these people are mainly male, retired, with coronary heart disease, low left ventricular ejection fraction, and a comorbidity, mainly chronic kidney disease, diabetes mellitus, and obesity.

Keywords: chronic heart failure, coronary heart disease, cardiology, left ventricular ejection fraction

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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Conformity with the principles of ethics. All patients signed an informed consent to participate in the study. The study was approved by the local Ethics Committee at Research Institute for Complex Problems of Cardiovascular Diseases.

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Клинико-анамнестические особенности пациентов в зависимости от величины фракции выброса левого желудочка: результаты регистрового исследования

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РЕЗЮМЕ

Цель. Изучить клинико-анамнестические особенности пациентов, наблюдающихся в центре хронической сердечной недостаточности на базе регионального сосудистого центра по данным соответствующего регистра.

Материалы и методы. В настоящее исследование вошли данные 802 пациентов из Кузбасского регистра пациентов, наблюдающихся в центре помощи больным с хронической сердечной недостаточностью, включенных за период с 2020 по 2022 г. Средний срок наблюдения составил 1,6 ± 0,7 лет.

Результаты. По данным настоящего регистра, в гендерной структуре пациентов с хронической сердечной недостаточностью преобладали мужчины – 612 (76,3%) ($p < 0,001$). Наибольшую группу исследуемых составляли пациенты с низкой фракцией выброса левого желудочка (менее 40%) – 546 человек. Данная категория была и более тяжелой по функциональному классу хронической сердечной недостаточности (New York Heart Association), преобладали больные с функциональным классом III–IV ($p < 0,001$).

При анализе коморбидной патологии выявлено, что наиболее распространенными являлись: хроническая болезнь почек (скорость клубочковой фильтрации по CKD-EPI менее 60 мл/мин/1,73 м^2) – 614 (76,5%) человек и ожирение (индекс массы тела более 30 кг/м^2) – 334 (41,6%) пациента. Сахарный диабет 2-го типа был зарегистрирован у 193 (24%) пациентов. Анализ этиологии хронической сердечной недостаточности показал, что основными причинами сердечной недостаточности в группах низкой и промежуточной фракций выброса являлись ишемическая болезнь сердца и комбинированные причины, в группе с сохранной фракцией выброса – ишемическая болезнь сердца и аритмогенная причины.

Заключение. Таким образом, оценивая клинико-анамнестические особенности пациентов с сердечной недостаточностью, можно говорить о том, что это лица преимущественно мужского пола, пенсионного возраста, с ишемической болезнь сердца и низкой фракцией выброса левого желудочка, а также имеющие коморбидную патологию, преимущественно хроническую болезнь почек, сахарный диабет и ожирение.

Ключевые слова: хроническая сердечная недостаточность, ишемическая болезнь сердца, кардиология, фракция выброса левого желудочка

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 НИИ КПССЗ.

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INTRODUCTION

Reducing mortality in chronic heart failure (CHF) among the population currently remains one of the most important and relevant issues in cardiology and healthcare in general. Patients with CHF are characterized by a progressive course of the disease, which ultimately leads to multiple hospitalizations due to decompensation of CHF, prolonged medical and expensive surgical treatment, and high levels of disability and mortality [1]. According to various estimates, the prevalence of CHF is approximately

1–2% in the general population, and it steadily increases, reaching more than 10% among people over 70 years of age [2].

Considering the growing number of elderly people and the progressive growth of cardiovascular pathology, the prevalence of CHF is also increasing annually [3]. In this regard, a global strategic task is to find ways to detect CHF at earlier stages of the disease, including by understanding the clinical and anamnestic characteristics of patients with CHF [4]. To resolve this issue, an epidemiological study EPOCHA-CHF was conducted from 2002 to 2017 in the European part of Russia. Its findings made it possible to estimate the prevalence and mortality rate of CHF and create a clinical portrait of a patient with CHF [5].

In the course of evolution of knowledge about the pathophysiology of CHF, it has been proven that CHF can develop not only with reduced, but also with normal left ventricular ejection fraction (LVEF). According to the EPOCHA-CHF project in Russia, 71% of patients with CHF had LVEF of more than 60% [6]. An important goal of register studies is to create effective methods for monitoring the outpatient stage of treatment, drug therapy, and physical and psychological rehabilitation of patients, that are impossible without knowledge about the characteristics of the patient cohort. In recent years, CHF centers have been established in outpatient settings, which proved their effectiveness in reducing the risk of overall and cardiovascular mortality and decreasing the number of re-hospitalizations [7]. In the meantime, the effectiveness of this approach to management of patients with CHF requires additional study, including research from the standpoint of different CHF phenotypes.

The aim of the study was to investigate the clinical and anamnestic features of patients followed up in the CHF center at the Regional Vascular Center according to the data of the corresponding register, depending on the CHF phenotype.

MATERIALS AND METHODS

The study included data from the Kuzbass Register of Patients Followed up at the Center for Chronic Heart Failure. The register includes data of patients with heart failure after discharge from Kuzbass Clinical Cardiology Center named after L.S. Barbarash or patients with newly diagnosed heart failure in the outpatient or inpatient setting registered in the CHF center database. The register was launched on 26.08.2020. The register was managed in accordance with the provisions of the Declaration of Helsinki and was approved by the local Ethics Committee. All patients signed an informed consent to be followed up at the CHF center.

The register was a database which served as the basis for a prospective, cohort, observational study of adult patients. The only inclusion criterion for patients was to be followed up at the CHF center at the Kuzbass Clinical Cardiology Center named after L.S. Barbarash. When maintaining the register, all requirements of the Federal Law of 27.07.2006 No. 152-FZ "On Personal Data" were met. During the analysis, all patient data were marked and anonymized.

Clinical data were recorded in a specially designed patented electronic form when entered in the register and then at regular intervals during follow-up from medical and outpatient records. Basic information about patients included demographic data, social status, history of the underlying disease, concomitant diseases, vital signs, clinical, instrumental and laboratory parameters, doses of cardiovascular drugs, and follow-up diaries. Baseline socio-demographic data were self-reported by patients. For newly enrolled patients, monthly follow-up checkpoints took place during the first 3 months. They involved telephone calls by a nurse who filled out follow-up diaries and, if necessary, in-person visits to the CHF center with an examination by a cardiologist. Then examinations were carried out every trimester.

The study included data of 802 patients included in the Kuzbass Register of Patients Followed up at the CHF Center from 2020 to 2022. The median follow-up was 1.6 ± 0.7 years.

Statistical analysis was carried out using the Statistica 10.0 (Statsoft, USA) and SPSS 11 software packages. Normality of distribution was assessed using the Kolmogorov – Smirnov test. Quantitative variables were presented as the mean and the standard deviation $M \pm \sigma$. Continuous variables with normal distribution were compared using the Student's *t*-test. To compare continuous variables with non-normal distribution, the nonparametric Mann – Whitney *U*-test was used. To compare three or more quantitative variables, the rank-based Kruskal – Wallis *H*-test was applied, followed by a pairwise comparison of groups using the Mann – Whitney test with the Bonferroni correction to estimate the *p* value. Discrete variables were compared using the χ^2 test with the Yates' continuity correction. If one of the compared groups was small, the two-tailed Fisher's test (F test) was

used. The differences were considered statistically significant at two-tailed $p < 0.05$.

RESULTS

According to the register, men prevailed in the gender profile of patients with CHF – 612 (76.3%) men, 190 women (23.7%), $p < 0.001$. The average age of the participants was 63.5 ± 11.2 years; men were younger than women: 62 ± 10.9 years and 67.5 ± 11.8 years, respectively, $p < 0.05$. The analysis of the place of residence showed that 626 (78.1%) patients were urban residents, and 176 (21.9%) participants were rural residents. The distribution of patients with a confirmed diagnosis of CHF by gender and age is shown in Figure 1.

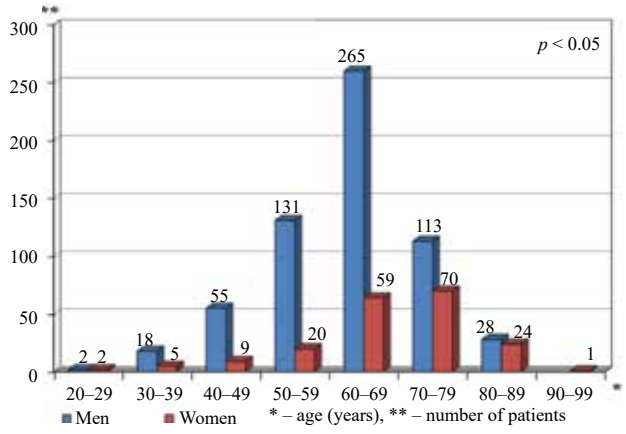

Fig. 1. Gender and age characteristics of patients with CHF

According to echocardiography findings, the average LVEF measured by the Simpson method was 37 ± 15.4% in the general sample. Patients with LVEF < 40% prevailed (Fig. 2).

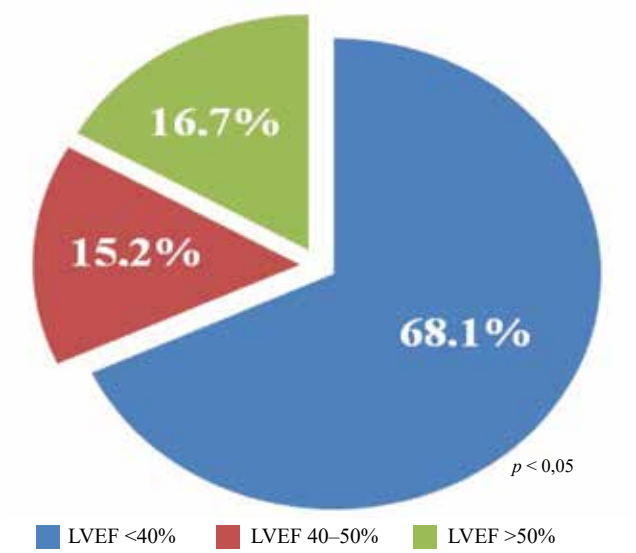

Fig. 2. Distribution of patients depending on LVEF: HFrEF – heart failure with reduced ejection fraction, HFmEF – heart failure with midrange ejection fraction, HFpEF – heart failure with preserved ejection fraction, *n* – number of patients, *p* – statistical significance

Distribution of the patients by stages and severity of CHF (NYHA functional class) depending on LVEF is presented in Table 1. The largest group was represented by patients with reduced LVEF (less than 40%) – 546 people. This category was also characterized by a more advanced functional class of CHF; patients with NYHA III–IV CHF prevailed, $p < 0.001$.

Peculiarities of gender distribution depending on the HF phenotype were identified. Thus, in the group with reduced LVEF, male patients prevailed; in the groups with midrange and preserved LVEF, there were more women.

Table 1

Stages and NYHA functional class of chronic heart failure depending on the phenotype, *n* (%)

Parameter	HFrEF (< 40%), *n* = 546 (%)	HFmEF (40–49%), *n* = 122 (%)	HFpEF (≥ 50%), *n* = 134 (%)	χ^2; *p*
Stage by the Strazhesko – Vasilenko classification				
Stage I CHF	5 (0.9)	18 (14.7)	74 (55.2)	299.431; <0.001
Stage IIA CHF	359 (65.8)	69 (56.5)	43 (32.1)	50.578; <0.001
Stage IIB CHF	182 (33.3)	35 (28.7)	17 (12.7)	22.213; <0.001
NYHA functional class				
NYHA I	–	–	5 (3.7)	25.082; <0.001
NYHA II	59 (10.8)	33 (27.0)	106 (79.1)	270.366; <0.001
NYHA III	379 (69.4)	39 (32.0)	19 (14.2)	161.800; <0.001
NYHA IV	108 (19.8)	50 (41.0)	4 (3.0)	57.389; <0.001

The analysis of comorbid pathology in the studied patients revealed that the most common were chronic kidney disease (CKD) (glomerular filtration rate of less than 60 ml / min / 1.73 m² according to the CKD-EPI equation) – 614 (76.5%) patients and obesity (body mass index of more than 30 kg / m^2) – 334 (41.6%) patients. Type 2 diabetes mellitus was reported in 193 (24%) patients.

The analysis of the dependence of comorbidity on the CHF phenotype showed that in patients with reduced LVEF, CKD predominated; as expected, such patients more often had an implantable cardioverter – defibrillator (ICD) implanted. In patients with preserved LVEF, diabetes and chronic obstructive pulmonary tuberculosis (COPD) were more common, and in the group with midrange LVEF – prior stroke and anemia (Table 2).

Table 2

Anamnestic factors in patients with CHF depending on the disease phenotype, *n* (%)

Parameter	HFrEF (< 40%), *n* = 546 (%)	HFmEF (40–49%), *n* = 122 (%)	HFpEF (≥ 50%), *n* = 134 (%)	χ^2; *p*
Men	459 (84.0)	84 (68.8)	69 (51.5)	67.573; <0.001
Type 2 diabetes mellitus	112 (20.5)	39 (32.0)	42 (31.3)	8.806; 0.013
Obesity	223 (40.8)	62 (50.8)	49 (36.5)	5.792; 0.056
Prior stroke	74 (13.5)	31 (25.4)	24 (17.9)	10.783; 0.05
COPD	18 (3.3)	5 (4.1)	12 (8.9)	8.279; 0.016
CKD (≥Stage 3A)	482 (88.2)	60 (49.1)	72 (53.7)	131.652; <0.001
Anemia	42 (7.6)	31 (25.4)	10 (7.4)	35.183; <0.001
Implanted ICD	69 (12.6)	7 (5.7)	2 (1.5)	17.828; <0.001

The analysis of the etiology of CHF showed that more than half of the patients – 452 (56.3%) were diagnosed with coronary heart disease (CHD). It is worth noting that all patients with CHD in the study had arterial hypertension, and, therefore, these pathologies were considered together and did not belong to the "two or more causes" group. Among patients with CHD, 387 (85.6%) people suffered myocardial infarction, and 65 (14.4%) people were diagnosed with ischemic cardiomyopathy. Only in 4 (0.5%) patients, the cause of CHF development was isolated hypertension. Dilated cardiomyopathy (DCM) was the cause of CHF in 70 (8.7%) patients, atrial fibrillation or flutter – in 36 (4.5%) patients, previous myocarditis – in 13 (1.6%) cases, valvular defects – in 8 (1.0%) patients. In almost every fourth patient (219 (27.4%) cases), the cause of CHF was a combination of two or more pathologies, while coexisting CHD (angina and / or post-infarction cardiosclerosis) and persistent or permanent forms of atrial fibrillation were the most common comorbidities in 182 (83.1%) patients.

The analysis of the etiology of CHF depending on the disease phenotype showed that the main causes of heart failure in the groups with reduced and midrange LVEF were CHD and combined causes, while in the group with preserved LVEF, CHF mostly resulted from CHD and arrhythmogenic causes.

Changes in the drug therapy received by the patients prior to and during the follow-up at the CHF center are presented in Table 4.

Table 3

Etiology of CHF depending on the disease phenotype, *n* (%)

Parameter	HFrEF (< 40%), *n* = 546 (%)	HFmEF (40–49%), *n* = 122 (%)	HFpEF (≥ 50%), *n* = 134 (%)	χ^2; p
CHD	315 (57.7)	44 (36.1)	93 (69.4)	30.092; <0.001
PICS, *n* (% – from CHD)	297 (94.3)	37 (84.1)	53 (57.0)	28.012; <0.001
ICM, *n* (% – from CHD)	18 (5.7)	7 (15.9)	40 (43.0)	102.946; <0.001
Arrhythmogenic	17 (3.1)	5 (4.1)	14 (10.4)	13.550; 0.002
DCM	39 (7.1)	23 (18.9)	8 (6.0)	18.699; <0.001
Valvular defects	5 (0.9)	3 (2.5)	–	4.026; 0.134
Postmyocardial	7 (1.3)	4 (3.2)	2 (1.5)	2.510; 0.26
Hypertension	–	–	4 (3.0)	20.040; <0.001
Two and more causes	163 (29.9)	43 (35.2)	13 (9.7)	26.581; <0.001

Note. PICS – post-infarction cardiosclerosis, ICM – ischemic cardiomyopathy.

Table 4

Frequency of prescription of drug therapy, *n* (%)			
Parameter	Drug therapy prior to the follow-up at the CHF center, *n* = 802	Drug therapy during the follow-up at the CHF center, *n* = 802	χ^2; *p*
ACEI	459 (57.2)	573 (71.4)	35.313; <0.001
ARB	79 (9.8)	98 (12.2)	2.293; 0.130
ARNI	30 (3.7)	124 (15.4)	63.470; <0.001
BAB	652 (81.3)	706 (88.0)	14.001; <0.001
Statins	586 (73.1)	654 (81.5)	16.432; <0.001
MCRA	468 (58.4)	493 (61.4)	1.622; 0.203
Diuretics	540 (67.3)	681 (84.9)	68.191; <0.001
Antiarrhythmic drugs	139 (17.3)	172 (21.4)	4.344; 0.038
Antiplatelets	406 (50.6)	504 (62.8)	24.391; <0.001
OAC	117 (14.6)	166 (20.6)	10.302; 0.002
CCB	290 (36.2)	361 (45.0)	13.033; <0.001
SGLT2i	64 (7.9)	417 (52.0)	370.023; <0.001

Note. ACEI – angiotensin-converting enzyme inhibitor, ARB – angiotensin receptor blocker, ARNI – angiotensin receptor – neprilysin inhibitor, BAB – beta-adrenergic blocker, MCRA – mineralocorticoid receptor antagonist, OAC – oral anticoagulant, CCB – calcium channel blocker, SGLT2i – sodium – glucose cotransporter 2 inhibitors.

Prior to the follow-up at the CHF center, only 30% of patients were followed up by a cardiologist. No more than 46% of patients with HFrEF received optimal drug therapy, which involved the simultaneous use of renin – angiotensin – aldosterone system (RAAS) inhibitors, BAB, and MCRA. Only in 3% of cases, dose titration to the target dose was performed. As seen from Table 4, the follow-up at the CHF center led to increased adherence to therapy with RAAS inhibitors, SGLT2i, diuretics, antiarrhythmic drugs, and OAC. Still, an unsatisfactory number of patients adhered to optimal drug therapy.

DISCUSSION

The analysis of the data obtained revealed that men prevailed among patients with CHF, and the men / women ratio was almost 3:1. However, according to the results of the EPOCHA-Decompensation-CHF study, there were significantly more women participating in the study than men: 56.8 and 43.2%, respectively, $p = 0.001$ [6]. Therefore, the issue of gender distribution in CHF remains open. In a number of studies, researchers tried to answer the question of whether gender inequality was associated only with the prevalence of male patients or whether there were other reasons for this phenomenon [8–10]. It was noted that a smaller number of women may be due to the predominance of females in the cohort of patients with HFpEF, better subjective and clinical tolerability of CHF, and, therefore, low demand for medical care and low detection of CHF [11].

The data obtained in the present study partially support this theory. Thus, in the group with HFrEF, men prevailed, and in the group with HFpEF, women predominated. The analysis of age-related characteristics, as expected, showed that the number of patients with CHF naturally increased with age, reaching a maximum in the age group of 60–69 years ($p < 0.05$). Further, after 69 years of age, a sharp decrease in the number of male patients was registered, which was associated with a natural population decline. For female patients, this trend was observed only starting from 80 years of age – due to longer life expectancy. Early incidence of CHF in men was explained by the manifestation of CHD as the main cause of CHF at a younger age compared to women [12]. Therefore, the data obtained once again emphasize the importance of measures for primary and secondary prevention of CHD, including from the standpoint of reducing CHF morbidity and mortality.

It is important that about 70% of patients with CHF are elderly – this category of patients is characterized by the presence of multiple comorbid pathologies, which significantly aggravates the course of CHF and may be a contraindication to modern high-tech treatment methods, such as implantation of devices and heart transplantation [13–15].

When analyzing the place of residence, it was shown that the vast majority of patients were urban residents.

The difference in the distribution of those studied between urban and rural residents was explained by lower availability of medical care for rural residents and the existence of only first aid stations in some territories. One way to solve this problem may be the on-site work of cardiology teams, which has already been implemented in Kuzbass.

The analysis of the distribution of CHF phenotypes showed that the predominant group, as expected, included patients with HFrEF, which corresponds to the data of other studies. Thus, according to various references, in the general population of patients with CHF, the prevalence of HFrEF is about 50% and HFmEF – 10–25% [16, 17]. Severe NYHA III–IV CHF is mainly characteristic of patients with HFrEF and HFmEF, while NYHA I–II CHF is more often detected in patients with HFpEF. The results show that identifying individuals with HFpEF remains a pressing issue. It is known that long-term HF without a clear clinical presentation and a lack of timely treatment may in the long term have a more significant negative impact on the prognosis than timely detected HFrEF [18–20].

CHD still remains the main cause of CHF, which is confirmed in the present study. It is largely due to previous myocardial infarction and ischemic cardiomyopathy that a group of patients with HFrEF is formed [21–23]. Almost always (100% according to the data of this study), CHD is accompanied by hypertension, whose contribution to the development of CHF should not be underestimated. Coexisting CHD and hypertension were the etiological cause of CHF in the majority of patients (56.3%), which is in line with the results of studies conducted in other countries. An epidemiological study conducted in the Republic of Belarus found that coexisting CHD and hypertension were the cause of CHF development in 65.5% of those studied [25]. The small number of studied patients in the hypertension group of CHF etiology may be explained by outpatient follow-up of these patients by internal medicine services, without follow-up at the CHF center.

Analyzing the features of drug treatment, the positive dynamics during the follow-up at the CHF center is worth noting. As the results show, one of the important advantages of such follow-up is increased compliance of prescribed therapy with existing clinical guidelines and patient adherence. Identification of patients with HFrEF who adhere to optimal drug therapy, based on registry data, makes it possible to optimize compilation of waiting lists for such types of high-tech care as ICD implantation and orthotopic heart transplantation.

CONCLUSION

Assessing the clinical and anamnestic features of patients with heart failure followed up at the Kuzbass CHF center, it can be said that these people are mainly male, retired, with coronary heart disease, low left ventricular ejection fraction, and a comorbidity, mainly chronic kidney disease, diabetes mellitus, and obesity. This cohort of patients requires specific follow-up and management strategies aimed at increasing adherence, timely revascularization, social support, dynamic remote monitoring, and a multidisciplinary approach.

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Authors' contribution

Parfenov P.G. – conception and design, supervision of the study, statistical processing of the data, drafting of the manuscript. Yurkina A.V., Golubovskaya D.P., Shuster S.Y., Dren E.V. – compilation of the database, statistical processing of the data. Guselnikova Y.I. – review of literature. Lebedeva N.B., Pecherina T.B. – conception and design, supervision of the study, final approval of the manuscript for publication.

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Application of Charlson Comorbidity Index to assess prognosis of 18-month mortality in patients with acute myocardial infarction

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ABSTRACT

Aim. To evaluate the prognostic value of the Charlson Comorbidity Index (CCI) for predicting 18-month all-cause mortality and develop a nomogram for predicting 18-month mortality in acute myocardial infarction (MI) patients.

Materials and methods. The prospective, single-center, observational study included 712 consecutive patients with acute MI undergoing coronary angiography within 24 hours after hospitalization. The primary endpoint was 18-month all-cause mortality. The logistic regression analysis was adopted to identify independent prognostic factors. A nomogram for predicting the endpoint was developed using the multivariate analysis. The discriminative ability of the CCI and a nomogram were evaluated using the receiver-operating characteristic (ROC) curve analysis.

Results. Of the patients, 61% were male, median age was 65 years (interquartile range (IQR) was 56–74years). Median CCI was 4 (IQR: 3–6) points. The mortality rate was 12.1% at 18 months with the area under the curve (AUC) of 0.797 for CCI (95% confidence interval (CI): 0.746–0.849; $p < 0.001$). The multivariate analysis revealed that CCI (odds ratio (OR) 1.28; 95% CI 1.08–1.52; $p = 0.004$), age (OR 1.06; 95% CI 1.02–1.09; $p = 0.002$), and three-vessel coronary artery disease (OR 2.60; 95% CI 1.36–4.98; $p = 0.004$), incorporated into the nomogram, were independent predictive factors of an adverse outcome. The nomogram showed good discrimination in predicting 18-month mortality in patients with acute MI (AUC = 0.819; 95% CI: 0.767–0.870; $p < 0.001$; sensitivity 65.1%; specificity 88.2%.).

Conclusion. CCI was independently associated with and moderately predicted 18-month mortality in patients with acute MI. The proposed nomogram facilitated early identification of high-risk patients, allowing for the implementation of more effective treatment strategies and reducing acute MI mortality.

Keywords: Charlson Comorbidity Index, comorbidity, mortality, myocardial infarction, nomogram

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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Conformity with the principles of ethics. All patients signed an informed consent to participate in the study. The study was approved by the Ethics Committee at the Institute of Medicine of RUDN University.

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Применение индекса коморбидности Чарльсона для оценки прогноза 18-месячной смертности у пациентов с острым инфарктом миокарда

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РЕЗЮМЕ

Цель. Оценить прогностическую способность индекса коморбидности Чарльсона (ИКЧ) для прогнозирования 18-месячной смертностиу пациентов с острым инфарктом миокарда (ИМ) с применением номограммы, разработанной на основе математического анализа.

Материалы и методы. В проспективное одноцентровое наблюдательное исследование были включены 712 последовательных пациентов с острым ИМ, которым выполняли коронарографию в течение 24 ч с момента госпитализации. Первичной конечной точкой исследования была принята смерть от всех причин в течение 18 мес наблюдения. Для выявления независимых прогностических факторов риска наступления смерти применялся логистический регрессионный анализ. На основе результатов многофакторного анализа была разработана номограмма для прогнозирования клинического исхода. Дискриминационная способность ИКЧ и номограммы была оценена с помощью логистической регрессии, в качестве инструмента оценки его диагностической способности применялся метод ROC-анализа (ROC-анализ).

Результаты. Среди пациентов доминировали мужчины (61%), медиана возраста составила 65 лет (интерквартильный размах [ИКР] 56–74 года). Медиана ИКЧ составила 4 (ИКР: 3–6) балла. Смертность в течение 18 мес составила 12,1%, с площадью под ROC-кривой для ИКЧ 0,797 (95%-й доверительный интервал [ДИ] 0,746–0,849; $p < 0,001$). Многофакторный анализ показал, что ИКЧ (отношение шансов [ОШ] 1,28; 95%-й ДИ 1,08–1,52; $p = 0,004$), возраст (ОШ 1,06; 95%-й ДИ 1,02–1,09; $p = 0,002$), трехсосудистое поражение коронарных артерий (ОШ 2,60; 95%-й ДИ 1,36–4,98; $p = 0,004$), включенные в номограмму, были независимыми предиктивными факторами неблагоприятного клинического исхода. Номограмма продемонстрировала хорошую дискриминационную способность прогнозирования 18-месячной смертности у пациентов с острым ИМ (площадь под ROC-кривой 0,819; 95%-й ДИ 0,767–0,870; $p < 0,001$; чувствительность 65,1%; специфичность 88,2%).

Заключение. ИКЧ независимо ассоциировался и умеренно предсказывал смертность в течение 18 мес у пациентов с острым ИМ. Предложенная номограмма облегчила раннюю идентификацию пациентов с высоким риском событий, что позволило внедрить более эффективные стратегии лечения и снизить смертность при остром ИМ.

Ключевые слова: индекс коморбидности Чарльсона, инфаркт миокарда, коморбидность, смертность, номограмма

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 комитетом по этике Медицинского института РУДН.

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INTRODUCTION

Cardiovascular diseases (CVDs) are the most common cause of mortality worldwide, which substantially contributes to loss of good health and excessive healthcare costs [1, 2]. Acute myocardial infarction (MI) is one of the most common causes of death from CVDs. Despite improvements in the diagnostic, treatment, and preventive strategies, CVD remains the main cause of death in Europe, with coronary artery disease (CAD) being the most common cause of CVD mortality [2]. Growing aging population has resulted in higher prevalence of comorbidities, particularly among patients with acute MI [3] and is associated with an increased risk of mortality and future cardiovascular events [4, 5]. Therefore, determining key risk factors and implementing appropriate clinical recommendations can make a significant contribution to saving the lives of individuals with acute MI and comorbidities.

The Charlson Comorbidity Index (CCI) is a well-established surrogate marker of comorbidity, validated to predict a risk of adverse outcomes in patients with acute MI [6]. Despite frequent presence of comorbidities in MI patients, their role in the long-term prognosis after acute MI has been poorly studied.

The aim of the study was to evaluate the prognostic value of CCI for predicting 18-month all-cause mortality and develop a nomogram for predicting 18-month mortality in patients with acute MI.

MATERIALS AND METHODS

The present study is a prospective, single-center, observational study. It included all consecutive patients aged > 18 years admitted with acute MI to Vinogradov City Clinical Hospital (Moscow, Russia) from January 2017 to December 2018. The patients underwent coronary angiography (CAG) within 24 hours after hospitalization. The exclusion criteria were type 3, 4, or 5 MI. The diagnosis of acute MI was made according to the Third universal definition of MI [7]. All patients signed an informed consent to participate in the study. The study was approved by the local Ethics Committee at the Institute of Medicine, Peoples' Friendship University of Russia, and complied with the Declaration of Helsinki.

Data collection and clinical outcomes. Variables of interest included clinical characteristics, cardiovascular risk factors, comorbidities, physical examination findings, blood test results, and imaging data (electrocardiography, echocardiography, and CAG). Cardiac troponin I levels were measured using the Access 2 Immunoassay System (Beckman Coulter, USA), with 99th percentile upper reference limit being 0.02 ng / l. The CCI was calculated using baseline data by summing all comorbidity scores (available online) [8, 9]. The Global Registry of Acute Coronary Events (GRACE) 2.0 score was used to stratify risks for MI patients [10]. Anemia was defined as a hemoglobin concentration of less than 120 g / l for men or less than 130 g / l for women [11]. Multivessel CAD was defined as the presence of ≥ 70 % stenosis in two or more major coronary arteries of ≥ 2.5 mm in diameter detected during direct subtraction angiography [12].

The primary endpoint was 18-month all-cause mortality, both in-hospital and out-of-hospital, that was recorded in patient electronic medical records and death registers.

Statistical analysis. Statistical analysis was performed using the IBM SPSS Statistics 25.0 (SPSS Inc., Chicago, IL, USA) and R software (version 3.6.3) software. Quantitative variables were presented as the mean and the standard deviation for normally distributed data or as the median (*Me*) and the interquartile range (IQR) for non-normally distributed data. Qualitative variables were presented as absolute values and percentages. Categorical variables were compared using the Chi-square and Fisher's exact tests, while continuous variables were compared by the unpaired Student *t*-test and Mann – Whitney *U*-test. The logistic regression analysis was used to identify factors associated with 18-month

mortality. The odds ratios (OR) and 95% confidence intervals (CI) were presented. Based on the estimated factors from the multivariate logistic regression model, a nomogram was constructed to assess the risk of 18-month death (rms package in R). The ROC analysis was used to assess the discriminating ability of the CCI and the nomogram in relation to 18-month mortality [13]. A two-tailed *p* value of 0.05 was chosen as a threshold for statistical significance for all tests.

RESULTS

A total of 712 consecutive patients were included in the study; 434 patients (61%) were male. The median age of patients was 65 (IQR: 56–74) years; 47.8% of patients were hospitalized with ST elevation. The proportion of patients < 44 years, ≥ 45 and < 59 years, ≥ 60 and < 74 years, ≥ 75 and < 90 and ≥ 90 years was 8.84%, 5.8%, 28.1%, 42.6%, 23%, and 0.6%, respectively. During the follow-up period, 86 patients (12.1%) died.

The baseline characteristics of the patients are shown in Table 1. In the group of diseased patients, more elderly patients, female gender, ST elevation, arterial hypertension, CAD, prior MI, prior heart failure (HF), prior stroke, atrial fibrillation, chronic kidney disease (CKD), anemia, Killip class II–IV acute heart failure, three-vessel CAD, and higher CCI and GRACE scores were more common. Values for systolic blood pressure (BP), hemoglobin, creatinine, and left ventricular ejection fraction (LVEF) were lower in the group of deceased patients. There were no significant differences between the groups in prior myocardial revascularization, peripheral artery disease (PAD), chronic lung disease (asthma and / or chronic obstructive pulmonary disease), gastric and duodenal ulcer, troponin level, and the frequency of percutaneous coronary intervention (PCI).

The median CCI score was 4 (IQR: 3–6, range 0–13). The distribution of CCI scores in all the participants was shown in Fig.1. Among components of CCI, past history of MI was the most frequent comorbidity (21.8%) followed by diabetes mellitus (21.1%), chronic lung diseases (asthma and chronic obstructive pulmonary disease) (16.2%), dementia (9.3%), gastric and duodenal ulcer (9.1%), and CKD (8.6%) (Table 2).

Table 1

Basic characteristics of 712 patients with myocardial infarction

Parameter	Patient population, *n* = 712	Survived patients, *n* = 626	Deceased patients, *n* = 86	*p*
Age, years, *Me* (IQR)	65 (56; 74)	64 (55; 71.2)	76.5 (67.7; 83.2)	<0.001
Women, *n* (%)	278 (39)	227 (36.3)	51 (59.3)	<0.001
ST elevation, *n* (%)	340 (47.8)	289 (46.2)	51 (59.3)	0.022
Arterial hypertension, *n* (%)	634 (89)	552 (88.2)	82 (95.3)	0.046
CAD, *n* (%)	328 (46.1)	267 (42.7)	61 (70.9)	<0.001
Prior MI, *n* (%)	155 (21.8)	125 (20)	30 (34.9)	0.003
Prior myocardial revascularization, *n* (%)	85 (11.9)	77 (12.3)	8 (9.3)	0.483
Prior HF, *n* (%)	57 (8.0)	44 (7.0)	13 (15.1)	0.017
Diabetes, *n* (%)	150 (21.1)	125 (20)	25 (29.1)	0.066
Prior stroke, *n* (%)	51 (7.2)	36 (5.8)	15 (17.4)	<0.001
History of atrial fibrillation, *n* (%)	73 (10.3)	57 (9.1)	16 (18.6)	0.012
CKD, *n* (%)	61 (8.6)	44 (7.0)	17 (19.8)	<0.001
PAD, *n* (%)	26 (3.7%)	20 (3.2)	6 (7.0)	0.114
Chronic lung disease, *n* (%)	115 (16.2%)	95 (15.2)	20 (23.3)	0.062
Gastric and duodenal ulcer, *n* (%)	65 (9.1%)	57 (9.1)	8 (9.3)	1.0
CCI, score, *Me* (IQR)	4 (3; 6)	4 (3; 5)	6 (5; 8)	<0.001
Anemia, *n* (%)	189 (26.5)	145 (23.2)	44 (51.2)	<0.001
Chest pain, *n* (%)	658 (92.4)	583 (93.1)	75 (87.2)	0.078
Shortness of breath, *n* (%)	124 (17.4)	102 (16.3)	22 (25.6)	0.047
Killip class II–IV, HF *n* (%)	160 (22.5)	115 (18.4)	45 (52.3)	<0.001
Systolic blood pressure, mm Hg., *Me* (IQR)	140 (120; 159)	140 (120; 160)	130 (110; 150)	0.019

Table 1 (continued)

Parameter	Patient population, $n = 712$	Survived patients, $n = 626$	Deceased patients, $n = 86$	p
Diastolic blood pressure, mm Hg., *Me* (IQR)	80 (76; 89)	80 (77; 90)	80 (70; 80)	0.021
Troponin I, ng / ml, *Me* (IQR)	0.39 (0.09; 2.85)	0.39 (0.09; 2.86)	0.42 (0.09; 2.50)	0.996
Hemoglobin, g / l, *Me* (IQR)	136 (123; 147)	138 (125; 148)	122 (105; 136.5)	<0.001
Creatinine, mcmol / l, *Me* (IQR)	94 (80; 107)	69 (55; 84)	52.5 (41; 66.5)	0.014
LVEF, %, *Me* (IQR)	45 (40; 54)	45 (40; 54)	40 (34; 50)	<0.001
No lesion / stenosis of CA < 50%, *n* (%)	73 (10.3)	69 (11)	4 (4.7)	0.086
Three-vessel CAD, *n* (%)	390 (54.8)	320 (51.1)	70 (81.4)	<0.001
PCI, *n* (%)	566 (79.5)	499 (79.7)	67 (77.9)	0.671
GRACE, score, *Me* (IQR)	117 (98; 141)	113 (96; 134)	149 (128.5; 171.25)	<0.001

Note. AIDS – acquired immunodeficiency syndrome

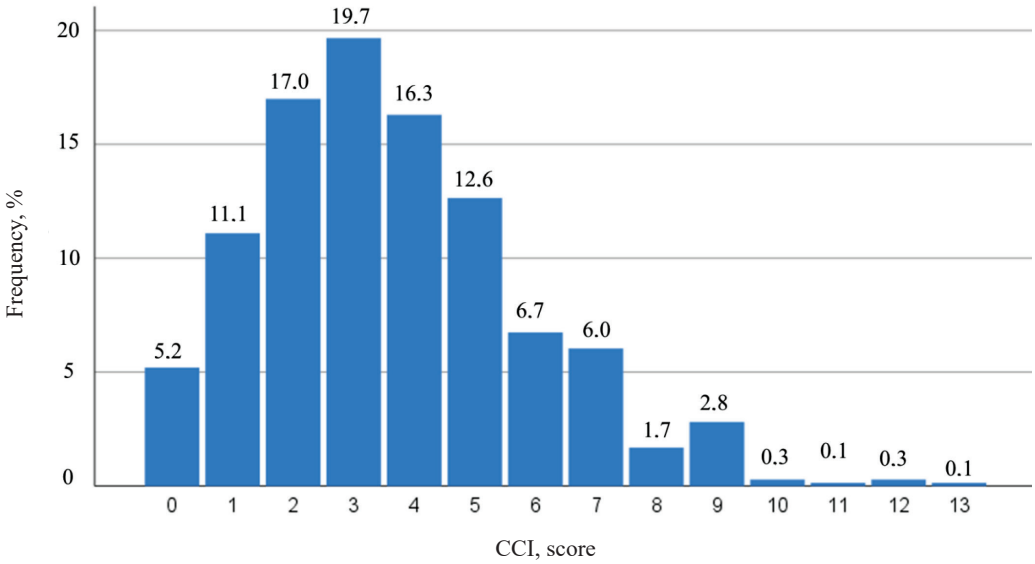

Fig. 1. Distribution of CCI scores among the patients

Table 2

Frequency of comorbidities for each CCI category		
Comorbidity	Number	Frequency
Prior MI	155	21.8
Diabetes (mild to moderate)	150	21.1
Chronic lung disease	115	16.2
Dementia	66	9.3
Gastric and duodenal ulcer	65	9.1
CKD	61	8.6
Prior hospitalization for HF	57	8.0
Stroke	51	7.2
Cancer	27	3.8
PAD	26	3.7
Liver disease	4	0.6
AIDS	1	0.1
Rheumatic disease	0	0
Hemiplegia or paraplegia	0	0

The univariate analysis showed that CCI score, age, female gender, ST elevation, systolic BP, diastolic BP, atrial fibrillation, anemia, Killip class HF, creatinine level, LVEF, and three-vessel CAD were associated with 18-month all-cause mortality ($p < 0.05$) (Table 3). The multivariate analysis revealed that CCI score (OR 1.28, 95% CI 1.08–1.52, $p = 0.004$), age (OR 1.06, 95% CI 1.02–1.09, $p = 0.002$), and three-vessel CAD (OR 2.60, 95%CI 1.36-–4.98, $p = 0.004$) were independently associated with the primary endpoint.

The analysis of ROC curves for CCI predicting 18-month all-cause mortality showed that the area under the curve (AUC) for CCI was 0.797 (95% CI: 0.746–0.849, $p < 0.001$). The sensitivity and

specificity were 69.8% and 78.4%, respectively with the cut-off CCI value being >5 points (Fig. 2).

Based on the estimated variables in the multivariate model, a nomogram was developed to predict the risk of 18-month mortality in patients with acute MI (AUC = 0.819, 95% CI: 0.767–0.870, $p < 0.001$, sensitivity 65.1%, specificity 88.2%) (Fig. 3). The risk of death within 18 months in patients with acute MI was assessed according to the following equation:

$$\text{Risk} = = {}^{1}/_{(1 + e^{-z})},$$

where Z = –7.984 + 0.341 x CCI + 0.051 x age + 0.967 x three-vessel CAD.

Instruction for use: a total score is summed from the score of each factor on the corresponding axis with drawing a vertical line to the "Points" axis. Summing scores of all factors and drawing a vertical line to the "Risk of 18-month mortality" line determine the individual's risk of death within 18 months.

Table 3

Univariate and multivariate analysis of the risk factors in predicting 18-month all-cause mortality

Parameter	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*p*	OR (95% CI)	*p*
CCI, score	1.65 (1.47–1.84)	<0.001	1.28 (1.08–1.52)	0.004
Age, years	1.09 (1.07–1.12)	<0.001	1.06 (1.02–1.09)	0.002
Women	2.56 (1.62–4.06)	<0.001	1.35 (0.73–2.51)	0.336
ST elevation MI	1.70 (1.07–2.69)	0.023	1.33 (0.76–2.34)	0.318
Systolic BP ≤ 115 mm Hg	2.37 (1.43–3.95)	0.001	2.84 (1.09–7.38)	0.032
Diastolic BP ≤ 70 mm Hg	1.88 (1.45–3.10)	0.012	2.14 (0.82–5.61)	0.121
History of atrial fibrillation	2.28 (1.24–4.19)	0.008	1.32 (0.64–2.75)	0.453
Anemia	3.47 (2.19–5.51)	<0.001	1.67 (0.93–2.99)	0.083
Killip class II–IV ≥ 2	2.67 (2.05–3.45)	<0.001	1.22 (0.66–2.26)	0.523
Creatinine level ≥ 115 mcmol / l	1.83 (1.08–3.08)	0.023	1.09 (0.55–2.13)	0.811
LVEF ≤ 40%	3.16 (1.93–5.17)	<0.001	1.79 (0.97–3.32)	0.063
Three-vessel CAD	4.18 (2.38–7.36)	<0.001	2.60 (1.36–4.98)	0.004

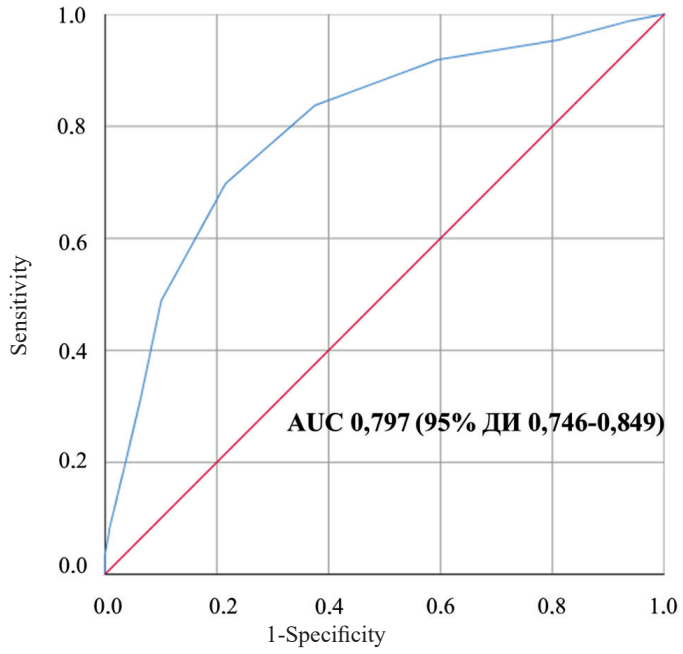

Fig. 2. The analysis of the ROC curve for CCI for predicting 18-month all-cause mortality in patients with acute MI

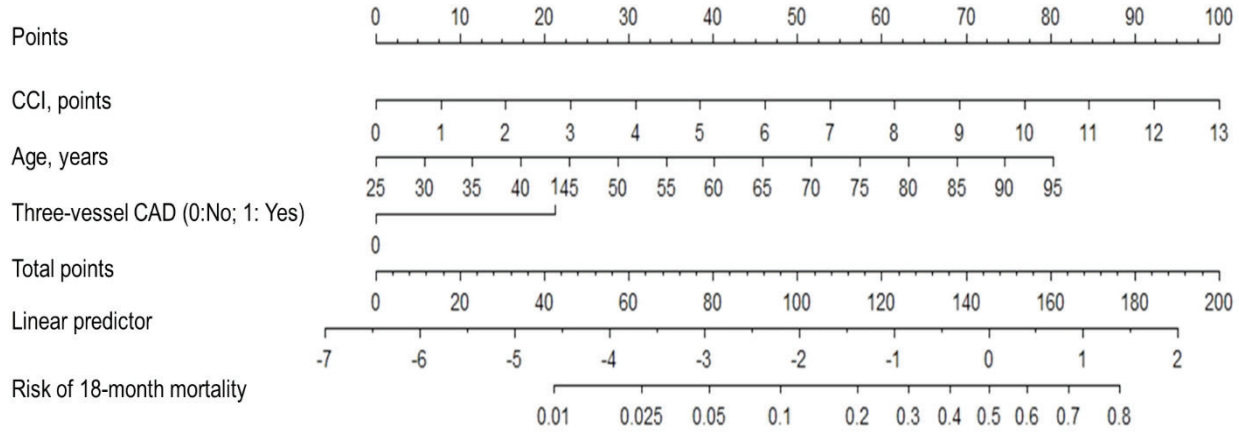

Fig. 3. Nomogram for predicting 18-month all-cause mortality in acute myocardial infarction: Charlson Comorbidity Index, age, and three-vessel coronary artery disease

DISCUSSION

The obtained results were used to build a predictive model for this population. In our study, male patients prevailed, which is consistent with the findings from previous studies [6, 14, 15]. Arterial hypertension had higher prevalence in our study (89%) than in the works by J. Sanchis et al. including 1,017 non ST-elevation acute coronary syndrome (NSTE-ACS) patients [16] and J.E. Núñez et al. in a cohort of 1,035 acute MI patients [17], where arterial hypertension accounted for 65 and 61.4% of the total population, respectively.

When analyzing the comorbidity profile in CCI, past history of MI (21.8%) and diabetes mellitus (21.1%) were the most frequent comorbidities, which is consistent with the results of the study by D. Radovanovic et al. including 30,711 patients with acute coronary syndrome (ACS) from 69 Swiss hospitals in the AMIS Plus registry [6]. In their study, prior MI and diabetes mellitus were reported in 18 and 14.7% of cases, which is concordant with the study by M. Hautamäki involving 1,576 patients in the MADDEC (Mass Data in Detection and Prevention of Serious Adverse Events in Cardiovascular Disease) study, where prior MI accounted for 22%. In contrast to the findings obtained by J.E. Núñez et al. [17], diabetes mellitus without target organ damage (21.5%) was the most common CCI category, followed by a history of acute MI (17.6%), chronic obstructive pulmonary disease (8.6%), stroke (6.6%), HF (6.4%), PAD (5.5%), and kidney disease (4.1%).

Previous studies have confirmed the positive correlation between CCI and adverse outcomes in patients with MI [3, 17–19]. In a prospective, multicenter, observational study involving 29,620 acute MI patients, 46.8% had comorbidities [6]. CCI was an independent predictor of in-hospital mortality: CCI = 1 had OR of 1.36 (95% CI 1.16–1.60; $p = 0.001$), CCI = 2 had OR of 1.65 (95% CI 1.38–1.97; $p < 0.001$), and CCI ≥ 3 had OR of 2.20 (95% CI 1.86–2.57; $p < 0.001$). With a combination of CCI, age, and gender in the ROC analysis, the AUC was 0.761 (95% CI 0.74–-0.773) for predicting in-hospital mortality. In contrast, using CCI alone yielded the AUC of 0.670 (95% CI 0.656–0.685) for in-hospital mortality. After adjustments for age, the AUC improved to 0.83 (95% CI 0.80–0.86) for predicting 12-month mortality.

M. Schmidt et al. examined the association between comorbidity and mortality in 234,331 patients with first-time hospitalization for MI from 1984 through 2008 [3]. The authors reported an association between comorbidity and 30-day mortality (adjusted hazard ratio (aHR) 1.35 (95% CI 1.26–1.45) for moderate (CCI = 1) and 1.96 (95% CI 1.83–2.11) for very severe comorbidity (CCI ≥ 3)), as well as 12-month mortality (aHR 1.83 (95% CI 1.68–2.00) for moderate and 3.89 (95% CI 3.58–4.24) for severe comorbidity). In another study [19], the overall aHR for all-cause mortality was 1.39 (95% CI 0.90–2.14) and 2.33 (95% CI: 0.79–6.84) for mild comorbidity, 2.05 (95% CI: 0.69–6.06) for moderate comorbidity, and 1.07 (95% CI: 0.64–1.80) for severe comorbidity. In a study involving 1,035 consecutive acute MI patients, J.E. Núñez et al. found that a higher CCI score independently predicted mortality or acute MI within 30 days and one year [17].

The analysis of factors associated with fatal outcomes revealed that CCI, age, and three-vessel CAD were independent predictors of 18-month mortality in patients with acute MI. In our study, the proportion of the elderly accounted for two-thirds of all patients. The clinical significance of CCI in this patient cohort was demonstrated in previous studies [20–22]. Given that comorbidity burden increases with age, particularly in elderly patients who become more vulnerable and frailer, comorbidity indices become more significant for this age group [23].

In a prospective, cohort study including 520 elderly (≥ 80 years) patients hospitalized with NSTE-ACS, CCI was independently associated with mortality or readmissions (HR 1.15, 95% CI (1.06–1.26); $p = 0.001$) within 6 months of follow-up [21]. In another prospective, cohort study involving 715 NSTE-ACS patients, CCI was a prognostic factor of readmission for HF after 2-year follow-up (OR = 1.2 (95%CI: 1.04–1.3) [24].

The severity of CAD detected by CAG also has a prognostic value in acute MI [25]. The prevalence of significant multi-vessel CAD was common in patient with acute MI, both with NSTE-ACS and ST elevation ACS, and accounted for 40–50% of cases [26–28]. Three-vessel CAD served as a prognostic factor within the CADILLAC risk score (the Controlled Abciximab and Device Investigation to Lower Late Angioplasty Complications), predicting 30-day and 12-month mortality for acute MI after PCI [29]. This score integrated age, LEEF, renal insufficiency, three-vessel CAD, and post-procedural Thrombolysis in Myocardial Infarction (TIMI) flow.

For risk stratification after acute MI, CCI was incorporated in several scores for predicting adverse outcomes [30, 31]. In a study of 1,202 ACS patients, the addition of CCI to the GRACE score improved the prediction of future cardiovascular events and mortality [32], while CCI was shown to be one of the strongest predictors of non-cardiovascular mortality in patients undergoing PCI [33]. The results from the National Readmissions Database revealed that CCI ≥ 3 was the foremost predictor of 30-day readmission among patients with NSTE-ACS [34]. The current analysis calls attention to the synergistic prognostic impact of both comorbidities and angiographic variables that are potentially modifiable or that require specific intervention.

Our study has several limitations. Firstly, it is an observational study, and inherent limitations like non-randomization and unmeasured confounding factors cannot be eliminated. However, well-designed observational studies can still provide valid results without systematically overestimating outcomes, compared to randomized controlled trials. Secondly, a small sample size, single-center nature of the study, and a lack of nomogram validation reduce the power of our model for clinical implementation. Thirdly, CCI was designed over 30 years ago, and clinical definitions of certain conditions, like CKD, have evolved significantly. This may make it impractical to accurately assess their impact on comorbidity burden and prognosis using the traditional CCI format.Top of Form

CONCLUSION

CCI demonstrated a moderate predictive capacity for 18-month mortality in acute MI patients. Age, CCI, and three-vessel CAD were independently associated with 18-month mortality. The nomogram-based risk assessment model facilitated early identification of high-risk patients, allowing for the implementation of more effective treatment strategies and reducing acute MI mortality.

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Molecular targets for metastasis-directed therapy in malignant tumors

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ABSTRACT

Over the past two decades, targeted therapy has actively developed and, demonstrating impressive clinical results, has gained an increasingly important role in the treatment of cancer. This was facilitated to a large extent by an in-depth understanding of the mechanisms of cancer development, and mainly, the discovery of molecular targets. Despite the fact that targeted therapy can radically change the results of treatment and the prognosis of the disease course in some cancer cases, its effectiveness is sometimes replaced by drug resistance, in others.

The authors of the lecture analyzed and systematized therapeutic approaches to addressing a number of important molecular targets that are key for implementing a specific stage in human tumor pathogenesis. These include maintaining chronic proliferative signaling, promoting evasion of cell growth suppressors, inducing angiogenesis, forming immune surveillance, and activating invasion and metastasis. The lecture presented targeted therapy drugs used in the Russian Federation, including antibody-based drugs and small molecule tyrosine kinase inhibitors. It also analyzed mechanisms of molecular interaction between these drugs and their targets, as well as possible factors for developing resistance and ways to overcome these resistance mechanisms.

Keywords: EGFR, HER2, VEGF, BCR-ABL1, CDK4/6, CTLA-4, PD-1, c-Met

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Молекулярные мишени таргетной терапии злокачественных новообразований

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РЕЗЮМЕ

В течение последних двух десятилетий таргетная терапия активно развивается и, демонстрируя впечатляющие клинические результаты, завоевывает все большую роль в терапии онкологических заболеваний. В значительной мере этому способствовало углубленное понимание механизмов развития рака и главным образом открытие молекулярных мишеней. Однако таргетная терапия способна радикально изменять результаты лечения и прогнозы течения заболевания в одних онкологических контекстах, в других же эффективность сменяется лекарственной устойчивостью.

В лекции проанализированы и систематизированы терапевтические подходы нацеливания на ряд важнейших молекулярных мишеней, которые являются ключевыми для осуществления конкретного этапа в многостадийном процессе патогенеза опухолей человека: поддерживающих хроническую пролиферативную передачу сигналов, способствующих уклонению от супрессоров клеточного роста, обеспечивающих индукцию ангиогенеза, формирующих иммунный надзор и активирующих инвазию и метастазирование. Представлены применяемые в России таргетные терапевтические препараты на основе антител и низкомолекулярные ингибиторы тирозинкиназ, проанализированы механизмы молекулярного взаимодействия препаратов и мишеней, а также возможные факторы развития резистентности и способы преодоления резистентных механизмов.

Ключевые слова: EGFR, HER2, VEGF, BCR-ABL1, CDK4/6, CTLA-4, PD-1, c-Met

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

Targeted therapy is a novel and most promising method for drug treatment of malignant tumors. Over the past two decades, in-depth investigation of cancer development mechanisms, including the discovery of molecular targets, has contributed to the impetuous development of targeted medicine. As per the hypothesis proposed by Hanahan and Weinberg, the primary hallmarks of cancer include maintenance of proliferative signaling, evasion of cell growth suppressors, induction of angiogenesis, evasion of immune surveillance, activation of invasion and metastasis, and resistance to cell death. Tumor cell genome mutation is defined as a fundamental feature that initiates malignant transformation [1, 2].

Currently, oncologists have a wide range of targeted therapeutic agents in their arsenal. These drugs are directed at molecular targets that are key to a definite stage in human tumor pathogenesis. Generally, targeted therapy is based on the use of low-molecular-weight tyrosine kinase inhibitors (ending in the suffix -ib) (Table 1) and monoclonal antibodies (ending in the suffix -mab) (Table 2). Tumor targets can represent receptor tyrosine kinases and their ligands (Figure).

Receptor tyrosine kinases (RTKs) are transmembrane proteins consisting of three parts, namely an extracellular domain that functions as a receptor for site-specific binding to ligands, a transmembrane domain, and a catalytic intracellular tyrosine kinase domain. The tyrosine kinase domain provides phosphorylation of substrates (transferring the phosphate residue of adenosine triphosphate (ATP) to the tyrosine residue of specific cellular target proteins for serine or threonine kinases). Ligand binding to the tyrosine kinase receptor leads to constitutive

activation of downstream signaling pathways. Therefore, tyrosine kinase receptors transmit signals from extracellular ligands to downstream signaling effectors, involving mainly serine / threonine kinases or other important proteins, such as RAS (retrovirus-associated DNA sequences).

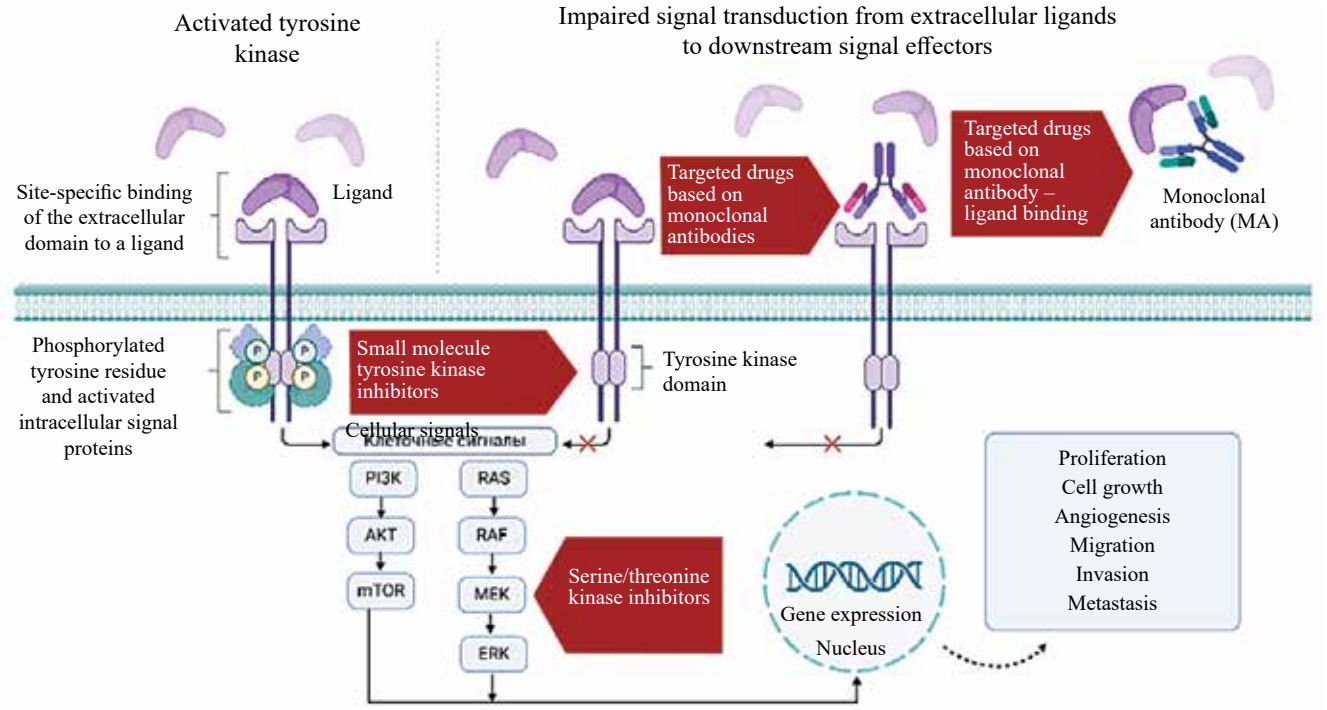

Figure. Methods of targeted therapy

Many kinases, being regulators of normal cellular processes in a healthy organism, such as angiogenesis, proliferation, differentiation, survival, and migration, are also crucial for cancer formation and progression. As a result of an oncogenic mutation or translocation, target kinase proteins can be overexpressed, intercept downstream signaling pathways, be resistant to regulatory mechanisms, and perform functions that promote tumor formation and growth. Both well-established in clinical practice and novel targeted drugs are directed at targets that are critical to the pathogenesis of tumor development.

Tyrosine kinase inhibitors (TKIs) can function by competing for ATP in the ATP binding pocket of the kinase in an active (TKI type I) or inactive conformation (TKI type II); perform interactions beyond the ATP binding site, causing allosteric inhibition of kinase activity (TKI type III); form an irreversible covalent bond with the active center of the kinase, most often by reacting with a nucleophilic cysteine residue (TKI type IV, irreversible inhibition) [3, 4]. TKIs have a cytostatic effect, leading to inhibition of tumor growth.

Monoclonal antibodies bind to the extracellular domain of the target receptor and act through a variety of mechanisms, including antibody-dependent cellular cytotoxicity, internalization followed by degradation, or inhibition of receptor dimerization [5, 6]. Monoclonal antibodies are divided into chimeric (-ximab), humanized (-zumab) or fully human (-umab). Monoclonal antibody-based drugs are used separately or in conjugation with a cytotoxic agent (trastuzumab emtansine, trastuzumab deruxtecan, brentuximab vedotin, enfortumab vedotin).

The aim of this lecture was both to analyze therapeutic approaches to directing a few important molecular targets of malignant neoplasms and to consider the problem of resistance to targeted drugs and strategies to overcome these resistance mechanisms. The material of the lecture would be of interest to researchers and specialists in the field of targeted therapy and oncology.

TARGETING CELL STRUCTURES THAT SUSTAIN CHRONIC PROLIFERATIVE SIGNALING

Normal cells require mitogenic growth signals mediated by transmembrane receptors to transfer from a quiescent state to a phase of active proliferation. On the contrary, tumor cells are capable of autonomously maintaining chronic proliferation. Sustained proliferation is carried out by increasing the amount of growth factor ligands, increasing the expression of receptor proteins on the surface of tumor cells, stabilization of the receptor in the dimeric state, and activation in the absence of ligand.

Epidermal growth factor (EGF) and its receptors with tyrosine kinase activity, members of the ErbB family: EGFR (HER1, ErbB-1), HER2/c-neu (ErbB-2), and Her 3 (ErbB-3), are common and well understood growth factors. ErbB family receptors ensure proliferation, survival, and differentiation of normal cells, while they are overexpressed in a variety of malignancies. Thus, EGFR (HER1) overexpression is found in non-small cell lung cancer, disseminated prostate cancer, head and neck cancer, glioma and glioblastoma, and also in colorectal and pancreatic cancers. HER2/c-neu overexpression is most commonly found in breast cancer (15–20% of cases) and less frequently in ovarian, gastric, prostate, and pancreatic neoplasms. The relevance of HER3 overexpression in malignant tumors is not as high as that of HER1 and HER2. This phenomenon can be observed in breast, ovarian, gastric, and prostate cancers [7].

Studying the crystal structure revealed that the extracellular domain of ErbB family receptors consists of four subdomains, of which domains I and III are involved in ligand binding, and domains II and IV are involved in intramolecular interactions and provide autoinhibition. EGF receptors usually are in an inactive monomeric state as a compact "bound" conformation determined by intramolecular binding between domains II and IV. These receptors are activated upon binding to a ligand and subsequent dimerization. To accomplish this process, domains I and III form a ligand-binding pocket and expose subdomain II, allowing for dimerization between identical receptors (homodimerization) or with other family members (heterodimerization). However, unlike other family members, HER2 exists in a stable open conformation that enables dimerization without ligand binding.

For many tyrosine kinase receptors, the transition to an active kinase conformation is provided by autophosphorylation of the activation loop in the kinase domain. Nevertheless, for ErbB receptors, the transition to an active conformation is mediated by the formation of a kinase domain dimer, in which one kinase allosterically activates the other. Next, kinase domains catalyze the phosphorylation of tyrosine residues (outside the kinase domain in the C-terminus), creating binding sites for proteins or enzymes involved in downstream signal transduction. The interaction of EGF receptors with extracellular ligand triggers a cascade of biochemical reactions through the RAS-RAF-MAPK, PI3k/AKT, and phosphoinositide-specific phospholipase C (PLCγ) signaling pathways [7, 8].

Currently, there are three generations of EGFR/HER1-directed TKIs approved for the treatment of non-small cell lung cancer (NSCLC). The use of first-generation reversible ATP-conjugating TKIs (gefitinib, erlotinib, icotinib) and second-generation TKIs (afatinib and dacotinib) with irreversible inhibition has significantly improved the efficacy of the therapy compared to classical chemotherapy. At the same time, despite a favorable initial response to therapy, in the majority of patients, tumor progression slows down after 9–14 weeks. Tumor progression is caused by the development of resistance, which in about half of the cases is due to the second-site T790M mutation in exon 20 [1].

The irreversible third-generation TKI, *osimertinib*, covalently binds to cysteine at the ATP interaction site of the EGFR intracellular domain, increasing the efficacy of therapy against the T790M mutation by nearly 200 times and has demonstrated potential efficacy in the treatment of brain metastases. Another advantage of this drug is significant reduction of cutaneous and gastrointestinal toxicity compared to wild-type TKIs. The development of resistance caused by a point mutation of cysteine at position 797 (C797X), which is the binding site for osimertinib, remains an urgent and unresolved problem. In addition, mutations of other amino acid residues of EGFR, EGFR amplification, and EGFR-independent resistance, in particular activation of bypass signaling pathways for signal transduction, may be observed during treatment.

EGFR/HER1-directed antibodies approved for treatment in the Russian Federation include cetuximab (chimeric IgG1 monoclonal antibody) and panitumumab (recombinant human IgG2 monoclonal

antibody). As a result of binding to EGFR, the antibodies prevent intracellular ligand-mediated tyrosine kinase phosphorylation, leading to inhibition of downstream signaling pathways, including RAS-RAF-MAPK and PI3K-Akt/mTOR pathways. Drugs in this group are used for the therapy of metastatic colorectal cancer (panitumumab) and head and neck cancer (cetuximab).

Lapatinib is a dual tyrosine kinase inhibitor that blocks the activity of the HER1 and HER2 tyrosine kinases by interacting with the ATP-binding site of the intracellular receptor domain. Lapatinib is approved for the therapy of HER2-positive breast cancer (BC).

HER2-directed antibodies approved for BC therapy include trastuzumab and pertuzumab. Trastuzumab, as a HER2-directed humanized IgG1monoclonal antibody, binds to subdomain IV, suppresses the intracellular PI3K and MAPK signaling pathways, and activates the immune response. Pertuzumab is a monoclonal antibody that, by binding to subdomain II of HER2, inhibits the dimerization of HER2 with other receptors of EGFR, HER3, and HER4. Clinical studies have indicated that trastuzumab in combination with pertuzumab provides a more effective blockade of the HER signaling pathway.

A new class of targeted therapy in cancer treatment, monoclonal antibody-drug conjugates, have proven to be highly effective with acceptable systemic toxicity. Such conjugates consist of three main parts, namely a monoclonal antibody, a chemical linker, and a cytotoxic agent. Highly active tubulin inhibitors (auristatin analogs and maitansin analogs); DNA damaging compounds (duocarmazine, calicheamicins, and pyrrolobenzodiazepines), RNA polymerase II inhibitors (amanitin), and topoisomerase I inhibitors (deruxtecan, govitecan) are used as cytotoxic compounds [9, 10]. In addition to effects mediated by cytotoxic agents, monoclonal antibodies may exhibit intrinsic antitumor activity, such as blocking target antigens and triggering antibody-dependent immune responses [11].

Consequently, for patients whose disease progresses after therapy with a combination of trastuzumab and pertuzumab, trastuzumab emtansine is the standard second-line treatment. Trastuzumab emtansine (T-DM1) consists of an IgG1 monoclonal antibody against HER2 фтв trastuzumab conjugated via a non-degradable thioester linker to the cytotoxic agent DM1, an inhibitor of microtubule tubulin polymerization.

Trastuzumab deruxtecan (T-DXd*)* is a new HER2-directed antibody conjugate with a drug and consists of humanized IgG1monoclonal antibody, with the same amino acid sequence as in trastuzumab, conjugated to the cytotoxic agent deruxtecan. Deruxtecan bound to the antibody via a maleimide tetrapeptide linker cleaved by cathepsins inhibits DNA topoisomerase I. The increased activity of cathepsins in malignant cells and in the tumor microenvironment allows for targeted release of a cytotoxic drug [12]. Thus, after binding to HER2 on tumor cells, T-DXd undergoes internalization and intracellular cleavage of the linker by lysosomal enzymes; and upon release, the cytotoxic agent DXd penetrates into the nucleus and causes DNA damage and apoptotic cell death.

Despite impressive advances in antibody – drug conjugate therapy, limitations of its use include congenital and acquired resistance and toxicity of the drugs. In particular, trastuzumab deruxtecan more often causes myelosuppression and interstitial lung disease, in particular pneumonia, compared to trastuzumab emtansine. At the same time, trastuzumab emtansine is characterized by a higher risk of hepatotoxicity, cardiotoxicity, and thrombocytopenia compared to trastuzumab deruxtecan [13].

Another target that enables for intense and unregulated cell growth is the chimeric protein BCR-ABL1. BCR-ABL1 promotes malignant degeneration and enhanced proliferation of hematopoietic cells in the bone marrow with their subsequent entry into the blood. An abnormal Philadelphia chromosome (Ph) carrying the BCR-ABL1 oncogene forms in a blood stem cell as a result of reciprocal translocation. In this process, a part of chromosome 9 containing the ABL gene translocates to chromosome 22 and joins the BCR gene. The BCR-ABL1 oncogene encodes the chimeric BCR-ABL1 protein in isoforms that vary in size depending on the specific breakpoint in the BCR gene, less often – on variations in the ABL1 breakpoint.

The three most common BCR-ABL1 isoforms are identified by protein molecular weight as p210 (e13a2 or e14a2, 210 kDa), p190 (e1a2, 190 kDa), and p230 (e19a2, 230 kDa) [14]. Expressed in approximately 95% of patients, p210 is a characteristic feature of chronic myeloid leukemia (CML) [15, 16]. Between 20 and 30% of patients with acute lymphoblastic leukemia (ALL) are also BCR-ABL1 positive (Ph+ ALL). These patients tend to have a worse prognosis compared to BCR-ABL negative patients. More than two thirds of patients with Ph-positive ALL express a

shorter p190 isoform, while only a third express p210 [17, 18]. The fused protein BCR-ABL1 increases the activity of ABL kinase, leading to the formation of a GRB2/GAB2/SOS complex and subsequent activation of the PI3K/AKT, MAPK, and JAK/STAT signaling pathways [19]. Reduced demand in growth and apoptotic factors and improved viability and proliferation are observed in hematopoietic cells transformed with the BCR-ABL1 gene [20, 21].

Therapies targeting mutant BCR-ABL1 include TKIs, which function by competing for ATP at the ABL kinase ATP-binding site, which leads to inhibition of tyrosine phosphorylation of proteins involved in signal transduction. Therapy with imatinib, the first-generation TKI, has improved patient outcomes by demonstrating long-term cytogenetic remission. However, some patients did not respond to imatinib, while others experienced disease progression after treatment failure was identified. The main cause of resistance to imatinib is point mutations in the BCR-ABL kinase domain, which are found in ≥ 50% of patients. Mutations in the kinase domain can interfere with the binding of imatinib, which leads to drug resistance [22, 23].

Most of these imatinib-resistant BCR-ABL mutations are inhibited by dasatinib, nilotinib, and bosutinib (second-generation TKIs), with the exception of T315I. The following mutations are relevant for the selection of second-generation TKIs: V229L (nilotinib therapy is preferred), F317L/V/I/C, T315A (bosutinib or nilotinib therapy), Y153H and E255K/V, F259V/C (bosutinib or dasatinib therapy) [24]. The T315I mutation, which replaces the amino acid threonine (Thr315) with isoleucine (Ile315) at position 315 in the kinase domain of ABL1, is the most resistant to drug inhibition. This is due to several factors, including loss of hydrogen bonding between the T315 side chain at the drug – target binding site, changes in the topology of the ATP binding pocket, and an increase in its own kinase activity [22, 23, 25, 26]. For these reasons, the T315I variant of the mutant kinase is difficult to inhibit with ATP mimetics.

Asciminib and ponatinib, the third generation of TKIs (imatinib- and dasatinib-resistant), have been developed to overcome the T315I mutation. These drugs are indicated for patients with chronic myeloid leukemia or Ph+ acute myeloid leukemia who are resistant and / or intolerant to imatinib, dasatinib or nilotinib, or who have the T315I mutation. Unlike second-generation TKIs, ponatinib does not form hydrogen bonds with the T315 side chain in the ABL kinase domain (ABL-1). Instead, asciminib has a different binding site and is the first STAMP (specifically targeting the ABL myristoyl pocket) inhibitor to suppress BCR-ABL1 kinase activity by interacting with the myristoyl pocket [24, 26].

AIMING AT THE TARGETS THAT PROMOTE EVASION OF CELL GROWTH SUPPRESSORS

Besides maintaining stable signals of proliferative activity, tumor cells must evade mechanisms that inhibit proliferation. The action of cyclin-dependent protein kinases (CDKs), activated by their interaction with D-type cyclins, controls the transition from one phase of the cell cycle to another. For example, the transition from G1 to S phase of the cell cycle occurs when CDK4 and CDK6 bind to cyclin D1, leading to the phosphorylation of retinoblastoma-associated protein 1 (Rb1). When CDK4 and CDK6 are inhibited, Rb1 is dephosphorylated, and the progression of the cell cycle is inhibited [27].

In about 75% of cases of disseminated breast cancer, the tumors are HER2-negative but have high levels of estrogen and / or progesterone receptors. HER2-targeted therapy is not indicated for such patients. Patients with hormone-sensitive tumors often have overexpression of cyclin D1, which plays a key role in cell cycle regulation. The efficacy of cyclin-dependent inhibitors, such as abemaciclib, palbociclib, and ribociclib, depends on sustained activation of the cyclin D1-CDK4/6 complex. These inhibitors block Rb phosphorylation and arrest the cell cycle from G1 to S phase, leading to apoptosis of tumor cells [28].

AIMING AT THE TARGETS INDUCING ANGIOGENESIS

The progressive growth of the tumor and the development of metastases are accompanied by a continuous increase in the number of new blood vessels [29], which is triggered by the angiogenic switch. This process involves a shift in the balance of pro- and anti-angiogenic factors toward activators, leading to an increase in tissue vascularization [30]. Hypoxia as a result of rapid tumor growth is the main driver for the production of angiogenic inducers. It is interesting to note that the activation of angiogenesis has been observed in the early stages of carcinogenesis, preceding the appearance of solid tumors, suggesting that its induction is a discrete process in carcinogenesis [29].

Vascular endothelial growth factor (VEGF) is a strong pro-angiogenic factor. Signaling proteins, such

as VEGF, as well as basic fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF), bind to cell surface receptors on vascular endothelial cells and regulate the angiogenic switch [31, 32].

Multi-targeted inhibitors represent TKI therapy, which aims at promoting angiogenesis in the tumor microenvironment. The drugs specified below have been approved for the treatment of thyroid carcinoma. Vandetanib is a TKI targeting angiogenic VEGFR-2, EGFR, and RET tyrosine kinases involved in tumor growth, progression, and angiogenesis. Lenvatinib is a multikinase inhibitor of VEGF1-3 and FGF1-4 receptors. Regorafenib, a diphenylurea multikinase inhibitor targeting angiogenic (VEGFR1-3, TIAM), stromal (PDGFRβ, FGFR), and oncogenic (KIT, RET and RAF) tyrosine kinase receptors, has also been approved for the treatment of metastatic colorectal cancer. Axitinib, a TKI with an affinity for angiogenic VEGF receptors (1, 2 and 3), PDGFR and c-KIT, is approved for the treatment of renal cell carcinoma. Sunitinib is a multikinase inhibitor approved for the treatment of gastrointestinal stromal tumors, renal cell carcinoma, and neuroendocrine tumors of the pancreas with activity against more than 80 kinases, including platelet growth factor receptors (PDGFRa and PDGRFß), vascular endothelial growth factor (VEGRF1, VEGRF2 and VEGRF3), KIT, FLT, CSF-IR, and RET. Sorafenib is another TKI approved for the treatment of hepatocellular carcinoma, renal cell carcinoma, and thyroid cancer. Bevacizumab and ramucirumab are IgG1 monoclonal antibodies that selectively bind to VEGF, inhibit its biological activity, and are approved for the treatment of tumors of various localizations.

TARGETING IMMUNE CHECKPOINTS

Normally, cells with oncogenic potential, due to genetic and epigenetic changes, are recognized as foreign by immune cells and destroyed by NK cells, dendritic cells, and T lymphocytes. However, cancer cells can effectively evade immune surveillance by reducing surface human leukocyte antigen (HLA) expression, tumor-associated antigens on their surface, or directly blocking T lymphocyte activation through clonal deletion or anergy.

Immune checkpoints are receptors and their ligands that regulate the immune response. Immune checkpoints that suppress T lymphocyte activation pathways include cytotoxic T lymphocyte-associated glycoprotein 4 (CTLA-4), programmed cell death receptor 1 (PD-1), and its ligands (PD-L1 and PD-L2). CTLA-4, in particular, is thought to regulate T lymphocyte proliferation during the early stages of an immune response, primarily in the lymph nodes. On the other hand, PD-1 suppresses T cell activity during later stages of immune response development, primarily in peripheral tissues [33]. Monoclonal antibodies targeting these molecules are used in therapy to inhibit immune checkpoints. Blockade of these receptors or ligands can prevent acquired peripheral tolerance to tumor antigens and restore an effective anti-tumor immune response.

To date, there are two main types of immune checkpoint antibodies, namely mAbs targeting CTLA-4 and mAbs targeting PD-1 and / or its ligand PD-L1. The first approved drug for the treatment of unresectable or metastatic melanoma is ipilimumab, which targets CTLA-4. Pembrolizumab and nivolumab, which target PD-1, have also been approved to treat patients with advanced melanoma. These drugs have since been approved to treat tumors in other sites, including non-small cell lung cancer, renal cell carcinoma (RCC), squamous cell carcinoma of the head and neck, and Hodgkin's lymphoma [34].

It should be noted that most patients do not respond to monotherapy with drugs of this class, and cases of primary and acquired resistance to treatment are not uncommon, leading to loss of immunity and tumor progression. Double blockade of immune checkpoints through the combined use of CTLA-4 and PD-1 inhibitors is a more effective therapeutic strategy and shows a synergistic anti-tumor effect. Anti-CTLA-4 and anti-PD-1 agents enhance the anti–cancer immune response through different but complementary mechanisms as they act at different times and in different sites throughout the evolution of T cells. One well-known combination is the use of ipilimumab and nivolumab.

AIMING AT THE TARGETS ACTIVATING INVASION AND METASTASIS

The spread of tumor cells from a primary lesion to other organs and tissues is a major cause of cancer-related mortality. According to the metastatic cascade theory, this process involves successive stages, which can be divided into the stages described below. The first stage is physical dissemination of tumor cells away from the original site, which involves transformation of the tumor cells and their invasion deep into the adjacent tissues. This includes intravasation, migration of cells into lymphatic and vascular capillaries, and extravasation to sites of release. The next stage is

colonization and growth of new tumors, leading to the development of microscopic metastases with the potential for progression to larger tumors.

Epithelial – mesenchymal transition (EMT) is the process by which epithelial cells undergo morphological and functional transformation and acquire mesenchymal properties, including increased invasiveness, mobility, and migration. Inducers of EMT include transforming growth factor beta (TGFβ), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), insulin-like growth factors (IGFs), etc.

The hepatocyte growth factor receptor (c-Met) is involved in the initiation and development of various types of cancer in humans, and it mediates the proliferation, migration, and invasion of tumor cells. The interaction of c-Met with its ligand, HGF, activates downstream PI3K/Akt and Ras/MAPK signaling pathways. This triggers cytoskeletal restructuring and various cellular reactions, such as cell migration, mitogenesis, morphogenesis, proliferation, invasion, and angiogenesis.

Small-molecule C-Met inhibitors can be divided into selective and multikinase ones. Capmatinib is a selective ATP-competitive TKI that targets C-Met, including the mutant variant produced by skipping exon 14 (METex14 mutation). Skipping exon 14 results in a truncated C-Met receptor lacking the regulatory domain, which reduces its negative regulation and consequently reduces the possibility of degradation of the C-Met protein, leading to its stable activation and oncogenesis [35]. Multikinase TKIs, specifically those with C-Met inhibition, include crizotinib (targeting ALK, ROS1, C-Met, and RON), which is used to treat patients with ALK+ or ROS1+ NSCLC, and cabozantinib (targeting MET, VEGF, GAS6 (AXL), RET, ROS1, TYRO3, MER, KIT, TRKB, FLT3, and TIE-2) to treat RCC. Amplification of the MET gene has been associated with acquired resistance to therapy with EGFR family agents. MET activation has also been shown to increase VEGF-A expression, which is a promoter of angiogenesis and endothelial cell growth.

Monoclonal antibody therapy is divided into antibodies against c-Met (such as onartuzumab and emibetuzumab) and antibodies against HGF (such as ficlatuzumab and rilotumumab), which are FDA-approved but have not yet been approved in Russia [36].

Table 1

Tyrosine kinase inhibitors registered in the Russian Federation, classified according to the target

Mechanism of action	Target (kinases)	Name of drug	Indications
Inhibition of overexpressed or mutant protein (angiogenesis)	VFGFR1, VFGFR2, VFGFR3	Axitinib (Axitinib, Inlita®)	Renal cell carcinoma
	VEGFR1 (FLT1), VEGFR2 (KDR) and VEGFR3 (FLT4), FGFR1, FGFR2, FGFR3, FGFR4, PDGFRa, and tyrosine kinase receptors KIT and RET	Lenvatinib (Lenvatinib, Lenvima®)	Thyroid cancer
	VFGFR1, VFGFR2, VFGFR3, PDGFR α and β, FGFR1, FGFR2, FGFR3	Nintedanib (Vargatef®)	Non-small cell lung cancer
	TIE, KIT, RET, RAF-1, BRAF, BRAFv600E, PDGFR, FGFR, CSF1R	Regorafenib (Stivarga®)	Colorectal cancer, gastrointestinal stromal tumors
	Multikinase (>80 kinases), including: PDGFRα, PDGRFß, VEGRF1, VEGRF2 and VEGRF3, KIT, FLT, CSF-IR, RET.	Sunitinib (Flutrixan, Sunitinib, Sunitinib-Amedart, Sunitinib-Himrar, Valeotinib, Sutent®, Sunitinib-Promomed)	Gastrointestinal stromal tumors, renal cell carcinoma, neuroendocrine tumors of the pancreas
Inhibition of overexpressed or mutant protein (angiogenesis, proliferation)	VEGF, EGFR/HER1	Vandetanib (Caprelsa)	Thyroid cancer
Inhibition of overexpressed or mutant protein (proliferation)	EGFR/HER1, HER2, HER3, and HER4	Afatinib (Giotrif®, Gefitinib-Promomed)	Non-small cell lung cancer with EGFR mutations
	EGFR/HER1, HER2, HER3, and HER4	Gefitinib (Gefitinib, Valkyra®, Gefitinib-Tl, Getinex®, Geftessa, Langerra)	Non-small cell lung cancer with EGFR mutations
	EGFR/HER1, HER2, and HER4	Dacomitinib (Visimpro®)	Non-small cell lung cancer with EGFR mutations

Table 1 (continued)

Mechanism of action	Target (kinases)	Name of drug	Indications
Inhibition of overexpressed or mutant protein (proliferation)	EGFR/HER1, HER2	Lapatinib (Brestocer, Lapatinib-Promomed, Lapatinib-Himrar, Tyverb®)	HER2+ breast cancer
	EGFR/HER1	Osimertinib (Osimertinib, Retezmo™)	Non-small cell lung cancer with EGFR mutations (deletions in exon 19 or L858R substitutions in exon 21, with the T790M mutation)
	EGFR/HER1	Erlotinib (Erlotinib, Erlater, Erlotinib-Tl)	Non-small cell lung cancer
Inhibition of overexpressed or mutant protein (evasion of cell growth suppressors)	CDK4/6	Abemaciclib (Zenlistik)	HR+ and HER2- breast cancer
	CDK4/6	Palbociclib (Itulsi)	HR+ and HER2- breast cancer
	CDK4/6	Ribociclib (Risarg)	HR+ and HER2- breast cancer
Inhibition of intracellular kinases (downstream signaling pathways)	MEK	Binimetinib (Mektovi)	Melanoma
	MEK1/2	Cobimetinib (Cotellic®)	Melanoma with BRAF V600 mutations
	MEK1/2	Selumetinib (Coselugo)	Plexiform neurofibroma
Inhibition of intracellular kinases (downstream signaling pathways)	BRAF	Vemurafenib (Zelboraf®)	Melanoma with the BRAF V600 mutation
	BRAF	Encorafenib (Braftovi)	Melanoma with BRAF mutations, colon cancer with BRAF mutations
Inhibition of overexpressed or mutant protein and intracellular kinases	c-CRAF, BRAF, mutant BRAF, KIT, FLT-3, RET, RET/PTC, VEGFR1, VEGFR2, VEGFR3, and PDGFRß	Sorafenib (Sorafenib, Sorafenib-Amedart, Effaronix®, Nexavar®, Sorafenib-Promomed)	Hepatocellular carcinoma, renal cell carcinoma, and thyroid cancer
Inhibition of overexpressed or mutant protein	Bruton's tyrosine kinase (BTK)	Zanubrutinib (Brukinza®)	Mantle cell lymphoma
	Bruton's tyrosine kinase (BTK)	Ibrutinib (Ibrutinib-Nativ)	Mantle cell lymphoma
	Bruton's tyrosine kinase (BTK)	Acalabrutinib (Calquence®)	Chronic lymphocytic leukemia / small cell lymphocytic lymphoma, mantle cell lymphoma
Inhibition of overexpressed or mutant protein	BCR-ABL1, c-KIT	Imatinib (Imatinib, Neopax®, Imatinib-Teva, Imatinib Grindex)	Ph+ chronic myeloid leukemia, Ph+ acute lymphoblastic leukemia
	BCR-ABL1, BCR-ABL1 mutant forms except for T315I mutation, c-KIT, Eph, PDGFß	Nilotinib (Nilotinib, Nilotinib-Promomed)	Ph+ chronic myeloid leukemia
	BCR-ABL1, BCR-ABL1 mutant forms except for 315I mutation, Src family (including Src, Lyn and Hck), c-KIT, Eph, PDGFß	Dasatinib (Dasatinib, Mirsonib, Dasatinib-nativ, Dasatinib-Himrar)	Ph+ chronic myeloid leukemia, Ph+ acute lymphoblastic leukemia
	BCR-ABL1, BCR-ABL1 mutant forms except for T315I mutation, Src family (including Src, Lyn, and Hck)	Bozutinib (Bozutinib, Bozulif)	Ph+ chronic myeloid leukemia
	BCR-ABL1, BCR-ABL1 mutant forms (including T315I mutation)	Asciminib (Scemblix)	Ph+ chronic myeloid leukemia, chronic myeloid leukemia with T315I mutation
	BCR-ABL1, BCR-ABL1 mutant forms (including T315I mutation)	Ponatinib (Iclusig®)	Ph+ chronic myeloid leukemia, Ph+ chronic myeloid leukemia with T315I mutation, Ph+ acute lymphoblastic leukemia
Inhibition of overexpressed or mutant protein	ALK	Alecetinib (Alecensa®)	ALK+ non-small cell lung cancer
	ALK	Ceritinib (Zykadia®)	ALK+ non-small cell lung cancer

Table 1 (continued)

Mechanism of action	Target (kinases)	Name of drug	Indications
Inhibition of overexpressed or mutant protein	ALK, ROS1 TYK1, FER, FPS, TRKA, TRKB, TRKC, FAK, FAK2, and ACK.	Lorlatinib (Lorviqua®)	Thyroid carcinoma, ALK+ non-small cell lung cancer
Inhibition of overexpressed or mutant protein (proliferation, invasion-metastasis, etc.)	ALK, ROS1, c-Met, RON	Crizotinib (Xalkori®)	ALK+ or ROS1+ non-small cell lung cancer
Inhibition of overexpressed or mutant protein (invasion-metastasis)	MET	Capmatinib (Tabrecta)	Non-small cell lung cancer with the METex14 mutation.
Inhibition of overexpressed or mutant protein (invasion-metastasis, angiogenesis, etc.)	METH, VEGF, GAS6 (AXL), RET, ROS1, TYRO3, MER, KIT, TRKB, FLT3, and TIE-2.	Cabozantinib (Cabometyx®)	Renal cell carcinoma
Inhibition of overexpressed or mutant protein (genome mutation)	PARP1, PARP2, and PARP3	Olaparib (Lynparza®)	Ovarian, fallopian tube, or peritoneal cancer with mutation of the BRCA gene
Inhibition of overexpressed or mutant protein	TRKA, TRKB and TRKC encoded by the NTRK1, NTRK2, and NTRK3 genes	Larotrectinib (Vitrakvi®)	Solid tumors expressing the NTRK fusion gene
	TRKA, TRKB, and TRKC encoded by the genes NTRK1, NTRK2 and NTRK3, ROS1	Entrectinib (Roslitrek®)	Solid tumors expressing the NTRK fusion gene ROS1- positive non-small cell lung cancer

Table 2

Classification of targeted drugs based on monoclonal antibodies (including conjugates of monoclonal antibodies with cytotoxic agents) registered in the Russian Federation

Mechanism of carcinogenesis	Target	Name of drug	Indications
Proliferative signal transduction	EGFR	Cetuximab (Erbitux®)	Metastatic colorectal cancer, head and neck cancer
		Panitumumumab (Vectibix)	Metastatic colorectal cancer with wild-type RAS genes in the tumor
	HER2	Pertuzumab (Perjeta®)	Breast cancer
		Trastuzumab (Herceptin®, Herticad®, Trasimera®)	Breast cancer
		Trastuzumab Deruxtecan (Enchertu)	Breast cancer
		Trastuzumab Emtansine (Cadsila®)	Breast cancer
Angiogenesis	VEGF	Bevacizumab (Avastin®, Avegra® Biocad, Versavo®, Stibevara®)	Metastatic colorectal cancer
		Ramucirumab (Ciramza®)	Gastric cancer, gastroesophageal junction adenocarcinoma, non-small cell lung cancer, metastatic colorectal cancer, hepatocellular carcinoma
Immunotherapy: immune checkpoints	CTLA- 4	Ipilimumab (Yervoy®)	Melanoma
		Tremelimumab (Imjudo)	Non-small cell lung cancer, bladder cancer, head and neck cancer, liver cancer and hemoblastosis
	PD-L1	Avelumab (Bavencio®)	Merkel cell carcinoma
		Atezolizumab (Tecentriq®)	Locally advanced or metastatic urothelial cancer
		Durvalumab (Imfinzi®)	Non-small cell lung cancer

Table 1 (continued)

Mechanism of carcinogenesis	Target	Name of drug	Indications
Immunotherapy: immune checkpoints	PD-1	Nivolumab (Opdivo®)	Melanoma, non-small cell lung cancer, advanced renal cell carcinoma
		Pembrolizumab (Kitruda®, Pembroria)	Melanoma
		Prolgolimumab (Forteca®)	Melanoma
Immunotherapy	CD19, CD3	Blinatumomab (Blincyto®)	Acute lymphoblastic leukemia
	CD20	Obinutuzumab (Gazyva®)	Chronic lymphocytic leukemia
		Ofatumumumab (Bonspree®)	Chronic lymphocytic leukemia
		Rituximab (Redditux®, Rituxara®, Acellbia®, Mabthera®)	B-cell non-Hodgkin's lymphomas
	CD20, CD3	Mosunetuzumab (Lansumio®)	Follicular lymphoma
	CD22	Inotuzumab Ozogamicin (Bisponsa)	CD22-positive B-cell acute lymphoblastic leukemia
	CD30	Brentuximab Vedotin (Adcetris®)	CD30-positive Hodgkin's lymphoma
	CD33	Gemtuzumab Ozogamicin (Mylotarg®)	CD33-positive acute myeloid leukemia
	CD38	Daratumumumab (Darzalex)	Multiple myeloma
		Isatuximab (Sarcliza®)	Multiple myeloma
	CD79b	Polatuzumab Vedotin (Polayvi®)	Diffuse B-large cell lymphoma
	GD2	Dinutuximab Beta (Carziba)	Neuroblastoma
	SLAMF7	Elotuzumab (Emplicity®)	Multiple myeloma
Proliferation, migration, and adhesion	Nectin-4	Enfortumumab Vedotin (Padtsev Onco)	Non-muscle invasive bladder cancer

CONCLUSION

The development of novel targeted drugs with low toxicity and the ability to bypass resistance mechanisms is a major challenge for medicine in the XXI century. In this regard, it is essential to understand the mechanisms of malignancy and identify the key targets in the development of a particular tumor.

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Frailty syndrome and its features in Parkinson's diseas

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ABSTRACT

Frailty syndrome is common in older adults around the world, and its emergence is associated with an increase in life expectancy. The lecture shows the multifactorial nature of the syndrome: changes in physical health, social and psychological factors, gender characteristics, and age. The classic diagnosis of the syndrome consists in assessing physical weakness according to the Fried phenotype. The modern view of the problem complements the diagnosis with indices of weakness to characterize multifactorial development and the use of digital wearable technologies for long-term health monitoring.

The lecture provides a detailed justification of the effect of comorbidity on the development of frailty syndrome. Frailty syndrome is difficult to diagnose in Parkinson's disease with possibly high prevalence. There are few studies on frailty syndrome in Parkinson's disease, probably due to the similarity of their symptoms.

The lecture identifies possible risks of frailty syndrome in Parkinson's disease: the influence of various forms of Parkinson's disease, gender, cognitive and functional disorders, polypharmacy, and levodopa doses. The role of a multidisciplinary rehabilitation team and independent physical activity in Parkinson's disease and frailty syndrome is shown.

Keywords: frailty syndrome, diagnosis, comorbidity, Parkinson's disease

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Синдром старческой астении и его особенности при болезни Паркинсона

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РЕЗЮМЕ

Синдром старческой астении распространен во всем мире, и его появление связано с увеличением продолжительности жизни. В лекции показаны мультифакторность формирования синдрома: изменения физического здоровья, социальные и психологические факторы, а также гендерные особенности и влияние возраста. Классическая диагностика синдрома состоит в оценке физической слабости по фенотипу Фрайд. Современный взгляд на проблему дополняет диагностику индексами слабости для характеристики мульти-

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факторности развития и применением цифровых носимых технологий для длительного мониторирования функциональных показателей пациента.

Дано развернутое обоснование влияния коморбидности на развитие старческой астении. Особенностью синдрома старческой астении при болезни Паркинсона является трудность его диагностики при возможно высокой распространенности. Исследования старческой астении при болезни Паркинсона малочисленны, вероятно, из-за схожести симптомов болезни и синдрома.

Определены возможные риски возникновения старческой астении при болезни Паркинсона: влияние различных форм болезни Паркинсона, пола, когнитивных и функциональных нарушений, полипрагмазии, дозы леводопы. Показана роль комплексной мультидисциплинарной реабилитации и самостоятельной физической активности при сочетании болезни Паркинсона и синдрома старческой астении.

Ключевые слова: синдром старческой астении, диагностика, коморбидность, болезнь Паркинсона

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

Worldwide, the pace of population aging is fast and much faster than it used to be in the past. For example, in China in 2021, the elderly population comprised 14.2%, being the largest in the world in absolute terms. By 2050, the elderly population in China will increase to 395 million people, which will exceed the current population of the United States by 1.2 times. At the same time, the total fertility rate (the number of births per 1 woman during the reproductive period) was 6:1 in 1955 and 1:3 in 2020 [1].

The demographic change associated with population aging has a comprehensive impact on the life of society, since in addition to physiological changes during aging, which can reduce functional and adaptive capabilities to varying degrees, an elderly person often has a number of diseases with concomitant drug therapy, unfavorable lifestyle features, and social conditions [1–4]. These factors contribute to an increase in the healthcare burden, disability, inability to self-care, and the need for social care. Frailty syndrome can be attributed to an extreme combination of factors.

The syndrome is characterized by a progressive decrease in strength, strength endurance, functional reserve, increased vulnerability of the body to stress factors, dependence on other people during daily activity, and adverse outcomes [2]. The situation is complicated by the fact that a decrease in the birth rate and an increase in the elderly population lead to a decrease in the number of middle-aged working people and an increase in working elderly people, including those with concomitant diseases and a decline in the functional status [5]. Taking all this into account, the leaders of many countries cannot but plan to increase the retirement age in order to maintain economic stability. In this regard, a new term “the expected duration of a healthy working life” has appeared [5].

In order to find a consensus, the UN General Assembly declared 2021–2030 the Decade of Healthy Aging. During this decade, various activities are planned to improve the lives of older people today and in the future. These include early detection and therapy of frailty syndrome, as well as preventive measures among the elderly and youth and creation of a model of healthy aging [1].

The aim of this lecture was to bring to the attention of the medical community frailty syndrome, its manifestations, and the difficulty of diagnosing the syndrome in Parkinson’s disease.

EPIDEMIOLOGY AND PATHOGENESIS OF FRAILTY SYNDROME

The prevalence of frailty syndrome is high worldwide [3]. It ranges from 4 to 59% among the elderly. The syndrome is widespread in the elderly population not only in high-income countries with longer life expectancy, but also in low-income countries, for example, in Brazil and Vietnam [1–4, 6]. Social and economic inequality leads to different incidence of the syndrome. Even in one country, the incidence may vary. For example, the syndrome

occurs in European countries more often in the south than in the north, in accordance with the geographical grade of the prevalence of concomitant pathology [7].

Most often, the cycle of frailty syndrome formation includes poor nutrition in terms of quality and / or quantity, a progressive decrease in muscle mass (sarcopenia), and decreased strength, mobility and activity of daily living. Age-related type 2 muscle fiber atrophy responsible for exercise training is the main reason for a decrease in physical capabilities. However, not every elderly person of the same age is diagnosed with frailty.

Frailty syndrome is a multifactorial syndrome that includes various factors: physical, psychological, and social. The interaction between these factors determines the severity of the syndrome. Concomitant diseases, such as deforming arthrosis, Parkinson's disease, diabetes mellitus, and others contribute to its emergence [8, 9]. Frailty syndrome is provoked by both diseases and lifestyle features. Social isolation, living in a nursing home, taking medications without considering side effects, polypharmacy, and a sedentary lifestyle accelerate the development of the syndrome [10, 11].

Women are more likely to suffer from frailty syndrome [12]. Despite their long life expectancy, unlike men of the same age, they sometimes have several diseases, but not critical. This dissonance is designated as the male – female health survival paradox. Men are more likely to suffer from life-threatening diseases, such as coronary heart disease and stroke. In addition, men are more likely to suffer from type 2 diabetes mellitus, non-specific lung diseases, chronic obstructive bronchitis, peptic ulcer, and Parkinson's disease. Women of the same age are more likely to have non-fatal diseases that are associated with a worse quality of life: migraine, musculoskeletal pathology, autoimmune diseases [7, 13]. Cancer prevails in men until old age, and in women from the age of 75.

The frequency of frailty increases with the age of an elderly person [10]. Since the life expectancy of men is shorter than that of women, the frequency of the syndrome increases as women get older.

DIAGNOSIS AND CLINICAL VARIANTS OF FRAILTY SYNDROME

An experienced doctor with knowledge of the syndrome intuitively understands that a patient has frailty syndrome. Patients move slowly and get tired very quickly. They complain that they cannot perform basic household chores. Currently, there is no unanimous opinion on the diagnosis of the syndrome. Doctors use different assessment scales. Assessment of the criteria that are pre-defined for the Fried frailty phenotype is most commonly used to diagnose weakness [2, 14].

The Fried frailty phenotype includes the following criteria: weight loss of more than 5 kg per year, hand grip strength, fatigue in the last month (the physician either asks a patient about their symptoms or uses questionnaires to assess them), slow gait speed (less than 4 meters in 5 seconds), and daily physical activity [2]. A patient is considered frail if at least three criteria are positive and pre-frail if one or two criteria are positive [15]. The Fried criteria are criticized because they evaluate only physical weakness.

The new concept of frailty syndrome interprets the syndrome as a multiple domain consisting of biological, social, psychological, and functional disorders [15]. Therefore, in addition to evaluating the Fried frailty phenotype, physicians use other scales. For example, the Rockwood Clinical Frailty Scale includes 7 points. The EFS scale (Edmonton Frail Scale) consists of 9 components. Ten additional cognitive components have been added to the FI-CGA (Frailty Index Comprehensive Geriatric Assessment). The Tilburg Frailty Indicator aims at assessing physical, psychological, and social condition of an elderly person. The scales include questions about education, nutrition, medications taken, episodes of falling and identify disorders of cognitive status, bladder function, etc. [15, 16].

In addition to scales, modern instrumental diagnosis is used, including digital wearable technologies separately or in combination with platforms, and video cameras. It is especially important to diagnose daily activity, including walking, climbing stairs, washing, dressing, eating, going to bed [16, 17]. Daily activity can be combined with a task that involves a cognitive component. Inertial sensors of wearable technologies on the waist and lower extremities based on the operation of an accelerometer or gyroscope are used to describe gait. Additional inertial sensors on the waist help diagnose balance disorders [16–19].

The area of the stabilogram of an elderly person obtained during monitoring is interrelated with the criteria of the Fried frailty phenotype. For diagnosis, built-in sensors are used in objects surrounding an elderly person in the form of an eChair (electronic chair), ePOD (electronic carpet) and others, combining diagnostic capabilities of wearable technology

with other parameters of the Internet of Things. The characteristic of gait includes an increase in the time of the standing phase on two legs, as this compensates for the lack of balance during movement, an irregular pattern, and a decrease in gait speed. The Stand up and Walk test is evaluated using inertial sensors and 3D anatomical motion data [17–20]. Parameters obtained during long-term monitoring undergo machine learning in the form of big data and form a phenotype model that includes kinetic parameters. Balance and gait parameters in combination with the history of falls and vector machine learning with the formation of a frailty index model are used to assess falls [19, 20].

Gait speed is the main indicator in the frailty index, all other data are integrated with this parameter. There is currently no accurate predictive model of frailty syndrome. Exergame technology is the future of digital medicine. It is an interactive gaming platform that is a diagnostic and training site for investigating the presence and reversibility of frailty syndrome [16].

Despite the fact that the main age-related changes in body composition occur in power muscle fibers, the syndrome affects various tissues and systems: nervous, endocrine, immune and musculoskeletal [17]. The connection between cognitive impairment and physical frailty is widely known. However, comorbidity does not imply the concept of cognitive frailty in the form of dementia. Cognitive frailty is defined as the simultaneous presence of frailty syndrome based on the Fried frailty phenotype and cognitive impairment without dementia [15, 21–25].

Cognitive frailty is divided into two subtypes: reversible and potentially reversible. Reversible cognitive frailty includes an incomplete Fried phenotype – pre-frailty and subjective cognitive impairment and / or positive biomarkers. Potentially reversible frailty is more severe cognitive frailty, including mild cognitive impairment and the Fried phenotype in which all the criteria are positive. The next stage of development involves the adverse effects of cognitive frailty: falls, disability, Alzheimer's disease, non-Alzheimer's dementia, and death. The prevalence of cognitive frailty is 10.7–22.0% in clinical studies and 1.0–4.4% in population-based studies. Understanding the physiological relationship between cognitive impairment and physical frailty will help guide the development of geriatrics and neurology to create a model for the treatment of two pathological conditions [21].

In cognitive frailty, multimorbidity is observed due to chronic inflammation with changes in immune markers, the endocrine system, and oxidative stress, which negatively affect the nervous system and muscle tissue. For example, the lack of interleukin 10 (IL-10) increases the expression of the inflammatory mediator, nuclear factor NF-kV, reduces muscle metabolism, and negatively affects information processing speed. Lack of growth hormone leads to a combined decrease in brain mass and muscle mass. Elderly women, who are often diagnosed with frailty syndrome, have low ghrelin levels, which results in decreased muscle strength, endurance, and impaired memory and information processing speed [21].

Frailty syndrome can be associated with orthostatic hypotension. Orthostatic hypotension occurs in 30% of people over 65 years of age [26]. The frequency of orthostatic hypotension in the elderly population depends on comorbidity. The pathology is most often observed in Parkinson's disease, multiple system atrophy, dementia with Lewy bodies. Orthostatic hypotension is associated with falls, heart failure, stroke, decreased quality of life, and an increased risk of death [27]. Physical frailty and orthostatic hypotension increase the likelihood of adverse effects. The homeostasis of maintaining blood pressure includes the integrated activity of the nervous system, heart, blood vessels, and muscles. Age-related changes in these organs and systems lead to a decrease in baroreflex, hypoperfusion of the brain, retina and muscles, dizziness, and loss of consciousness [26, 27].

Frailty syndrome may be accompanied by pain syndrome [28]. Chronic pain occurs in 66% of the elderly and is three times more common in women than in men. The most common complaint among the elderly is lower back pain (33% of people over 65 years of age). The cause of the pain can also be deforming osteoarthritis, which occurs in 12% of the world's elderly population, or rheumatoid polyarthritis occurring in 1–2%. Pain syndrome accompanies malignant neoplasms, neurodegenerative diseases, diabetes mellitus, kidney and liver diseases [28].

Depression lowers pain tolerance, while neuroinflammation is considered as a common pathogenetic step in the development of depression and pain. Patients suffering from depression complain of a variety of common pains. The severity of frailty syndrome is aggravated in such cases [28]. Conversely, physical frailty is a predictor of depression. In 30.3% of patients with frailty syndrome without depression, the disease is subsequently diagnosed. Long–term

depression is a heavy burden for the patient and society due to its chronic course and relapses. The peculiarity of depression in the elderly is mood disorders of various etiology, combined with high comorbidity, cognitive impairment, and physical suffering.

According to literature data, frailty occurs in 6.8% of elderly patients with depression; in nursing homes the disease combination was noted more often – from 10.7 to 40% [29, 30]. The severity of frailty syndrome negatively correlates with the remission duration of depression [29]. On the other hand, severe syndrome affects the frequency of remissions and increases the risk of depression: 75% of patients with severe depression have frailty syndrome [29]. The pathophysiological mechanisms of the syndrome in depression are diverse, including low-grade inflammation, deficiency and qualitative eating disorders, especially vitamin D deficiency, low physical activity, sarcopenia, and age-related hormonal changes. Additional factors include the number of psychoactive medications taken, lack of education, loneliness, lack of socialization, and lack of Internet access at home [2]. The relationship between psychosocial factors and the occurrence of physical frailty in depression requires further study [30]. Apathy, lack of hygiene, low physical activity, and poor nutrition affect the development of depression in frailty syndrome.

Despite the general development mechanisms, the concept of physical frailty in mental distress did not go any further, since the diagnostic signs of the two pathologies overlap, even though initially no criteria in the Fried phenotype are associated with depression. Obviously, while treating patients with a combination of physical frailty and depression, it is difficult to determine cause-and-effect relationships in order to identify the primary pathology. However, there is a positive aspect in such cases, as treatment of one pathology makes the manifestations of the other less pronounced [30].

The literature presents data on the genetic predisposition to the development of frailty syndrome [31]. However, studies have not found a link between changes in candidate genes encoding IL-6, tumor necrosis factor (TNF), insulin-like growth factor-1 (IGF-1) and the onset of the syndrome, which is due to the fact that frailty syndrome is a multifactorial disease that depends on lifestyle, genetic and epigenetic factors. Epigenetic mechanisms include chromatin remodeling during aging. The genes at the 9p21-23 locus are susceptible to this process, which may be the cause of the syndrome [32].

PARADOXES OF FRAILTY SYNDROME IN PARKINSON'S DISEASE

Parkinson's disease is a neurodegenerative disease that is not the result of aging, but the risk of its occurrence increases with age. Old age contributes to the progression of the disease and a decrease in response to levodopa, a drug used to treat Parkinson's disease. The incidence of frailty syndrome in people with Parkinson's disease is high, according to the literature, from 32.6 to 55.3% [33].

Nevertheless, scientists try not to include patients with Parkinson's disease in scientific studies on the syndrome, since the clinical symptoms of the pathologies are similar. This is the paradox of diagnosing frailty syndrome in Parkinson's disease [14, 34]. Often, the concomitant syndrome is misinterpreted as aggravation of the underlying pathology [14, 34]. Usually, female patients who have got Parkinson's disease and are frail are older than men and have greater disturbances in the everyday life activity [13, 33, 35]. Women suffer from the syndrome twice more often than men, and the severity of the syndrome depends on the stage of Parkinson's disease: frailty syndrome is more common at the advanced stage of the disease [33, 35, 36].

Low physical activity and severe Parkinson's disease contribute to the emergence of frailty syndrome. These factors have got direct and indirect effects, with the latter being associated with changes in behavior, and quantity and quality of medications taken [33, 34, 37]. For example, manifestations of kinesiophobia could lead to low everyday life activity, since patients believe that any movement leads to a fall, injury, and increased pain. Patients with Parkinson's disease have high frequency of behavioral changes in the form of kinesiophobia [37]. Restriction of physical activity contributes to a poor physical condition, functional disability, and depression.

However, there is an opposite opinion about physical activity in Parkinson's disease: patients are more actively involved in physical education programs, with better commitment, regardless of all the Fried physical frailty criteria being positive [13]. In patients without Parkinson's disease, low physical activity leads to the development of frailty syndrome. Patients with Parkinson's disease have greater self-reported physical activity and are less susceptible to frailty syndrome [13].

The development of frailty syndrome is influenced not only by the duration, but also by the type of

Parkinson's disease. The syndrome is most often found in patients with predominance of postural instability and walking disorders than in those with tremor [33]. Although another paradox of the syndrome in Parkinson's disease is that tremor reduces protein synthesis in muscles. Thus, tremor may be a trigger for sarcopenia, the main age-related change leading to the onset of physical frailty [34, 36].

Polypharmacy is the use of more than 5 medications by patients with Parkinson's disease, including drugs that affect the central nervous system, dopaminergic therapy: carbidopa and levodopa, and dopamine receptor stimulators that increase the QT interval and interact with cytochrome P450, increasing the likelihood of frailty syndrome [33, 34]. High doses of levodopa increase the risk of motor complications in the form of dyskinesia, which further reduces physical activity of patients. A high dose of carbidopa or levodopa may trigger the syndrome, since taking drugs, while improving motor characteristics, does not affect postural stability and thus increases the risk of injury. In addition, carbidopa and levodopa increase the risk of osteoporosis and fractures [38].

The probability of the syndrome increases with additional intake of antidepressants and drugs that improve bladder function while increasing the anticholinergic load on the body [14]. Poor adherence to drug therapy and errors in drug intake are often the cause of hospitalization and delirium, which increases the likelihood of developing frailty syndrome [14].

Frailty syndrome in Parkinson's disease, like in other pathologies, is associated with aging, cognitive dysfunction, and orthostatic hypotension [15, 33, 39]. Inability to undertake purposeful activity is revealed in patients with Parkinson's disease, like in other neurodegenerative diseases. This disorder is a predictor of the development of frailty [15, 39]. It is more common in people living in social shelters, using additional care from a nurse or relative when performing necessary daily activities, and in hospital patients. Hospitalization of patients with Parkinson's disease and frailty syndrome is associated with an increase in 30-day mortality, an increase in the duration of hospitalization, and rehospitalization within a month [33, 35]. Fatigue, the most common criterion of the Fried phenotype in Parkinson's disease, is also a non-motor symptom of the disease. Fatigue reduces daily physical activity and contributes to the progression of frailty syndrome.

An additional examination of a patient with Parkinson's disease and suspected frailty syndrome includes filling out questionnaires to assess the risk of falls, screening using a cognitive scale, examining for orthostatic hypotension, and screening for fatigue and hallucinations [33, 40].

Early diagnosis and therapy of the syndrome in a patient with Parkinson's disease are necessary for successful therapy of comorbid pathology. Given the diagnostic paradox, it is very difficult to do this. However, the risk is increased in women with a functional status decline and cognitive impairment [41]. Therapy requires the interaction of a geriatrician, neurologist, physical rehabilitation doctor, and psychotherapist (psychiatrist). A multidisciplinary approach helps reduce the risk of polypharmacy and select an adequate dose of medications for Parkinson's disease, reducing its severity, as well as motor and non-motor symptoms.

Physical therapy is the leading non–drug method of treating Parkinson's disease [42]. The classic treatment of Parkinson's disease with frailty syndrome is strength training, since the rehabilitation process is primarily aimed at overcoming sarcopenia [36, 42, 43]. Another paradox is that the possibilities of strength training are limited. Strength training increases muscle rigidity, exacerbating one of the main symptoms of Parkinson's disease. The second component of physical therapy is functional training which focuses on real-life activities, adapting the patient to the disease. The patient becomes less dependent on symptoms and becomes active in everyday life. A psychotherapist adapts patients to adequate emotional tolerance of their condition, conducts depression therapy, and reduces the effect of depression on face, voice, and posture [42].

The problems of the elderly are recognized in many countries, regardless of their economic situation. In Russia, an additional payment and social services have been established for people over 80 years of age. Vietnam, a low-income country, also provides a similar payment [6]. Financial and social support is justified from an ethical point of view and is also a preventive measure, since it reduces the expenses of the state on the treatment and rehabilitation in neglected cases [44].

CONCLUSION

Despite the fact that research on frailty syndrome began 15–20 years ago, increasing attention to the problem has been paid in the last 2–3 years. It is due to the COVID-19 pandemic. The elderly population aged 65 and older was the most affected group by

COVID-19. However, during the pandemic, it turned out that some of the elderly were not affected by this infection, since they had nonspecific resistance and did not get sick even before the vaccine was developed. Another part of the elderly died of COVID-19. These elderly people suffered from frailty and comorbid diseases.

Old age is associated with a stereotype: illness, loneliness, and limited financial resources. There are also elderly people who lead an active lifestyle: working, taking care of themselves and even their family, engaged in sports and recreational activities, gardening, and personal life. A lifestyle that prevents diseases of the modern world and slows down the rate of sarcopenia and aging is of great importance in the development of frailty syndrome, although the article also considers the contribution of genetic factors [45]. Undoubtedly, early diagnosis and rehabilitation play an important role in preventing the spread of frailty syndrome.

First of all, it is necessary to familiarize practitioners with frailty syndrome and conduct a differential diagnosis so as not to attribute the symptoms of frailty syndrome to other relevant pathologies. Still, prevention is even more important, especially among young and middle-aged people, to stop early aging and the onset of diseases of the modern world [1, 46].

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The role of estrogens in mitochondrial metabolism

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ABSTRACT

Central organelles in cells are mitochondria, which are essential for many fundamental biological processes. In the course of evolution, mitochondria have been transformed into signaling centers in biological systems that can cause changes in the cell via secreted factors and affect physiology of humans and animals.

Along with performing many key functions for the cell, mitochondria have also evolved into active hubs that can both control cellular programs through interaction with other compartments, such as the endoplasmic reticulum, and affect tissues, determining the health of the body via mechanisms that we are only beginning to understand.

Keywords: estrogens, mitochondria, sex differences, malignant tumors

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Роль эстрогенов в метаболизме митохондрий

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РЕЗЮМЕ

В клетках центральными органеллами являются митохондрии, которые необходимы для многих фундаментальных биологических процессов. Митохондрии в процессе эволюции трансформировались в сигнальные узлы в биологических системах, способные вызывать изменения в клетке посредством секретируемых факторов и влияющие на физиологию человека и животных.

В дополнение к выполнению множества ключевых функций для клетки, митохондрии также превратились в активные центры, которые могут контролировать не только клеточные программы посредством взаимодействия с другими компартментами, например, с эндоплазматическим ретикулумом, но также воздействовать на ткани, определяя здоровье организма с помощью механизмов, которые мы только начинаем понимать.

Ключевые слова: эстрогены, митохондрии, половые различия, злокачественные новообразования

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INTRODUCTION

Sex differences in human morbidity are partly due to the number of endogenous sex steroids that are involved in the regulation of mitochondrial metabolism. Although the mechanisms and targets by which estrogens directly or indirectly regulate mitochondrial function are not fully understood, it is clear that estradiol (E2) regulates the metabolism and morphology of mitochondria through nuclear and mitochondrial-mediated events, including stimulation of transcription factors that bind to genomic and mitochondrial DNA.

E2 and other estrogens, as well as synthetic GPER1 agonists, regulate mitochondrial bioenergetics, fusion, and division processes. Estrogens control the expression of genes, which, in turn, regulate mitochondrial functions, such as metabolism, OXPHOS, apoptosis, UPR^{mt}, division, and fusion. The mechanism of these events involves binding of E2 and other estrogens by receptors – the estrogen receptor α (ERα) and the estrogen receptor β (ERß) to regulate the transcription of nuclear genes and signaling cascades. In addition, estrogens activate protein G – a protein related to GTPases and functioning secondary intermediaries in intracellular signaling, coupled with the GPER1 receptor, which also regulates intracellular signaling events, including through cross–interaction with endothelial growth factor (EGFR) [1].

ESTROGENS AND THEIR FUNCTIONS IN THE BODY

Estrone (E1) and estriol (E3) were first isolated in 1930–1931 from the urine of pregnant women by Edward A. Doisy. E2 was later isolated by Dr. Doisy from the follicular fluid of pigs [2]. Subsequently, the metabolism of E2, its tissue-specific capture, cloning of ERα, as well as the discovery and cloning of ERß were described [1].

There are three primary estrogens – E1, E2, and E3. E2 is considered as the most active type because it has the highest affinity for ERα and is the dominant estrogen in women of reproductive age. Estrogens E1 and E3 have lower affinity for ERα. E2 is synthesized in the ovaries, whereas E1 is synthesized from androstenedione in the adrenal cortex, and E3 is mainly of placental origin, although each of them can be synthesized from androgenic precursors depending on tissue expression of the aromatase CYP19 [3].

Postmenopausal obese and overweight women have a higher level of circulating estrogens produced by adipose tissue compared to slim women [4]. Estrogens bind the ERα and ERß, which are conservative nuclear receptors (NR) with high identity in the DNA-binding and ligand-binding domains [5]. In addition to the full-sized ERα and ERß, each receptor subtype has a variety of splicing variants. ERα and ERß were identified in the mitochondria of various cell types, where they bind mtDNA. It was found that ERα also indirectly interacts with nuclear DNA through direct (protein : protein) coupling with other transcription factors associated with DNA [6].

Currently, there is a great interest in understanding sex differences in the disease in order to personalize treatment. The National Institutes of Health (NIH) mandates each grant application to consider “gender as a biological variable.” The list of diseases showing sex differences is too long and includes, for example, hypertension [7], ischemic stroke and myocardial infarction [8], as well as neurodegenerative and neuropsychiatric diseases [9].

Each of these diseases is associated with mitochondrial dysfunction. Differences in the prevalence of diseases that are associated with higher E2 levels in premenopausal women include type 1 and type 2 diabetes (T1DM1 and T2DM). Premenopausal women have lower incidence of metabolic disorders, while postmenopausal women are more likely to develop diabetes mellitus, cardiovascular diseases, and kidney diseases than men [10].

Mitochondrial dysfunction is involved in many diseases, while it is known that defects in hundreds

of genes involved in the mitochondrial biology cause pathologies [11]. These defects cover mutations in the mitochondrial genome itself, in nuclear genes encoding mitochondrial components, and in genes belonging to various functional classes affecting the mechanism of mtDNA replication, mitochondrial division and fusion, oxidative phosphorylation (OXPHOS) or biosynthesis of iron – sulfur clusters [12, 13]. Mitochondrial dysfunction is associated with insulin resistance [14] and multiple endocrine disorders [15]. The existence of sex differences depends not only on estrogens and androgens, but also on genes encoded by sex chromosomes [16].

Experimental and clinical studies found that E2 increases fat oxidation, inhibits lipogenesis, and regulates immune system cells, such as B cells, T cells, natural killer (NK) cells, neutrophils, and macrophages [17]. More than 75% of autoimmune diseases are more common in women. Recent studies have shown a direct relationship between the expression of ERα in T cells in the development of T-cell-dependent colitis in mice and a decrease in T cell proliferation [18]. The role of estrogens and ERα in systemic autoimmune diseases, where B cells and T cells are affected, has been described [19].

Hepatocellular carcinoma (HCC) is more common in men than in women, since estrogens have a protective effect against the emergence and progression of HCC [20]. Sex-dependent differences in liver metabolism cover the expression of cytochrome P450 liver enzymes, as well as transcription factors (TF), including ERα, the aryl hydrocarbon receptor (AHR), the peroxisome proliferator-activated receptor (PPAR) α, and the farnesoid X receptor (FXR), leading to differences in drug responses and metabolism in men and women [21].

Non-alcoholic fatty liver disease (NAFLD) is more common in men than in premenopausal women, but increases in postmenopausal women [22]. With drug-induced liver injury (DILI), sex-dependent differences also manifest themselves: 41 drugs affect the liver, and DILI is detected predominantly in women (only in premenopausal women). Interestingly, drugs for the treatment of DILI in women have a more pronounced effect on mitochondria, which is associated with the formation of reactive metabolites and a greater potential for inhibition of mitochondrial transporters [23]. The model of immune-mediated DILI in BALB/c mice showed that the production of proinflammatory hepatic cytokines (interleukin (IL)-6) in females was higher than in males, and hepatitis in male mice was more severe. This fact suggests that E2 and IL-6 may be responsible for a decrease in the protective and regulatory function of T cells [24].

There are three factors that emphasize the deep-rooted biological links between mitochondria and gender as a biological trait. Firstly, mammalian mitochondria are inherited through the maternal lineage, which means that they are transmitted exclusively through the egg. In the course of animal experiments, it was suggested that transfer of mitochondria to female and male organisms has different effects on metabolism and life expectancy [25].

Secondly, it is an underestimated fact that the stage limiting the rate of synthesis of all sex hormones, including estrogens, progestins, and testosterone, occurs in mitochondria, located mainly in the ovaries and testes [26]. The first enzymatic stages of the synthesis of all steroids, which also include glucocorticoids and mineralocorticoids, occur in the mitochondrial matrix [27].

Thirdly, mitochondria contain receptors for sex hormones. Both ERß receptors and androgen receptor (AR) move into the mitochondrial matrix, where they interact with mtDNA and affect many areas of the mitochondrial biology [28, 29]. Thus, the mitochondria of the genital organs have such a molecular mechanism that contributes to the development of and canonical mechanisms for hormone perception of sexual differentiation.

At the same time, A. Junker et al. (2022) [30] showed that stable binary sex-dependent differences were determined in greater mitochondrial content in female urine and isolated leukocyte subpopulations and higher ROS production in male skeletal muscles. Other measurements showed inconsistent sex-dependent differences with large discrepancies in the strength and direction of research, experimental conditions (for example, metabolic substrates), and evaluated tissues.

MITOCHONDRIAL HORMONE-REGULATING FUNCTIONS

Mitochondria are tightly packed dynamic organelles of bacterial and endosymbiotic origin [31]. Mitochondria support life by converting metabolites of food fuel into ATP, CO_2, and H_2O, while releasing heat and providing adaptation to stress for survival. The origin of mtDNA from oocytes leads to hereditary disorders that are transmitted through the maternal

lineage [31]. Paternal mitochondrial transmission is extremely rare [32].

Mitochondria contain their own DNA of the mitochondrial genome in the matrix. The mitochondrial genome is inherited through the maternal lineage and exists in the form of circular double-stranded DNA consisting of 16,569 base pairs in humans [33]. Since the mitochondrial genome encodes a small number of mitochondrial genes, including transport RNAs, mitochondrial ribosomal RNAs, and protein subunits of complexes with an electron transport chain, many mitochondrial genes are encoded in the nucleus. Thus, coordination of transcription events between mitochondria and the nucleus is necessary to maintain metabolic homeostasis [34].

During ATP production, electron transport also generates reactive oxygen species (ROS), which damage macromolecules, including mtDNA, proteins, and lipids. Estrogens and androgens protect mitochondria from the degenerative effects of aging in a tissue-specific way through activation of the corresponding receptors [35]. ROS contributes to mitochondrial stress and abnormal protein conformation. Misfolded proteins and aggregates accumulate in the inner membrane space (IMS) and the mitochondrial matrix, which leads to activation of the mitochondrial unfolded protein response (UPR ^{mt}).

Regulation of E2 – ERα by means of UPR ^{mt} is known. Recent studies show that breast cancer cells co-opt mitohormesis, the process of increasing basal UPR^{mt} and reducing oxidative stress, leading to increased invasion and metastasis, and, therefore, to worse survival of breast cancer patients with the UPR^{mt} gene signature [36]. E2 – ERα increases the transcription of sirtuin 3 (SIRT3) localized in mitochondria, where it weakens ROS by deacetylation of manganese superoxide dismutase (MnSOD, SOD2) and interacts with forkhead box protein O3 (FOXO3A) to activate its translocation into the nucleus. Next, the expression of genes encoding PGC-1α, a coregulator necessary for transcription, and MnSOD takes place [37].

REGULATION OF APOPTOSIS IN MITOCHONDRIA BY HORMONES

Mitochondria not only produce energy, but are also the site of synthesis of all steroid hormones, including E2 in granulosa cells of the ovaries [38, 39]. The participation of steroid hormones in the regulation of apoptosis has been established, so E2 is able to inhibit apoptosis via a variety of mechanisms. Under mitochondrial stress, mitochondria also produce and secrete mitokines, for example, humanin, a stress-sensitive peptide encoded by the *MT-RNR2* gene in mtDNA, and fibroblast growth factor 21 (FGF-21), which regulates energy metabolism [40]. Estrogen deficiency disrupts regulation of the L-type Ca2+ channel, the ryanodine receptor, the sarcoplasmic / endoplasmic reticulum Ca2+-ATPase (SERCA), and the Na+ – Ca2+ exchanger, causing impairment of Ca2+ homeostasis, which leads to cardiovascular diseases [41].

Another recent review summarized sex-dependent differences in human skeletal muscles [42]. It was found that many mitochondrial functional genes are expressed differently, and this correlates with known inter-sex differences in the composition of muscle fibers in women with a higher percentage of type I muscle fibers with a more oxidative phenotype [1]. Differences in gene expression in skeletal muscles of men and women are partly due to epigenetic changes, including differences in DNA methylation, histone modifications, and microRNA expression [42].

Activation of ER and G-protein-coupled estrogen receptor (GPER) preserves mitochondrial function and reduces mitophagy after injury (ischemia / reperfusion) by signaling dependent on mitochondrial permeability and activation of mitogen-activated protein kinase (MEK) regulated by extracellular signal-regulated kinase (ERK), thus reducing apoptosis by preservation of the mitochondrial integrity. In this regard, the administration of estrogen in *in vivo* models before ischemia / reperfusion reduces the infarct size and improves myocardial contractility [43].

Sex-dependent differences in mitochondria and mitochondrial function in various organs were considered, mainly in rodents [44-46]. A recent study reported that the effect of gender on gene expression and mitochondrial metabolism in adipose tissue depended on the mouse lineage when studying 100 inbred mouse lines [47].

The role of mitochondrial ERα and ERß in the transcription of mtDNA genes and the function of mitochondria depend on the type of cells, which is consistent with their specific localization. A group of researchers found that retrograde signaling via activation of the ROS-AKT pathway in response to UPR^{mt} activates ERα and increases nuclear respiratory factor-1 (NRF-1) signaling [48]. However, there are still many unresolved questions about the protective effects of estrogens in mitochondria that have yet to be fully elucidated.

THE ROLE OF ESTROGENS IN THE REGULATION OF LPO – ANTIOXIDANTS SYSTEM

Mitochondrial metabolism inevitably leads to the formation of ROS, which, in turn, cause mitochondrial dysfunction. E2 is known to cause a decrease in ROS levels and increase the amount of antioxidant proteins, including superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2) and glutathione peroxidase (GPx) [49]. On the other hand, in the vascular network, GPER modulates ROS by reducing NADPH oxidase 4 (NOX4), prostaglandin – endoperoxide synthase 2 (PTGS2), and GPx1, as well as by increasing the amount of antioxidant proteins, such as SIRT3 and glutathione S-transferase Kappa 1 (GSTK1) [49]. Consequently, as described in several studies, women differ from men in the level of antioxidants localized in the mitochondria, thus producing fewer free radicals and, in turn, less oxidative damage to the heart [50].

In this regard, some studies reported that female mitochondria produce half as much hydrogen peroxide as male ones and have higher levels of mitochondrial reduced glutathione. However, the mechanism through which E2 exerts these effects, as well as the involvement of other cell organelles have not yet been fully elucidated [51].

Another interesting feature that may be related to the modulation of ROS is the participation of E2 in the regulation of Ca2+ levels. Two studies showed that in female OVX mice, mitochondria had a reduced ability to retain Ca2+, which was restored after the introduction of E2, thus improving normal contraction and relaxation of the heart [41]. Similarly, several studies found that the regulation of mitochondrial homeostasis was of key importance for attenuating the damaging effects of various pathological processes in cardiovascular diseases. Certain proteins, such as the peroxisome proliferator-activated receptor-γ coactivator 1 α (PGC-1α), AMP-activated protein kinase (AMPK), and several genes involved in the electron transport chain (ETC), are regulated by sex hormones and, more specifically, by estrogen signaling [49].

ERα and ERß have been identified in mitochondria and reported to regulate mtDNA transcription [6]. E2 increases the transmission of redox signals in MCF-7 breast cancer cells containing ERα. This process is considered as part of the oncogenic process in breast cancer and involves the activation of AKT signaling, which leads to the initiation of NRF-1 [52]. E2 rapidly increased the temporal localization of ERα in mitochondria in MCF-7 cells and stimulated the direct ERα – MnSOD interaction, which was found using confocal imaging and co-immunoprecipitation [53]. The mitochondrial localization of ERα and the ERα – MnSOD interaction were blocked by fulvestrant, which suggested the importance of ERα conformation for the described interactions. Induced migration of ERα into mitochondria in MCF-7 cells is considered as a non-genomic E2 response to increased MnSOD acetylation of K68, which leads to inhibition of MnSOD activity. It was reported that the E2 – ERα – MnSOD bond blocks the MnSOD – SIRT3 interaction, increasing the superoxide level and activating mTORC2 [53].

Mitochondria contain NR – thyroid hormone receptor (TR), androgen receptor (AR), retinoid X receptor (RXR), RAR, glucocorticoid receptor (GR), and gamma receptor activated by the peroxisome proliferator-activated receptor (PPARG, PPARy2) [54]. ERß was identified in the mitochondria of the human heart. A recent study reported that low levels of mitochondrial ERß (mitoERß) were associated with an increased risk of breast cancer recurrence [55]. Transfection of MCF-7 breast cancer cells using GST-ERß followed by GST pull-down identified HSPA9 (mitochondrial heat shock protein 70; also called GRP75) associated with ERß.

MALDI-TOF mass spectrometry identified ERß and HSPA9 in the purified complex, and knockdown and overexpression studies showed that HSPA9 moved ERß into the mitochondria of MCF-7. Transfection of triple-negative breast cancer (TNBC) cells MDA-MB-231 by mitochondria-directed ERß expression vector reduced cell proliferation, invasion, and migration *in vitro* and tumor formation *in vivo*. A higher level of ERß was determined in mitochondrial fractions from ectopic endometrial tissues compared to uterine fibroids or controls without lesions [56]. Given the uncertainty regarding the specificity of some antibodies to ERß [57], further studies on the localization and activity of ERß in mitochondria are required.

The naturally occurring variants of ERα splicing, ERα36 and ERα46, are the result of the use of a differential promoter and splicing, which leads to shortened forms of ERα devoid of N-terminal domains A and B, which make up AF-1. ERα36 also lacks the F-domain at the C-end of the full-size ERα66 and has a shortened LBD [58]. It was reported that ERα 36 is localized mainly in the mitochondria of the human

uterine leiomyoma (UtLM) and smooth muscle cell lines and interacts with inhibin (PHB) [59].

B.N. Radde et al. reported that E2 (10 nM) stimulated the baseline oxygen consumption rate (OCR) and baseline extracellular acidification rate (ECAR) in breast cancer cells MCF-7 and T47D lumen A (ERa+) and activated ATP-bound OCR, while not affecting the maximum mitochondrial reserve capacity. The authors suggested that E2 did not affect the tolerance to cellular stress in these cell lines [60]. Medroxyprogesterone acetate (MPA) inhibited the potentiation of E2 primary neurons of the rat hippocampus and mitochondrial reserve capacity of glial respiration *in vitro*, however, the mechanisms of this phenomenon have not been disclosed. In the meantime, a recent study showed that ERα knockout in CD4+ T cells reduced mitochondrial reserve capacity, and it was assumed that ERα regulated mitochondrial metabolism in T cells [18].

A search in PubMed for articles investigating the effect of estrogens on mitochondrial bioenergetics revealed relatively few results. Thus, one group of researchers found that overexpression of ERα in SK-N-BE(2) MYCN-amplified (MNA) neuroblastoma (NB) cells suppressed the growth of a tumor xenograft, blocking many processes associated with oncogenesis of NB [61].Glycolysis (measured as ECAR in the Seahorse bioanalyzer), maximum glycolytic capacity, and glycolytic reserve were significantly reduced in cells overexpressing ERα, and treatment with E2 and nerve growth factor had no additional effect on any of these parameters in NB cells [61].

Similarly, the baseline level of OCR, ATP-bound OCR, and mitochondrial bioenergetic reserve capacity were increased in ERα cells overexpressing SK-N-BE(2) MNA NB, which was partially mediated by suppression of fatty acid utilization. Overexpression of the ERß-labeled mitochondrial-targeting sequence in primary human endometrioid cells increased basal OCR and mitochondrial reserve capacity. ERß knockdown reduced the expression of NRF1, TFAM, MT-CO1, and MT-ATP6 transcripts in endometrioid cells and increased the anti-apoptotic protein BCL-2, thereby helping cells avoid mitochondrial apoptosis caused by oxidative stress [56].

Studies on female mice with muscle-specific Esr1 (ERα) ERα (MERKO) knockout showed that glucose homeostasis was impaired in mice of this line, and obesity was present in combination with aberrant mitochondrial morphology, increased ROS, impaired mitochondrial division, and an imbalance of calcium and ATP production. These data indicate a key role of the mitochondrial function of ERα in muscles. It was shown that the level of ERα was reduced in the muscles of women with metabolic syndrome. Transmission electron microscopy revealed elongated hyperfused mitochondria with an increased content of inactive diamine-related protein 1 (DRP1) phosphorylated by the inhibitory serine residue (SER 637).

The authors also observed an increase in regulator of calcineurin 1 (Rcan1) and calcineurin inhibitor leading to mitophagy disorders and increased ROS, which causes inflammation and insulin resistance [62]. The E2 – ERα bond was also necessary to maintain the number of satellite cells (muscle stem cells) in the muscles of female rodents and humans [63]. Indeed, in mice with ERα knockout (Esr1 -/-), a decrease in fatty acid oxidation in muscles, increased overall obesity, impaired mtDNA replication, mitophagy, and autophagy, failure of insulin signaling (including glucose utilization), high levels of H_2O_2 and superoxide, lipid accumulation, and inflammation were recorded. The authors noted the importance of identifying methods and selecting therapeutic agents for modulation of tissue-specific pathways regulated by ERα, which will adjust the energy balance and glucose homeostasis, especially in postmenopausal women [64].

Replacement therapy using E2 in female mice after ovariectomy improved the activity of mitochondrial complex I (CI) and decreed H_2O_2 in skeletal muscles, but increased CI-mediated H_2O_2 production and decreased the intensity of OXPHOS in the liver. The authors stated that "the mechanism(s) of tissue specificity of E2 effect on mitochondrial function remains unknown" [65]. At the same time, transcriptome profiling revealed microRNAs controlling glycolysis and oxidative metabolism in the muscle fibers of male mice [66]. The role of estrogens in the regulation of these microRNAs is still known.

Interestingly, studies examining miRNAs in skeletal muscles of homozygous twins with discordant use of hormone replacement therapy (HRT) revealed miR-182, miR-233, and miR-142-3p targeting IGF-R1, FOXO3A, and inflammatory signaling [67]. In another study, this group of scientists also identified E2 regulation of muscle energy pathways in women receiving HRT [68].

In mice with the ERα – MERKO knockout, a change in the morphology of mitochondria was demonstrated, and mitochondrial elongation occurred. In addition, impairment of mitochondrial division by pronounced

suppression of signal transmission took place. In fact, ERα deficiency leads to suppressed phosphorylation of DRP1, a key factor in mitochondrial division. That is, the mitochondrial dysfunction phenotype in muscles prevails in MERKO mice. Obviously, ERα is necessary to maintain mitochondrial function and protects against mitochondrial-related health disorders in women [69].

In vitro experiments using molecular methods determined that E2 increased the levels of mRNA transcripts MFN1, MFN2, OPA1, and DRP1, while reducing FIS1 during 4-hour treatment of MCF-7 cells, and these transcriptional responses were inhibited by antiestrogenic fulvestrant (ICI 182.780). The authors reported that E2 induced mitochondrial fusion in MCF-7 cells, reduced the expression of OXPHOS complex proteins, and increased ATP levels.

Similar results were recorded in T47D cells treated with E2. In addition, overexpression of ERß in T47D cells was found to increase the number of OXPHOS complex proteins and reduce division, while increasing fusion [70]. On the contrary, activation of the E2 – Erα pathway in MCF-7 cells increased phosphorylation of DRP1 at ser616 to induce DRP1 activity leading to mitochondrial division [71]. There was a need for ERα, since knockdown blocked E2 and induced phosphorylation of DRP1, but the authors did not evaluate whether this was mediated by genomic or non-genomic activation.

From the standpoint of aging, it was established that E2 protects against cellular aging and mitochondrial dysfunction. This fact was revealed in experiments using human umbilical vein cells and vascular smooth muscle cells in female C57BL/6 mice [72]. The ability of E2 to increase mitochondrial autophagy and maintain mitochondrial function, thereby slowing down aging is known. However, E2 does not modulate the microtubule-associated protein 1 light chain 3 (LC3), as well as the deficiency of the autophagy-related protein 7 (ATG7). Moreover, E2-mediated effects on mitochondrial autophagy were eliminated using either Unc-51-like kinase-1 (Ulk-1) or Ras-related protein Rab-9 (Rab9).

These results showed that E2-mediated mitochondrial autophagy is associated with Rab9-dependent alternative autophagy. In addition, E2 enhances the regulation of sirtuin 1 (SIRT1) and activates liver kinase B1 (LKB1), AMPK, and Ulk1, which indicates that the effect of E2 on the induction of Rab9-dependent alternative autophagy is mediated by the SIRT1/LKB1/AMPK/Ulk1 pathway. Compared with sham-operated mice, mice with ovariectomy (OVX) are characterized by reduced mitochondrial autophagy, increased mitochondrial dysfunction, and aging of the arteries, all of which was successfully blocked by E2 [72].

CONCLUSION

Mitochondria are organelles inherited through the maternal lineage, which have the most important tissue-specific functions, including hormone synthesis and energy production, affecting human development, health, and aging. However, it still remains unclear whether mitochondria of women and men are characterized by stable biological differences, which is a serious gap in knowledge. Solving this issue is of paramount importance for the development of clinically specific indicators of mitochondrial biology and the construction of comprehensive human health models that include mitochondrial bioenergetics.

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Urinary tract microbiota in patients with multiple sclerosis and neurogenic pelvic dysfunction

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ABSTRACT

Multiple sclerosis (MS) is a chronic progressive disease of the central nervous system common among young people. Neurogenic bladder often is a common symptom of the disease. Young people with MS often have to make treatment and family planning decisions at the same time.

The possibility of realizing reproductive plans is closely related to urological complications of the disease, high risk of urinary tract infections, and sexual dysfunction. In addition, disease modifying therapies for MS play a significant role in increasing the likelihood of infectious complications. Therefore, the issue of infection prevention in MS is critical. Effective personalized prevention of urogenital infections is possible with a clear understanding of the microbiota composition.

DNA sequencing methods have changed the conventional idea that normal urine is sterile and gave rise to the concepts of asymptomatic bacteriuria in healthy people. Moreover, data on the genitourobiome of patients with neurological diseases have recently emerged. Extended knowledge about the microbiology in the genitourinary system of neurological patients is necessary to unleash the capacity of health-preserving technologies.

The aim of the review was to integrate currently available data concerning the microbiocenosis of the lower urinary tract and vagina with underlying neurogenic pelvic dysfunction, including MS, as well as to present data on the association between closely located biotopes and the effect of MS therapy on the risks of developing genitourinary infections.

Keywords: multiple sclerosis, neurogenic bladder, vaginosis, microbiota

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Микробиота мочевых путей у пациентов с нейрогенной дисфункцией тазовых органов при рассеянном склерозе

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РЕЗЮМЕ

Рассеянный склероз (РС) является хроническим прогрессирующим заболеванием центральной нервной системы, распространенным среди лиц молодого возраста. Частый симптом болезни – это нейрогенная дисфункция тазовых органов. Молодым людям с диагнозом РС зачастую приходится одновременно принимать решения, связанные с лечением и планированием семьи.

Возможность реализации репродуктивных планов тесно связана с урологическими осложнениями заболевания, высоким риском инфекционных процессов мочеполовой системы, сексуальной дисфункцией. Кроме того, многие препараты, изменяющие течение РС, играют значительную роль в увеличении вероятности инфекционных осложнений. В связи с чем актуальной проблемой являются вопросы профилактики инфекций при РС. Эффективная персонализированная профилактика урогенитальных инфекций возможна при четком представлении о составе микробиоты.

Методы секвенирования ДНК позволили изменить классические представления о том, что здоровая моча стерильна. Были сформированы представления о бессимптомной бактериурии у здоровых людей, и в последние годы появляются сведения о генитоуробиоме пациентов с неврологическими заболеваниями. Расширение знаний о составе микроорганизмов мочеполовой системы неврологических пациентов необходимы для формирования потенциала здоровьесберегающих технологий.

Цель обзора: обобщение известных к настоящему времени сведений о микробиоценозе нижних мочевых путей и влагалища при нейрогенной дисфункции тазовых органов, в том числе при РС, представление данных о связи близко расположенных биотопов и влиянии терапии РС на риски инфекций мочеполовой сферы.

Ключевые слова: рассеянный склероз, нейрогенная дисфункция мочеиспускания, вагиноз, микробиота

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INTRODUCTION

Currently, the environment and the way it impacts the genetic factors are considered the basis for the development of autoimmunity in the human body. The composition of the gut microbiota and the gut – brain axis are being increasingly considered as a prerequisite for the development of immune-mediated conditions of the nervous system, including multiple sclerosis (MS). In the modern world, this concept is of great importance since microbial dysbiosis arises from the so-called Western diet, widespread use of antibiotics, and excessive sanitation.

Until recently, scientists have focused specifically on studying the gut microbiota in MS patients. Recently, research has been conducted on other loci in the host organism. It has been shown that oral cavity microorganisms might affect the level of inflammation in MS, thereby contributing to the mechanisms of the disease [1]. It should be noted that data on the composition and functioning of other biotopes are scarce or even lacking. At the same time, it is suggested that neighboring loci may be closely connected. It is likely that changes in the composition and functional activity of the microbiota in one location can lead to dysfunction in nearby areas.

Bowel and bladder dysfunction is part of the varied MS clinical pattern and is reported in 50–90% of patients [2]. On average, 6 years after the disease onset, urinary tract infections (UTIs) are diagnosed in lower urinary tract (from 13 to 74%) and 8% in upper urinary tract (from 0 to 25%), while chronic renal failure and urosepsis are some of the leading causes of death in patients with MS. At the same time, obstructive and mixed forms of urination disorders bring the highest level of threat due to progression of infectious inflammation and are significantly more common in MS.

The aim of this review was to summarize the present knowledge about the composition of the urinary

tract microbiota in patients with neurogenic bladder, especially in MS, and to interpret the risks of infectious diseases of the genitourinary system. The information search was conducted both in Russian and English using keywords, such as multiple sclerosis, urinary microbiota, vaginitis, neurogenic bladder, and lower urinary tract infections. For the information search, PubMed and elibrary.ru databases were used. A total of 251 publications (clinical studies, meta-analyses, randomized controlled trials, systematic reviews) over the past 15 years were identified and 46 relevant articles on the problem under study were selected.

MICROBIOTA OF THE LOWER URINARY TRACT

Until late, urine culture tests proved urine to be sterile, and the availability of bacteria therein was referred to an inflammatory response and UTIs. Yet, modern highly sensitive diagnostic tests established that the urinary tract of a healthy person is not sterile throughout its entire length. The year 2010 marked the emergence of a new understanding of asymptomatic bacteriuria (AS) owing to the studies by D.E. Nelson et al. [3] and a group of Russian scientists under the supervision of M.I. Kogan [4]. Their findings on the presence of bacteria in the urine of healthy men and women was confirmed by H. Siddiqui et al. in 2011 by metagenomic sequencing [5].

A.J. Wolfe et al. studied urine sampled from healthy women by various methods. The presence of bacteria in these samples was assessed by urine culture test, light microscopy, and *16S* rRNA gene sequencing. Urine samples obtained from the urethra by spontaneous voiding contained bacteria both from urinary and genital tracts. Microorganisms identified in urine samples simultaneously collected using a transurethral catheter and suprapubic aspiration had similar properties and additionally contained nonculturable bacteria, which allowed the authors to conclude that the bladder was not sterile [6].

In later years, new information has come to light regarding urinary microbiota in healthy individuals and in a number of diseases and conditions: overactive bladder [7], urinary incontinence [8], chronic prostatitis [9], interstitial cystitis [10, 11], bladder and prostate cancer [12–14], urolithiasis [15]. Genomics techniques revealed the existence of a microbial community in the bladder, which standard culture test failed to determine.

Thus, all people normally have bacteriuria, and given the fact that not only bacteria, but also other microorganisms (viruses, fungi) are found in the urinary tract, it is now more justified to use the concept of "urinary tract microbiome" instead of the term "bacteriuria". The urinary normobiome differs genderwise. Men have a significantly higher relative content of *Corynebacterium* bacteria, while women have a higher content of *Lactobacillus*. Moreover, given high importance of the microbiome, some scientists even suggest using the term "urinary tract dysbiosis" instead of "urinary tract infection" (UTI) [16].

Despite the fact that a large number of studies are dedicated to the composition of the bladder microbiome [17–19], no data are available on colonization of the upper urinary tract, due to obvious technical difficulties. It has been suggested that microorganisms are present in the urinary tract throughout its entire length [6]. This discovery is crucial. Excessive and unjustified attempts to sanitize the genitourinary tract for AS cause resistance to antimicrobial medications, which in turn leads to inadequate treatment of UTIs, increased morbidity, and mortality.

TRANSLOCATION MECHANISM OF MICROORGANISM MIGRATION

As of today, a growing number of researchers are attempting to study the microbial biocenosis of the genitourinary tract both in healthy individuals and in patients with UTI in terms of its relationship with the large intestine microbiota. This approach is aimed at elaborating the etiopathogenesis of infectious diseases in the genitourinary tract. In addition, it is assumed that anorectal disorders (in particular, constipation) may trigger the development of UTIs [20]. Given the fact that a number of neurological diseases, including MS, are characterized by pelvic dysfunction in the clinical presentation, urination disorders often coexist with bowel dysfunction.

Attempts to investigate the translocation mechanism of bacterial migration from the large intestine to the organs of the genitourinary system led to the conclusion that uropathogenic gut bacteria effectively colonize the urinary tract. For instance, healthy women with no UTI episodes in history had fecal *Escherichia coli* isolates in urine, which in general were closely related in the genomic pattern to fecal *E. coli* fecal isolates recovered from UTI patients [21]. When studying cases of UTI caused by *Klebsiella pneumoniae*, this microorganism was also reported to originate from the colon [22].

Yu. L. Naboka et al. presented data on significant correlation coefficients between microorganisms

recovered from urine and those from the colon, which indirectly confirms the translocation mechanism [23]. Alternatively, it is known that normal urine microbiome is characterized by the predominance of *Lactobacillus* in women and *Corynebacterium* in men. *Corynebacterium* microorganisms are common representatives of skin microbial flora in men, while vagina is colonized by various species of the *Lactobacillus* genus, which probably indicates the existence of translocation migration mechanisms between these loci. Therefore, the existence of a fecal – perineal – urethral transmission route of microbial flora to the genitourinary tract is still currently being discussed [24].

MICROBIOTA IN NEUROGENIC BLADDER

Data on the urinary tract microbiome in neurological patients with neurogenic bladder as part of their clinical pattern are scarce and refer to a small group of patients with traumatic spinal cord injury. A cross-over study aimed at examining urinary microbiome compared 27 patients with spinal injury and symptoms of neurogenic bladder with healthy volunteers, using 16S rDNA sequencing and the metaproteomics technique. It is worth noting that more than one technique of urinary diversion was used for 19 patients (intermittent catheterization, Foley catheter).

The top ten bacterial taxa predominant in abundance and variability in urine samples included Lactobacillales, Enterobacteriales, Actinomycetales, Bacillales, Clostridiales, Bacteroidales, Burkholderiales, Pseudomonadales, Bifidobacteriales, and Coriobacteriales. Moreover, Lactobacillales and Enterobacteriales comprised the two most abundant and variable taxonomic groups [25]. Clear *Lactobacillus* predominance was detected in the urine of healthy women in the control group. At the same time, a progressive decrease was noted in the abundance of representatives of normal vaginal microbial flora in women with neurogenic bladder with any type of voiding (whether spontaneous or using a catheter). It is likely that increasing time of catheter use of more than 3 months and an increase in the severity of neurogenic bladder affect the ability of *Lactobacillus* to colonize the urinary tract [25].

An alternative explanation for this phenomenon may be due to the proximity of the external urethra to the vaginal microbiocenosis, therefore bacteria of the *Lactobacillus* genus are considered as contaminants of the urinary tract. However, taking into account the work by A.J. Wolfe et al., who proved the presence of *Lactobacillus* representatives in urine sampled both by transurethral catheters and by suprapubic aspiration directly from the bladder, this assumption seems unlikely [6] *Lactobacillus* bacteria produce lactic acid and thereby control the growth of virulent bacteria incapable of surviving in a more acidic environment. It is suggested that the presence of *Lactobacillus* in the urethra and / or bladder may have a protective effect in both women and men. So, Q. Dong et al. confirmed the presence of *Lactobacillus* microorganisms in clean urine samples of healthy men [26].

This hypothesis has critical implications for individuals with neurogenic bladder. The need to use assistive technology increases the risk of UTI, while dysbiosis of the urinary tract provides a favorable environment for the growth of pathogenic microorganisms. Thus, representatives of *Lactobacillus* are considered as commensal microorganisms reflecting the state of eubiosis more often in women than in men. The microbiome in the risk group for developing UTIs may be characterized by a lower level or even obvious absence of *Lactobacillus*. Taken together, these data suggest that the clinical goal of sterile germ-free urine may not be optimal for the patient.

The microbiological pattern of neurogenic bladder varies depending on the method of urine diversion. Thus, when a catheter is used, *studies describe the predominance of other microorganisms from* the Lactobacillales family, like *Aerococcus* and *Enterococcus* bacteria in addition to a significant decrease in the *Lactobacillus* representation. Two species of aerococci, *A. urinae* and *A. sanguinicola*, may cause UTIs. In addition, the number of Enterobacteriaceae in the urinary microbiota of patients with neurogenic bladder increases with increasing duration of symptoms.

Similar data on the urinary microbiota composition in neurogenic bladder caused by spinal cord injury or *spina bifida* were obtained by E.S. Filippova et al. [27]. The authors noted a correlation between the results of urine culture test and 16S rRNA sequencing data. Consistent with earlier studies, the urobiome of these patients consisted of a variety of *Enterobacterales*. Representatives of *Escherichia*, *Klebsiella*, *Lactobacillus,* and *Enterococcus* were the most common microorganisms.

Metagenomic sequencing provides an opportunity to identify microorganisms undetectable in urine culture tests. In a number of patients, up to 21 genera

were revealed in the urine, namely Cellulomonad (*Cellulomonas* spp.), Prevotellaceae (*Prevotella melaninogenica, Prevotella* spp.), Flavobacteriaceae, and Bacillales Family X. Incertae Sedis, Gemella (*Gemella asaccharolytica*), Carnobacteriaceae (*Carnobacterium* spp.), Veillonellaceae (*Veillonella spp*.), Peptoniphilaceae (*Parvimonas* spp.), Sphingomonadaceae (*Sphingomonas* spp.), Pseudoalteromonadaceae (*Pseudoalteromonas* spp.), Moraxellaceae (*Acinetobacter* spp.), and Vibrionaceae (*Vibrio* spp.).

URODYNAMIC FUNCTIONAL STATUS OF THE BLADDER AND UROBIOME

The mechanisms underlying the interaction of the bladder and microbial flora are diverse and are the subject of scientific debate. One of them is disturbance in bladder wall blood supply due to high intravesical pressure. Tissue ischemia provides favorable conditions for gut microbial flora to colonize the mucosa. Moreover, impaired wall tropism leads to histologic changes in the detrusor, namely, the volume of muscle fibers decreases and connective tissue develops in their place. Such transformations involve a decrease in bladder plasticity and increased ischemia [27, 28].

Another issue related to neurogenic bladder is a high risk of vesicoureteral reflux in the context of high intravesical pressure. Such a disturbance in urodynamics, in turn, is a proven risk factor for UTI in patients with neurogenic bladder [27, 29]. Therefore, the prevention of UTIs in a patient with a neurological disease is based on correction of urodynamics, control of neurogenic detrusor overactivity, prevention of vesicoureteral reflux, and the choice of the optimal urine diversion technique. The place of probiotics in preventing UTIs is still yet to be discussed, since there is insufficient knowledge about the composition and functions of the urinary tract microbiota and a lack of clinical studies that would confirm the proven effectiveness of such a preventive approach.

One of the modern methods incorporating all preventive objectives is therapy for detrusor overactivity with botulinum toxin type A. In a pilot project, E.S. Filippova et al. observed that the qualitative composition of the urinary tract microbiome changed during botulinum therapy for neurogenic bladder. The authors noted a trend toward restoration of eubiosis, specifically, three women out of four demonstrated an increase in Lactobacillaceae and a decrease in Enterobacteriaceae bacteria family [27].

LOWER URINARY TRACT MICROBIOTA AND MULTIPLE SCLEROSIS

Many neurological diseases have pelvic dysfunction in their clinical pattern. However, in publications, special attention is paid to microbiological changes in urine in spinal injury and congenital pathology of the spine.

It has been established that *Escherichia coli*, *Pseudomonas aeruginosa,* and *Klebsiella pneumoniae* are most common in urine in MS. These data were obtained in studies using culture tests. We have not found data on the use of methods that allow for identifying nonculturable bacteria in the urine of patients with MS and neurogenic pelvic dysfunction. It is known that MS is characterized by high prevalence of UTIs and a high relapse rate [30].

Accordingly, in a prospective analysis of 798 MS clinical cases, every third person with primary progressive MS had a history of UTI; every fifth patient with relapsing – remitting MS (RRMS) experienced vaginitis or UTI; 40% of patients with secondary progressive MS had a history of UTI or vaginal infection [31]. However, there are no clear concepts concerning the composition of the lower urinary tract microbiocenosis in MS patients.

It should be noted that sometimes UTIs provoke increased clinical disease activity, aggravating MS [32]. In addition, pharmacological treatment for AS is still often used unnecessarily in clinical practice, even though experts currently emphasize that this approach has no evidence of clinical effectiveness. Since AS treatment causes a significant increase in more resistant strains of bacteria, it should be prescribed in exceptional cases for recurrent acute UTIs, prior to UTI treatment procedures, during pregnancy, or in patients requiring immunosuppression [32].

Lower urinary tract infection with multidrug resistant microbial flora, recurrent UTIs, and irrational use of antibiotics create another problem – antibiotic resistance. Moreover, UTIs in this group of patients are associated with high hospitalization rate and increased mortality. Unlike other neurological patients, those with MS represent a special population exposed to multiple risk factors for the development of infections, such as constant immunomodulatory treatment, episodes of high-dose glucocorticoid therapy for disease exacerbations, focal damage to the spinal cord and concomitant pelvic dysfunction, which often leads to the need for bladder catheterization.

MS patients receive lifelong treatments aimed at modifying the functioning of the immune system, the so-called MS disease-modifying therapy (DMT). In Russia, there are about 11 medicinal compounds registered; they vary by mechanisms of action and degree of influence on the immune system. DMTs in Russia include glatiramer acetate (GA), interferon β-1b and β-1a, teriflunomide (TFN), dimethyl fumarate (DMF), fingolimod, natalizumab (NAT), ocrelizumab, acrelizumab, cladribine, etc. The likelihood of the effect of DMTs on the lower urinary tract microbiota remains a matter of debate.

In 2020, J. Hellgren et al. suggested that the use of rituximab, a medication not registered in Russia for the treatment of MS, may increase the risk of developing UTIs [33]. C.G. Chisari et al. confirmed that the use of rituximab can increase the incidence of UTIs in MS [34]. However, these studies did not investigate the correlation between the duration of rituximab therapy and the incidence of UTIs. Later, M.A. Mesgarof et al. showed that the incidence of UTI increased proportionately to the increasing time of rituximab use [31].

Currently, there is a controversy regarding the use of DMTs and the risk of UTI. It was observed that UTIs were significantly more common in patients receiving alemtuzumab. In two clinical studies, the incidence of UTIs was higher with alemtuzumab compared to interferon β-1a and slightly lower (but not statistically significant) in another study [31, 35].

Some studies confirmed that treatment with interferon β-1b may increase the incidence of UTIs [32, 36]. On the other hand, the 2021 expert consensus on infectious complications during DMT stated that the interferon group did not increase the risk of UTI in MS patients, like the majority of other DMTs, such as GA, TFN, DMF, NAT, fingolimod, cladribine, and ocrelizumab [30]. However, in a more recent study, M.A. Mesgarof et al. obtained data on the significant effect of interferon β-1b therapy and its duration on the likelihood of developing UTI; in turn, interferon β-1a did not increase the risk of UTI [31].

In 2021, B.A. Cree et al. conducted a randomized clinical trial on the effectiveness and safety of fingolimod for the treatment of MS; GA was chosen as a reference-listed drug [37]. In their publication, the authors considered UTI as an adverse event of therapy in the GA group. In addition, M.A. Mesgarof et al. also found that exposure to GA increased the incidence of UTIs, while the duration of its use did not significantly affect this risk [31].

VAGINAL MICROBIOTA AND MULTIPLE SCLEROSIS

MS itself has no adverse effects on fertility, gestation, or childbirth, yet such patients should be especially careful when planning pregnancy. The decision on the possibility of carrying to term is affected by immunotropic therapy and the course of the disease and concomitant conditions, in particular neurourological disorders that increase the risk of UTI.

The composition of the vaginal biotope in MS patients is yet to be studied in detail. We have not found studies devoted to the quantitative and qualitative microbiological composition of the vagina in MS. However, similar to the concept of susceptibility of MS patients to infectious diseases, there is a problem of increasing incidence of bacterial vaginitis in women with MS. This is indicated by some studies and descriptions of clinical cases of infectious vaginitis in women receiving DMT [31].

A long-term follow-up program of patients receiving NAT in context of DMT proved infections to be the most common adverse events of therapy, of which UTIs developed in 0.3% of cases [38]. G.M. Makris et al. note that recurrent vaginitis should be considered as a possible side effect that occurs with long-term NAT treatment [39]. They described a clinical case of a patient with RRMS who suffered from persistent gynecological infections, and culture tests detected pathogenic microorganisms in vaginal secretions. The mentioned female patient received NAT treatment for three years. The chronic infectious and inflammatory condition led to the repeated use of both local and systemic antibacterial and antifungal drugs.

Previously, a number of authors also mentioned natalizumab as a risk factor for the development of vaginitis in MS women [40, 41]. However, the study by M.A. Mesgarof et al. revealed no association between MS treatment with this monoclonal antibody and the development of vaginitis [31].

The use of rituximab within anti-B-cell therapy for MS may also be accompanied by a suboptimal status of the vaginal microbial flora which underlies the disappearance of lactobacilli and increases the risk of infectious complications [42].

J.M. Lee et al., in their interpretation of adverse events of fingolimod therapy, in 2015 reported vaginitis to be a side effect of the drug [43]. M.A. Mesgarof et al. also concluded that fingolimod treatment increased the risk of vaginitis regardless of the duration of therapy [31]. However, more recent

studies have shown contradicting data; a number of studies have not noted an increase in the incidence of vaginal infections [44, 45].

Contemporary literature contains almost no evidence that GA is a risk factor for the development of vaginitis, with the exception of the study by M.A. Mesgarof et al., which demonstrated that long-term GA therapy may contribute to the infectious process in the vagina [31].

The study on the relationship between the type of MS and the incidence of vaginal infections and inflammatory diseases established that vaginitis most often occurred in RRMS, but no significant differences were observed [31].

It should be noted that most studies had a number of limitations related to the number of patients, the methods used, and the influence of concomitant factors.

CONCLUSION

Modern ideas about the urinary tract microbiota in patients with neurogenic bladder suggest the presence of asymptomatic bacteriuria with a pronounced decrease in the bacteria of the normal flora. Voiding dysfunction and its duration, as well as the urinary diversion method are recognized risk factors for UTI, but unanimity was not reached regarding the effect of other factors. Many issues remain unresolved regarding the genitourobiome of MS patients.

This category of neurological patients is a risk group for the development of infectious and inflammatory diseases of the genitourinary system. The probable translocation of microorganisms between loci and the relationship between the functional status of the bladder and the composition of the microbiome may become the basis for new preventive and treatment strategies to solve the problem of UTI, maintain reproductive health, and support family planning. Currently, further research of genitourinary microbiocenosis in MS patients is required to expand fundamental knowledge and improve the quality of medical care.

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Authors' contribution

Luzanova E.I. – conception and design, analysis and interpretation of the data. Karpova M.I., Abramovskikh O.S. – justification of the manuscript or critical revision of the manuscript for important intellectual content, final approval of the manuscript for publication. Chetvernina E.A., Kupriyanov S.V. – conception and design.

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Pyroptosis and its therapeutic potential

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ABSTRACT

The review examines present data on pyroptosis – a type of programmed cell death associated with infection with various pathogens. During pyroptosis. specific molecular complexes, inflammasomes, are formed, caspases are activated, and proinflammatory cytokines are produced.

We consider the mechanisms of pyroptosis activation, including canonical and non-canonical pathways, as well as methods for its detection in cells. The review substantiates the relevance of studying the role of pyroptosis in pathological processes in different tissues. We focus on the therapeutic potential of pyroptosis, including its role in the treatment of sepsis. Pyroptosis is involved in sepsis-induced tissue damage in various organs, so regulation of this type of cell death can serve as the basis for the development of innovative treatment methods.

Keywords: cell death, pyroptosis, gasdermins, inflammasome, caspases, therapy, sepsis

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Пироптоз и его терапевтический потенциал

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РЕЗЮМЕ

В обзоре рассмотрены современные сведения о пироптозе – форме запрограммированной гибели клеток, связанной с инфицированием различными патогенами. При этом образуются специфические молекулярные комплексы – инфламмасомы, происходит активация каспаз и выработка цитокинов, опосредующих воспаление.

Рассмотрены механизмы активации пироптоза, включающие канонический и неканонический пути, а также методы его выявления в клетках. Обосновывается актуальность исследования роли пироптоза в патологических процессах в разных тканях. Акцентировано внимание на терапевтическом потенциале пироптоза, в том числе при лечении сепсиса. Пироптоз вовлечен в вызванные сепсисом повреждения тканей разных органов, поэтому регулирование этой формы клеточной гибели может служить основой для разработки инновационных методов лечения.

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Ключевые слова: клеточная гибель, пироптоз, гасдермины, инфламмасома, каспазы, терапия, сепсис

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

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INTRODUCTION

The vital activity of cells includes a number of key processes, namely proliferation, differentiation, adaptation, reactive changes, etc. The terminal phase of cell life cycle is death, which is implemented by simultaneous or sequential involvement of certain biomolecules. The German researcher Karl Vogt was the first to mention cell death; he described the death of embryonic cells of the notochord in 1842 [1]. Since then, the understanding of cell death has expanded significantly. Scientists paid close attention to the study of forms of cell death only in the second half of the XX century, which contributed to the creation of the international Nomenclature Committee on Cell Death (NCCD).

Cell death occurs both under normal (physiological) conditions and during pathological histogenesis. It is one of the fundamental cytophysiological processes in all living organisms and is observed in embryonic development, during the normal functioning of tissues and organs, aging, immune (including autoimmune) responses, irreversible reactive changes, and various pathological processes. For a long time, experts considered non-programmed cell death (necrosis or necrobiosis) as the main form of cell death. Later, they discovered apoptosis, and more recently, autophagy-dependent cell death, neutrophil extracellular traps, ETosis, entotic cell death, ferroptosis, mitotic catastrophe, death through terminal differentiation, etc. [2–7].

One of the recently identified forms of proinflammatory cell death is pyroptosis, which occurs not only in leukocytes and macrophages, but also in other cells and affects the course of both normal and pathological histogenesis [8–10]. In recent years, its role in the pathogenesis of certain diseases has been discovered, and the possibilities of therapeutic effects on this process are under discussion [7, 11, 12].

HISTORICAL REFERENCE

The term “pyroptosis” takes its origin from the Greek roots “pyro” – fire, fever, and “ptosis” – fall. A. Zychlinsky et al. [13] described pyroptosis for the first time in the late XX century and revealed the death of macrophages infected with *Shigella flexneri*. In 1996, D.M. Monack et al. published a study describing the death of macrophages infected with *Salmonella enteric* (serotype *Typhimurium* – *S. typhimurium*) [14]. Due to some similarity in morphological manifestations and the absence of characteristic differentiation biomarkers discovered later, this form of cell death was mistakenly considered as apoptosis at that time.

Subsequently, scientists identified similarities and differences in the mechanisms of pyroptosis and apoptosis, which brought some clarity to the interpretation of the data obtained [15]. Further studies showed that this bacteria-induced cell death depends on the enzyme caspase-1 [16]. The work by S.M. Man and T.-D. Kanneganti [17] confirmed the importance of caspase-1 and also revealed that *S. flexneri* cannot induce pyroptosis in macrophages that have a knockout of this enzyme. In 2001, B.T. Cookson and M.A. Brennan [18], having discovered this form of programmed cell death in macrophages infected with *S. thyphimurium*, called it pyroptosis.

In 2002, special molecular intracellular protein complexes, inflammasomes, were discovered and it was found that caspase-1 is one of their components [19, 20]. Further studies of inflammasomes showed that these structures are of great importance in the development of pyroptosis. In 2008, S.L. Fink et al. [21] found that in pyroptosis, DNA was fragmented and the cell membrane was damaged, which was accompanied by a release of intracellular contents, initiating an inflammation. Laboratory mice are the

most common laboratory animals on which pyroptosis has been studied. In 2011, in an experiment on mice, N. Kayagaki et al. [22] found that caspase-11 can induce macrophage death in mice, and this process is similar to pyroptosis mediated by caspase-1 in humans. In contrast to pyroptosis, which involves caspase-1 (the so-called canonical pathway), the authors designated capase-11-dependent pyroptosis as non-canonical. Pyroptosis, which involves human caspase-4 and caspase-5, is also called non-canonical.

MECHANISMS OF PYROPTOSIS

The main goal of pyroptosis is to induce strong inflammatory responses that protect the body from bacterial infection [23]. Probably, for this reason, this phenomenon is best studied in cells whose main function is protective (for example, leukocytes and macrophages). It has been established that pyroptosis inhibits the intracellular replication of microorganisms and activates immune cells to destroy pathogens [24, 25]. Activation of pyroptosis occurs in response to a wide range of effects, primarily infection (contamination) with pathogenic microorganisms. Based on its mechanism, pyroptosis can be canonical and non-canonical. The differences between the two types are not significant, since both pathways result in the formation of transmembrane gasdermin pores in the plasma membrane and disruption of the salt and water homeostasis in the cytoplasm.

The formation of gasdermin pores in the cell disrupts the water and ion balance, ultimately leading to cell death [23]. With the discovery of the gasdermin group of proteins, which in humans includes six proteins, the scope of research on pyroptosis has expanded significantly. All gasdermins (with the exception of the gasdermin protein pejvakin) play different roles in pyroptosis [26, 27]. Nowadays, gasdermin D is the most studied one [23]. It has two domains, an N-terminal domain and a C-terminal domain (GsdmD-N and GsdmD-C, respectively), connected by a peptide linker.

Only the N-terminal domain is considered as an effector domain and can form transmembrane pores [23, 28–30]. Human caspase-1 (canonical pyroptosis pathway) or human caspase-4 and 5 and caspase-11 in mice (non-canonical pyroptosis pathway) split gasdermin D into two domains in the cytoplasm [31, 32]. Embedding into the cell membrane, GsdmD-N selectively binds to its lipids and forms a gasdermin transmembrane pore, releasing cellular contents, including proinflammatory cytokines and the so-called danger signals (alarm signals) [23, 33–37]. There is evidence that GsdmD-N can not only perforate the cell membrane, but also takes part in the activation of cytokines, such as interleukin (IL)-18 and IL-1β [31, 32].

Caspases are a family of evolutionary conserved cysteine proteases [38, 39], which can be divided into two main groups, namely caspases-I (caspases 1, 4, 5, 13, 14) and caspases-II (caspases 2, 3, 6, 10). Caspase-1 substrates include cytokine precursors IL-1β, IL-18, and IL-33 [7, 40, 41].

The canonical pathway of pyroptosis develops when pathogen-associated molecular patterns (PAMPs) and the so-called danger signals or damage-associated molecular patterns (DAMPs) affect the cell. PAMPs include, for example, bacterial, viral, and fungal substances. DAMPs are released from damaged cells into the extracellular matrix and serve as potent proinflammatory factors [42]. Fragments of damaged cells including DNA, ATP, RNA, heat-shock proteins, fatty acids, etc., can act as DAMPs. It is proposed to single out metabolic disorders called homeostasis-altering molecular processes (HAMPs) into a separate group of pyroptosis activators [43–45]. The intracellular lipopolysaccharides (LPS) of gram-negative bacteria [23] initiate the non-canonical pyroptosis pathway.

The key sensors of PAMPs, DAMPs, and HAMPs are cellular receptors, which are called pattern recognition receptors (PRRs). In particular, these include toll-like (TLR), NOD-like (NLR), and Rig-1-like (RLR) receptors [11]. TLR are the most diverse. They are located both on the cell surface and in the cytoplasm and are represented on cells of different cell lineages. Known TLR ligands include various bacterial and fungal components, including LPS for TLR4, flagellin for TLR5, etc. [46]. Products of necrotic cells, heat-shock proteins HSP60 and HSP70, are the ligands of TLR2 and TLR4 [46]. It is known that human HSP60 acts on TLR4 to subsequently stimulate tumor necrosis factor (TNF) α and NO [47]. The mechanism of TLR action is to transmit a signal to the cell nucleus and activate nuclear factor (NF-kB), leading to the production of proinflammatory cytokines and chemokines (IL-1 α, IL-1β, IL-6, and other inflammatory mediators) [46, 48–50].

Activation of PRRs due to interaction with a pathogen causes the assembly of inflammasomes, which are necessary not only for pyroptosis, but also for the production of active forms of proinflammatory cytokines [7, 23]. The main components of

inflammasomes are PRRs, ASC (apoptosis-associated speck-like protein), and procaspase-1 [23]. Formation of inflammasomes ultimately leads to the maturation (activation) of caspase-1 (canonical pathway) or caspase-4, -5 (in humans) and caspase-11 (in mice) (non-canonical pathway). These enzymes cleave the gasdermin D protein, releasing its N-terminal domain.

The analysis of the literature indicates that it is inflammasomes that play a key role in the development of pyroptosis. Currently, we know more than 20 varieties of inflammasomes. Modern reviews by E.E. Garanina et. al (2020) and V.V. Klimov et al. (2023) [7, 11] describe in detail their molecular structure, mechanisms of activation, features of functioning, and regulation methods. It is emphasized that the mechanisms of component activation and assembly of inflammasomes need to be clarified and studied further.

METHODS FOR DETECTING PYROPTOSIS IN CELLS

It is known that different molecular mechanisms regulate cell death, which are accompanied by various changes at the morphological level [51]. Pyroptosis activates caspase-1- dependent nuclease, which leads to chromosome condensation [52]. We can observe enhanced pore formation in the cell membrane, but there is no disruption of the integrity of the mitochondrial membrane, which can be detected by electron microscopy [53]. Low-molecular-weight dyes, such as propidium iodide and ethidium bromide, can be used to detect pyroptosis [42]. Normally, the cell membrane is impermeable to these dyes, but during pyroptosis, these dyes penetrate through the damaged cell membrane are found in the cytoplasm.

Pyroptosis can also be detected by staining preparations with annexin V, but it does not make it possible to clearly distinguish pyroptosis from apoptosis [42]. To detect pyroptosis, it is advisable to use methods, such as flow cytometry, immunofluorescence staining of proteins of the gasdermin family, and determination of lactate dehydrogenase in the extracellular environment by Western blotting. The work by T.F. Sergeeva et al. (2015) proposes various methods for detecting caspase activation and DNA fragmentation [54]. In particular, the authors [54] propose methods for studying the caspase activation *in vitro*: immunohistochemistry, enzyme-linked immunosorbent assay, flow cytofluorometry, fluorescence imaging, fluorescence spectroscopy, FRET/FLIM imaging without a detailed explanation.

Characterizing the methods proposed by researchers for detecting pyroptosis, it should be noted that many of them are not strictly specific to this form of cell death, and we should continue the search in order to detect more accurate signs of differentiation.

PATHOLOGY AND THERAPEUTIC POTENTIAL OF PYROPTOSIS

In the process of studying pyroptosis, it became clear that this phenomenon has a dual meaning and can be both positive and negative [42, 51, 55, 56]. The positive value of pyroptosis is associated with the possibility of release of proinflammatory cytokines from the cells that produce them (macrophages and neutrophil granulocytes) through the transmembrane gasdermin pores of the cell membrane. Oligomerization of the N-terminal domains of gasdermin leads to pore formation, cell swelling, and release of cytoplasmic contents, including IL-1β, IL-33, and IL-18, which trigger the host inflammatory response associated with the inflammasome. Immunocytes recognize and eliminate bacteria that remain viable.

It has been shown that activated gasdermin can induce the formation of transmembrane pores not only in the cell membrane of human cells, but also in the membranes of bacterial cells, causing the death of microorganisms, such as *E. coli*, *L. monocytogenes*, *S. aureus* [34]. Therefore, pyroptosis has a protective function at an early stage of infection. Even though we considered pyroptosis as an exclusively pathological form of cell death, further research has shown that pyroptosis is a protective mechanism of the body that promotes the elimination of pathogens.

Along with the positive effect of pyroptosis, we should also take into account its role in the development of excessively pronounced inflammation and other pathological conditions. The discovery of inflammasomes, mediators of pyroptosis, and experimental confirmation that they are regulators of the secretion of proinflammatory cytokines made it possible to substantiate the leading role of inflammasomes in the development of many diseases [7]. Studies have shown that although pyroptosis can protect the body from microbial agents, its dysregulation leads to the development of autoimmune and autoinflammatory conditions [57, 58].

It is known that an excessive proinflammatory response or immunosuppression can lead to organ dysfunction or the development of secondary infection during the development of sepsis [59]. Although IL-1β, IL-18, and IL-33 are the only

known proinflammatory cytokines generated directly as a result of inflammasome activation, *in vivo* activation of the inflammasome can indirectly lead to the production of numerous other proinflammatory cytokines, including TNFα and IL-6, which causes the so-called cytokine storm and tissue damage to vital organs [60].

X. Zheng et al. [61] emphasize that excessive activation of pyroptosis will inevitably cause uncontrolled inflammation, which significantly accelerates the onset and development of sepsis, which indicates a poor prognosis. It has been shown that pyroptosis participates in septic damage to various cells: neurons and astrocytes of the brain [62], renal tubular epithelial cells [63], hepatocytes [64]. Elevated levels of IL-18 in the blood serum indicate severe sepsis and correlate with a poor prognosis [65, 66].

Many studies demonstrate that overactivated pyroptosis causes organ damage, and A. Sarkar et al. (2006) believe that in sepsis, pyroptosis can contribute to the development of another form of programmed cell death – apoptosis, thereby exacerbating inflammation and clinical manifestations of multiple organ dysfunction [67]. There are indications that pyroptosis is closely associated with atherosclerosis and diabetic nephropathy [31]. Cardiovascular diseases, especially atherosclerosis and myocardial infarction, are often accompanied by cell death and acute or chronic inflammation. The research by L. Wang et al. (2021) revealed that exosomes obtained from monocytes can contain the TXNIP-NLRP3 complex and transport it to macrophages of the myocardial connective tissue, subsequently promoting IL-1β and IL-18 production by them and aggravating inflammation [68].

An increasing number of studies are devoted to studying the role and molecular mechanisms of pyroptosis in sepsis-induced myocardial dysfunction (SIMD), which is a devastating complication of sepsis with a mortality rate of more than 50%. A small molecule called PSSM1443 can reduce the protein levels of active caspase-1, IL-1β, and IL-18 in SIMD mice by disrupting the TXNIP-NLRP3 interaction [68]. This indicates that inhibition of NLRP3 inflammasome activation is beneficial for the treatment of this cardiac pathology.

Proinflammatory caspases take part in endothelial cell pyroptosis, and caspase-11 takes part in the pathogenesis of sepsis-induced lung injury. In the experiment, mice with caspase-11 showed a decrease in inflammation and lung damage within 12 hours compared to mice in the control group, indicating the involvement of this caspase in the pathogenesis of sepsis-induced lung injury [69].

M. Kalbitz et al. (2016) found that number of the NLRP3 inflammasomes and the concentration of IL-1β were significantly increased in left ventricular cardiomyocytes in mice with experimental peritonitis [70]. At the molecular level, IL-1β matures through activation of the NLRP3 inflammasome, which can further cause atrophy and impair cardiomyocyte contractility and relaxation [71]. Analysis of the literature allows us to conclude that currently much attention is paid to the study of diseases associated with inflammasomes [7, 9, 42]. There is information in the literature about the role of pyroptosis in the tumor process. The induction of pyroptosis in tumor cells occurs with the activation of both innate and acquired immunity [42]. In this case lysis of the tumor cell and release of its contents into the intercellular space take place, and local inflammation occurs with the production of IL-1β and IL-18 by neutrophil granulocytes and macrophages, which helps attract immune system cells to the area of the primary tumor.

An extremely urgent task is to find effective ways to eliminate the adverse effect of pyroptosis in the development of pathological conditions. There is evidence that administration of certain substances can enhance or, conversely, suppress pyroptosis. Thus, in an experiment with the administration of the glutamine, pyroptosis of hepatocytes increased within 24 hours after experimental modeling of sepsis, but suppression of pyroptosis was observed after 72 hours [64]. The authors concluded that the regulation of pyroptosis cell death can serve as a basis for the development of treatment methods for certain diseases.

Based on the study of inflammasomes and pyroptosis, various research teams are developing innovative therapeutic approaches [9, 55]. In recent years, researchers have considered the possibility of using pyroptosis as a potential strategy for treating tumors and developing new anticancer drugs [72]. It is assumed that the activation of pyroptosis in tumor cells may be justified in the treatment of malignant neoplasms [42].

Recent studies have shown that $CD8^+$ T lymphocytes can suppress tumor growth by inducing pyroptosis and ferroptosis. R. Tang et al. (2020) concluded that pyroptosis, along with necroptosis and ferroptosis, represents a potentially new mechanism of immunogenic cell death [73]. Currently, promising

gasdermin D inhibitors are being developed for the treatment of a number of inflammatory diseases [23]. There is information about the effectiveness of drugs that are inflammasome inhibitors for the treatment of certain diseases [7]. One of them is rilonacept, which can bind IL-1α and 1β. C. Liu et al. (2021) found that Gly-Pro-Ala (GPA) peptide could significantly attenuate lung tissue damage in mice [74]. *In vitro* experiments showed that GPA peptide can protect alveolar macrophage from caspase-1-dependent pyroptosis.

CONCLUSION

The analysis of modern scientific literature indicates significant interest in studying the molecular mechanisms of pyroptosis and its therapeutic potential. Nowadays, we know the main manifestations of this type of programmed cell death, pattern recognition cell receptors, the structure and significance of various types of inflammasomes, and proteins of the gasdermin family. At the same time, many questions remain poorly researched, and the available answers to them are contradictory.

Pyroptosis in immunocompetent cells, neutrophil granulocytes, and macrophages has been studied quite well. Much less attention is paid to other cells, especially to representatives of the main cell lineages in the tissues of vital organs. Methods of pyroptosis detection in experimental and clinical conditions require further development. An effective solution to these issues is possible only through the interaction of specialists in different fields, namely morphologists, molecular biologists, biochemists, physiologists, pathologists, microbiologists, clinicians, etc.

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Authors' contribution

Odintsova I.A. – conception and design, drafting of the manuscript, critical revision of the manuscript for important intellectual content, final approval of the manuscript for publication. Chirsky V.S. – drafting of the manuscript, editing of the manuscript, final approval of the manuscript for publication. Slutskaya D.R. – search and analysis of literature on the mechanisms of pyroptosis. Andreeva E.A. – search

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Monkeys excluding apes as a model for studies on metabolic syndrome

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ABSTRACT

Aim. To summarize the results of research on metabolic syndrome in monkeys excluding apes and to conduct a comparison with humans.

A search for full-text publications in PubMed and Scopus databases was carried out using the following keywords: nonhuman primate, monkey, obesity, diabetes mellitus, metabolic syndrome, insulin, atherosclerosis, hypertension. Articles were selected that describe studies involving the following monkey species: cynomolgus monkeys (*Macaca fascicularis*), rhesus macaques (*Macaca mulatta*), baboons (*Papio* sp.), grivets (*Cercopithecus aethiops*), and common marmosets (*Callithrix jacchus*).

The development of various metabolic syndrome criteria was demonstrated in all monkey species reviewed. Many similarities with humans were revealed: macaques with obesity, insulin resistance, and type 2 diabetes mellitus demonstrated an increase in total cholesterol, triglycerides, and free fatty acids and a decrease in the concentration of high-density lipoprotein cholesterol. Obesity and insulin resistance were precursors to impaired carbohydrate metabolism. Blood pressure increased along with the progression of insulin resistance. The similarity of genetic and environmental risk factors between humans and monkeys is important in the development of metabolic syndrome.

The reviewed data suggest that the use of monkeys in biomedical research remains an indispensable resource for the study of pathogenesis and assessment of the efficacy and safety of new therapeutic strategies targeting clinically important metabolic diseases, including obesity, dyslipidemia, atherosclerosis, type 2 diabetes mellitus, and, possibly, other conditions associated with metabolic syndrome.

Keywords: monkeys, model, metabolic syndrome, obesity, diabetes mellitus, arterial hypertension

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Низшие обезьяны как модельный объект изучения метаболического синдрома

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РЕЗЮМЕ

Цель работы – обобщение результатов исследований по изучению метаболического синдрома на низших обезьянах, проведение сравнительной характеристики с человеком.

Осуществлен поиск полнотекстовых публикаций в базах данных PubMed, Scopus по ключевым словам: nonhuman primate, monkey, obesity, diabetes mellitus, metabolic syndrome, insulin, atherosclerosis, hypertension. Отобраны статьи, описывающие эксперименты с участием следующих видов обезьян: яванские макаки (*Macaca fascicularis*), макаки-резус (*Macaca mulatta*), павианы (*Papio sp.*), африканские зеленые мартышки (*Cercopithecus aethiops*), обыкновенные игрунки (*Callithrix jacchus*).

Развитие различных критериев метаболического синдрома показано у всех рассмотренных видов обезьян. При этом имеется много сходных с людьми черт: у макак с ожирением, инсулинорезистентностью и сахарным диабетом 2-го типа выявляются повышение уровня общего холестерина, триглицеридов и свободных жирных кислот, снижение концентрации холестерина липопротеидов высокой плотности. Предвестником нарушения углеводного метаболизма являются ожирение и инсулинорезистентность, по мере прогрессирования которой также увеличивается артериальное давление. В развитии метаболического синдрома немаловажным является сходство между людьми и обезьянами генетических и средовых факторов.

Возможность использовать обезьян в качестве биомодели человека остается незаменимым ресурсом для изучения патогенеза, оценки эффективности и безопасности новых терапевтических стратегий, направленных на клинически важные метаболические заболевания, включая ожирение, дислипидемию, атеросклероз, сахарный диабет 2-го типа и прочие состояния, ассоциированные с метаболической дисфункцией.

Ключевые слова: обезьяны, модель, метаболический синдром, ожирение, сахарный диабет, артериальная гипертензия

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

Источник финансирования. Работа выполнена в рамках реализации программы мегагрантов Правительства РФ по соглашению от 30.06.2022 № 075-15-2022-1110 и тематического плана Курчатовского комплекса медицинской приматологии.

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INTRODUCTION

Metabolic syndrome (MS) is a complex of symptoms that combines a number of metabolic disorders, namely insulin resistance, central obesity, atherogenic dyslipidemia, and arterial hypertension. Historically, G. Reaven proposed the term "syndrome X" in 1988, which was later called "MS" to differentiate from syndrome X in cardiology [1]. The first formal definition of MS was proposed in 1998, it was clarified several times, and currently there are main diagnostic criteria for MS (waist circumference or body mass index) and additional ones (fasting glucose level, impaired glucose tolerance, glycated hemoglobin level, low-density lipoprotein cholesterol level, and blood pressure) [2, 3].

When diagnosing MS, Russian experts use the criteria adopted by the Russian Scientific Society of Cardiology, according to which the main criterion for MS is abdominal obesity (waist circumference). Additional criteria (the presence of two or more) include arterial hypertension, increased triglyceride levels, decreased high-density lipoprotein cholesterol, increased low-density lipoprotein cholesterol, fasting hyperglycemia, and impaired glucose tolerance [4]. At the same time, MS is not considered as an independent nosological entity. The pathogenesis of MS includes a variety of genetic and acquired conditions that fall under the definition of insulin resistance and systemic chronic low-grade inflammation. If left untreated, MS is largely associated with an increased risk of developing type 2 diabetes mellitus (T2DM) and cardiovascular diseases [5]. The prevalence of MS ranges from 20 to 25% in adults and from 0 to 19.2% in children, and in patients with T2DM, it reaches 80% [6]. With age, the likelihood of developing metabolic disorders increases [7].

The widespread occurrence of MS and its particular clinical significance predetermine the active study of the pathological physiological mechanisms and morphological manifestations of MS. A large number of experimental models have been created on various animal species. Diabetes and obesity are modeled using high-calorie diets, chemicals, modification of the genetic apparatus of the cell, etc. [8, 9]. A special place among animals used to study the pathogenesis of MS is occupied by monkeys, which, along with humans, belong to the order of primates. This is due to a close genetic relationship with humans and similar physiological changes associated with obesity and metabolic disorders, similar life expectancy, and aging [10, 11].

The laboratory primate model should be considered as an important translational crossover between basic studies in rodent models and clinical studies in humans. Previously, the suborder of monkeys included the broad nosed monkeys (*Platyrrhina*), or New World monkeys, and the narrow nosed monkeys (*Catarrhina*), or Old World monkeys, which include the apes (*Hominoidea*) and humans. Recently, primates have been classified into the suborders *Strepsirrhini* and *Haplorhini*, the latter including tarsiers and monkeys [12]. In English-language literature, the term *Non-human Primates* is usually used, which should be translated from English to Russian as "primates other than humans" according to the taxonomy of the order, and is important to consider when analyzing scientific data.

However, a question arises whether it is possible to generalize such a diverse order when describing it as a human biomodel. It is probably possible but with certain limitations. Thus, it has been shown that waist circumference was positively correlated with systolic and diastolic blood pressure, glucose levels, and insulin resistance in chimpanzees of both sexes, and body weight was correlated with systolic and diastolic blood pressure in female chimpanzees and with triglyceride levels in male chimpanzees. Moreover, waist circumference is more associated with metabolic risk factors than body weight, especially in female chimpanzees [13]. In captive populations of aging adult chimpanzees, T2DM was described based on persistent fasting hyperglycemia, the presence of glycosuria, and the age of the disease onset.

However, cardiac pathology in humans and chimpanzees differs according to histopathological studies of the affected chimpanzee heart. Sudden cardiac death in chimpanzees (as well as gorillas and orangutans) is usually associated with diffuse interstitial myocardial fibrosis of unknown etiology, while in humans most cardiac diseases are known to be associated with atherosclerotic lesions of the coronary arteries. A typical human myocardial infarction caused by coronary thrombosis is rare in these monkey species, despite the human-like blood lipid profiles potentially associated with a high coronary risk. On the contrary, heart attacks in chimpanzees are probably associated with arrhythmias caused by the aforementioned myocardial fibrosis [14].

Apes are currently practically not used in biomedical research for ethical reasons [15]. Therefore, other species of the Haplorhini suborder are of greater interest. Monkeys exhibit the development of various age-related diseases, including cardiovascular

diseases, impaired glucose metabolism, redistribution and a general increase in the amount of fat [16]. The development of diabetes and obesity has been described in many monkey species. Among representatives of the Old World, diabetes develops in cynomolgus macaques (*Macaca fascicularis*), rhesus macaques (*Macaca mulatta*), baboons (*Papio sp*.), and African green monkeys or grivets (Cercopithecus aethiops) [17]. Among New World monkeys, diabetes has been described in common marmosets (*Callithrix jacchus*), squirrel monkeys (*Saimiri sciureus*), capuchins (*Cebus apella*) and tamarins (*Saquinus sp.*) [18, 19]. Obese cynomolgus macaques [14] and common marmosets have a number of metabolic parameters similar to those that determine MS in humans [18]. Below we will summarize the data on the criteria for MS in different species of monkeys excluding apes.

OBESITY, DYSLIPIDEMIA

Obesity can be induced in monkeys fed with a hypercaloric diet and is also often observed spontaneously, especially in rhesus monkeys, cynomolgus monkeys, African green monkeys and squirrel monkeys [20]. Hamilton et al. first reported characteristics of spontaneously obese middle-aged male monkeys in the early 1970s. Such rhesus macaques were characterized by hyperinsulinemia, hyperlipidemia, and with prolonged or severe obesity, the development of insulin-dependent DM was observed. It was shown that body weight was not a reliable indicator for determining the severity of obesity, and the amount of fat in an animal body was correlated best with waist circumference (r = 0.981) and the thickness of the skin fold on the anterior chest wall (r = 0.912). "Very obese" rhesus macaques of both sexes had a significant increase in fasting serum insulin levels, elevated insulin values after a glucose challenge, and insulin resistance [21].

In another study, in rhesus monkeys, waist circumference correlated best with body fat content (r = 0.90). There was also a strong linear relationship between waist circumference and plasma insulin levels (r = 0.66), impaired glucose tolerance (r = –0.53), but not with blood glucose levels, lipoprotein fractions or free fatty acids [22]. The pattern of abdominal fat distribution in the body of an obese person is similar [21].

Since obesity plays a key role in the progression of insulin resistance, MS, and T2DM, measuring body fat in animals is important [23]. Obesity is associated with increased levels of leptin in the blood of cynomolgus and rhesus macaques, as well as baboons. Leptin concentrations were often elevated in T2DM in rhesus and cynomolgus monkeys and were significantly correlated with body weight (r = 0.72). Moreover, leptin levels increased proportionately with insulin resistance and obesity in macaques, but decreased slightly with the development of T2DM and associated fat loss [17].

Leptin levels correlate positively with insulin concentration and body fat, with which adiponectin levels correlate negatively [14]. In a study on a population of African green monkeys of 98 males and 157 non-pregnant females, waist circumference was correlated with increased blood triglyceride concentrations. Moreover, females had a higher concentration of triglycerides than males and had a high risk of central obesity and a poor lipid profile [24]. Monkeys with diabetes with relative insulinopenia had elevated levels of cholesterol and triglycerides, which was associated with impaired activity of lipoprotein lipase, an insulin-dependent enzyme playing an important role in the catabolism of very low-density lipoproteins rich in triglycerides and to a lesser extent – low-density lipoproteins. In addition, in T2DM, the content of free fatty acids increases [17].

The development of obesity is accompanied by nonspecific tissue damage in the form of lipidosis and liver glycogenosis, as well as fatty infiltration of many organs [25]. Numerous studies of atherogenesis in monkeys exposed to a high-fat high-cholesterol diet demonstrated changes in the lipid profile similar to those in humans. Significant individual differences in the development of dyslipidemia and atherosclerosis were also established. In particular, no atherosclerotic changes were practically observed in some individuals, despite severe hypercholesterolemia [26]. The fatal fasting syndrome has been described in obese macaques, which is manifested by sudden death without previous signs of illness, often following short periods of anorexia or 20–30% body weight loss over a period of several days to two weeks. The pathogenesis of this syndrome has not been fully studied [27].

Baboons are considered as a model object for studying the genetics of obesity, in particular, genotyping and phenotypic characterization of a colony of baboons (more than 16,000 individuals traced in seven generations) is being carried out at the Southwest National Primate Research Center of the USA [28]. With increasing weight, animals showed an increase in body fat, waist circumference, and leptin concentration in the blood. Body composition analysis carried out using

the bioelectrical impedance method showed that when female baboons reach 20 kg (average adult weight is 19 kg), and males reach 38 kg (average adult weight is 31 kg), the amount of body fat is 20%. Many indicators of carbohydrate metabolism and obesity (body weight, insulin, glucose, C-peptide, triglycerides, adiponectin) are largely hereditary [28, 29].

FASTING HYPERGLYCEMIA, IMPAIRED GLUCOSE TOLERANCE, T2DM

As in humans, rhesus macaques, cynomolgus macaques, and baboons have an association of T2DM with age and body weight, mainly due to obesity. About 30% of cynomolgus macaques over 15 years of age have basal and / or postprandial hyperinsulinemia [17]. In rhesus monkeys, insulin sensitivity decreases with age [30]. However, obesity alone is not enough to predict the development of T2DM. T2DM is a progressive disease in macaques. It is initially characterized by normal glucose tolerance and insulin resistance with compensatory hyperinsulinemia. All monkeys with progressive development of T2DM were obese, but some obese monkeys maintained normal glucose tolerance [31].

Cynomolgus and rhesus monkeys have insulin resistance and hyperinsulinemia for a long time before the development of overt DM [32]. Subsequently, amyloid is deposited, and the number of β-cells decreases in the pancreatic islets. Insulin secretion cannot be maintained at an elevated level, the concentration of circulating insulin decreases, and impaired glucose tolerance develops. The mechanisms are identical to those in humans [33]. As obesity, insulin resistance and T2DM progress, postprandial glucose levels increase earlier than fasting glucose concentrations [17]. Glycation increases due to non-enzymatic binding of glucose to amino acid groups of proteins. As in humans, in monkeys with hyperglycemia, the blood levels of fructosamine (a product of albumin glycation) and glycated hemoglobin increase [10, 34].

Finally, when endocrine pancreatic function is insufficient, animals with T2DM may have elevated fasting insulin levels, but they cannot respond adequately to glucose administration, and fasting glucose levels increase. Calorie restriction and the use of oral hypoglycemic drugs are effective for some time, but over time, exogenous insulin is often required [35]. Grivets are susceptible to developing obesity and diabetes when kept in captivity. Females are especially at risk for central obesity and a poor lipid profile. Females with elevated levels of glycated hemoglobin had impaired glucose tolerance and central obesity, but not insulin resistance. A strong hereditary pattern was found suggesting the presence of a monogenic form of diabetes, such as MODY diabetes mellitus or mitochondrial diabetes [36].

The determination of reference values for biochemical parameters for all monkey species (Tables 1–3) used in biomedical research is still an important issue. For example, blood glucose concentrations may be affected by the status of the animal before blood collection, the procedure itself (stress, sedation, anesthesia), handling of blood samples (duration and temperature of storage, bacterial contamination), and the reliability of the determination method used. The tables provide data reflecting the differences and partial registration of indicators in different studies. In monkeys, fasting glucose concentrations are 20–30 mg / dl lower (conversion: 1.1–1.7 mmol / l) than in humans, and fasting glucose concentrations in the range of 100–126 mg / dl (conversion: 5.6–7.0 mmol / l) clearly indicate diabetes [17, 34]. Fasting glucose concentration differs depending on the stage of carbohydrate metabolism disorder and increases significantly in overt T2DM.

Table 1

Some anthropometric and biochemical parameters of *M. mulatta* monkeys with metabolic disorders

Diet	Number and characteristics of animals	Fasting glucose, mmol / l	HbA1c	TC, mmol / l	TG, mmol / l	Insulin, μU / ml	Waist circumference, cm	Ref.
Standard*	4 obese males	3.5 ± 0.2	–	3.67 ± 0.34	0.99 ± 0.17	164.7 ± 37.9**	74.9 ± 5.4	[21]
	4 obese females	3.3 ± 0.2	–	4.09 ± 0.23	0.91 ± 0.02	109.7 ± 16.3**	58.4 ± 3.1	
	3 males without obesity	3.2 ± 0.2	–	2.61 ± 0.36	0.42 ± 0.05	26.2 ± 11.8**	36.5 ± 1.6	
	3 females without obesity	2.9 ± 0.1	–	3.49 ± 0.62	0.52 ± 0.08	48.2 ± 11.5**	36.3 ± 4.2	
Standard*	18 males with MS	4.46 ± 0.21	–	3.31 ± 0.19	1.04 ± 0.15	58.9 ± 15.8	52.14 ± 2.35	[37]
	17 control males	3.90 ± 0.10	–	3.50 ± 0.16	0.58 ± 0.05	18.5 ± 3.6	41.97 ± 2.49	

Table 1 (continued)

Diet	Number and characteristics of animals	Fasting glucose, mmol / l	HbA1c	TC, mmol / l	TG, mmol / l	Insulin, μU / ml	Waist circumference, cm	Ref.
Standard***	«Full health» stage. 12 males, 3.0–8.9 years old	3.7 ± 0.1	–	–	–	42.0 ± 3.0	– (4-16% of body fat	[31]
	The stage of severe hyperinsulinemia. 6 males, 14.3–19.6 years old, obese	4.4 ± 0.2	–	–	–	415.0 ± 84.2	– (25–44% of body fat)	
	The stage of overt diabetes. 7 males, 14.8–21.3 years old, obese	10.8 ± 1.1	–	–	–	45.0 ± 5.1	– (18–30% of body fat)	

* body weight in obese males exceeded by 207% and in females – by 173% the body weight of animals without obesity. ** converted from pmol / l (1 pmol / l = 0.144 μU / ml). *** Among 42 males aged 3–28 years, weighing 5.0–31.7 kg (28 animals were initially obese), 8 stages of carbohydrate and lipid metabolism disorders were identified from the "full health" stage to the "overt diabetes" stage, the latter was characterized by weight loss and severe glycosuria.

Table 2

Some anthropometric and biochemical parameters of *M. fascicularis* monkeys with metabolic disorders

Diet	Number and characteristics of animals	Fasting glucose, mmol / l	HbA1c	TC, mmol / l	TG, mmol / l	Insulin, μU / ml	Waist circumference, cm	Ref.
High in carbohydrates and low in cholesterol	Control – 7 males and 5 females	3.06 ± 0.13*	–	3.52 ± 0.18*	2.22 ± 0.31*	12.8 ± 2.2	–	[17]
	5 males and 3 females with hyperinsulinemia	3.83 ± 0.25*	–	3.65 ± 0.33*	1.76 ± 0.16*	56.5 ± 10.4	–	
	3 males and 7 females with impaired glucose tolerance	3.44 ± 0.22*	–	3.91 ± 0.77*	1.89 ± 0.33*	15.1 ± 1.6	–	
	4 males, 1 female with hyperinsulinemia and impaired glucose tolerance**	4.95 ± 0.57*	–	3.36 ± 0.52 8	5.69 ± 1.71*	62.7 ± 13.5	–	
Standard*	24 control males	3.19 ± 0.09 *	3.8 ± 0.3 %	2.77 ± 0.1*	0.80 ± 0.10*	11.4 ± 0.9	–	[17]
	17 males and 8 females with type 2 diabetes mellitus	14.44 ± 1.21 *	10.7± 1.4 %	4.24 ± 0.39*	9.13 ± 1.24*	90.6 ± 18.5	–	

* converted from mg / dl (glucose: 1 mmol / l = 18.018 mg / dl; TC: 1 mmol / l = 38.66 mg / dl, TG: 1 mmol / l = 88.5 mg / dl). ** animals in this group were obese, exceeding by 40% the average body weight of animals in other groups.

Table 3

Some anthropometric and biochemical parameters of *Cercopithecus aethiops* monkeys with metabolic disorders

Diet	Number and characteristics of animals	Fasting glucose, mmol / l	HbA1c	TC, mmol / l	TG, mmol / l	Insulin, μU / ml	Waist circumference, cm	Ref.
Standard*	157 females, general population	3.35 ± 0.13*	5.,48 ± 0.15 %	3.96 ±0.07*	1.00 ± 0.05*	27.7 ± 1.7	37.8 ± 0.39	[24]
	Control, 4 females	3.36 ± 0.26*	5.27 ± 0.19 %	–	1.27 ± 0.27*	20.8 ± 5.2	38.37 ± 0.36	
	Impaired glucose tolerance, 3 females	5.81 ± 0.41*	8.30 ± 0.40 %	–	3.05 ± 1.04*	26.5 ± 2.8	43.33 ± 2.17	

* converted from mg / dl (glucose: 1 mmol / l = 18.018 mg / dl; TC: 1 mmol / l = 38.66 mg / dl, TG: 1 mmol / l = 88.5 mg / dl).

ARTERIAL HYPERTENSION, CARDIOVASCULAR PATHOLOGY

Diagnosis of spontaneous arterial hypertension is challenging in monkeys. Awake animals must be restrained during the procedure, which is a stressful factor that increases blood pressure. The use of sedatives is usually accompanied by changes in blood pressure. And the use of telemetry systems is limited by the need for surgical implantation and the service life of the system itself [38]. Spontaneous arterial hypertension develops in rhesus monkeys and grivets [39]. Two models of hypertension in baboons showed a doubling of the number of fatty streaks in the

abdominal aorta, iliofemoral artery, brachial artery, and coronary arteries after 13 months, regardless of plasma lipid levels [40].

Lesions of the main blood vessels cause death in people with T2DM and are associated with the progression of atherosclerosis leading to coronary heart disease and stroke [41]. Atherosclerotic manifestations are significantly more common in monkeys with spontaneous or drug-induced diabetes [26]. Cynomolgus macaques show an increase in blood pressure and inflammatory markers as they progress from insulin resistance to T2DM [19]. Rhesus macaques are some of many animal models for diabetic peripheral neuropathy and retinopathy [42], and glomerular dilation, glomerulosclerosis, and thickening of the glomerular basement membrane with hypertrophy have been described at the hyperinsulinemia stage of prediabetes [43]. Cynomolgus macaques with severe dyslipidemia, DM, and proteinuria showed left ventricular diastolic dysfunction with preserved ejection fraction and impaired cardiac reserve in dobutamine stress test, indicating the high translational potential of this model for humans [44]. This allows us to consider monkeys as a model for studying the role of insulin resistance in the development and progression of diabetic vascular diseases.

MS MODELING

In a study by a Chinese team [37], 408 adult *Macaca mulatta* males from three nurseries in China were screened. In accordance with the criteria for predisposition to MS in humans [3], the following ones were identified (three positive out of five): 1) blood pressure ≥ 120 / 75 mm Hg; 2) waist circumference ≥ 37 cm; 3) fasting plasma glucose ≥ 3.8 mmol / l; 4) fasting plasma triglycerides ≥ 0.45 mmol / l and above the 80th percentile; 5) HDL-C ≤ 1.10 mmol / l or below the 20th percentile. According to the selection method, the animals predisposed to metabolic syndrome had significantly higher blood pressure, fasting plasma glucose, waist circumference, and body weight than the controls. However, there were no differences between these groups in terms of triglycerides, HDL-C, LDL-C, total cholesterol or insulin [37].

After observing animals with a predisposition to MS, the authors proposed the following criteria to diagnose MS (≥ 3 positive out of five): 1) waist circumference ≥ 40 cm and waist-to-hip ratio ≥ 0.9; 2) fasting plasma glucose ≥ 4.40 mmol / l; 3) fasting plasma triglycerides ≥ 0.90 mmol / l; 4) HDL-C ≤ 1.55 mmol / l; 5) blood pressure ≥ 130 / 80 mm Hg. Eighteen monkeys met these criteria, among which one, four, and thirteen monkeys had a combination of 5, 4, and 3 positive MS criteria, respectively. The two most prevalent criteria were increased waist circumference (94%) and arterial hypertension (73%). The remaining criteria were found in combinations of varying frequency [37]. This pattern is similar to the one observed in humans [45].

DM MODELING

Historically, the most common methods for inducing diabetes have been partial or total pancreatectomy or the administration of alloxan, which causes rapid and complete loss of beta cells, soon after which hyperglycemia develops. The main factor limiting the use of alloxan is concomitant damage to the kidneys, adrenal glands, thyroid gland, pituitary gland, and liver [25]. A more specific beta cell toxin is streptozotocin, an antibiotic derived from *Streptomyces aromogenes*.

In monkeys, the administration of streptozotocin leads to severe hyperglycemia and dyslipidemia, which have some similarities with both type 1 and type 2 DM, however, changes in the pancreatic islets are more characteristic of type 1 DM [25]. The extent of damage to the islets of Langerhans varies, with some monkeys requiring more than one dose of streptozotocin to develop hyperglycemia. Animals do not develop insulin resistance unless it is combined with obesity or older age; correction insulin doses are 1.0–5.0 U / kg of body weight per day [46]. Plasma cholesterol and triglyceride levels increase slightly when hyperglycemia is adequately controlled [47].

DISCUSSION

The development of metabolic disorders in monkeys has many similarities with humans. Cynomolgus macaques with obesity, insulin resistance, and T2DM have the same changes in the lipid profile as patients with T2DM, namely increased total cholesterol, triglycerides, and free fatty acids and decreased HDL-C concentrations [17]. T2DM is a progressive disease in both monkeys and humans [31]. As insulin resistance progresses, blood pressure also increases. The association of MS with systemic inflammation is important. C-reactive protein synthesized by the liver during the acute-phase inflammatory response correlates with insulin resistance and obesity, as well as with an increased risk of developing T2DM and related cardiovascular diseases [48].

In cynomolgus macaques, the increase in C-reactive protein is stepwise when comparing control, insulin-resistant, and T2DM animals [17]. In grivets at high risk of MS, there is constant activation of the immune system associated with an increase in the proinflammatory cytokine interleukin (IL)-6 and mediated by resident gram-negative microbial communities that are formed in the visceral adipose tissue in both lean and obese individuals [49].

In addition to the similarity of pathogenetic characteristics in the development of MS, the similarity of genetic and environmental factors is also important. Since such factors are difficult to control in clinical practice, models of metabolic disorders in monkeys become unique in the context of studying the influence of genetic and environmental factors (diet, stress) on the development of obesity, DM, and MS. In controlled colonies of animals with a known pedigree, it becomes possible to study gene – environment interactions [24, 50].

Heredity has a strong effect on the body weight of baboons ($h^2 = 0.9$). Obviously, there appears to be a set of genes associated with insulin resistance that are also likely to affect obesity-related phenotypes, which confirms a common genetic basis for the development of insulin resistance and obesity [51]. Glucose transporter 4 (GLUT4) mRNA expression is under significant genetic influence and correlates with plasma insulin levels and body weight. This indicates a common genetic regulation of these phenotypic traits [28]. Diet is an important environmental factor influencing carbohydrate metabolism and obesity. Calorie restriction is beneficial for macaques since a positive effect is observed, namely a decrease in glucose and insulin levels and an increase in insulin sensitivity [52].

When keeping monkeys in captivity, it is routine practice to provide food *ad libitum*. Monkeys in groups have a certain social hierarchy (for example, a dominant or subordinate role), which determines differences in feeding behavior. The composition of the feed also matters. Many standard feeds (Purina, Teklad, etc.) use soybeans containing isoflavones (genistein, daidzein, etc.) as the main source of protein. Isoflavones are plant phytoestrogenic compounds that have an estrogenic or antiestrogenic effect, affecting the hormonal and metabolic parameters of animals. Adequate isoflavone intake level has not been established for monkeys [53].

Another environmental factor is stressful conditions of captivity. Thus, when kept in conditions of relative social crowding, grivets (*Chlorocebus aethiops*) and African red monkeys (*Erythrocebus patas*) coped significantly worse with glucose challenge test compared to their counterparts kept in smaller groups [17]. This factor may not be important for monkeys that live in large groups. For such species (for example, rhesus macaques), repeated reorganizations of the established social group are more stressful.

Primate models of MS are more adequate compared to rodents. For example, peroxisome proliferator-activated receptors (PPARs) have differences in expression levels and binding to regulatory elements in the apolipoprotein A1 (ApoA1) promoter between rodents and monkeys, which leads to opposite effects of PPAR agonists on ApoA1 production and HDL metabolism [54]. Thus, primate models have made a significant contribution to understanding the fundamental basis of the development of MS.

CONCLUSION

The ability to use monkeys as human biomodels remains an indispensable resource for studying the pathogenesis and assessing the efficacy and safety of new therapeutic strategies targeting clinically important metabolic diseases, including obesity, dyslipidemia, atherosclerosis, type 2 diabetes mellitus, and, possibly, other conditions associated with metabolic dysfunction.

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Authors' contribution

Orlov S.V., Uspensky Yu.P., Fominykh Yu.A., Panchenko A.V. – conception and design, analysis and interpretation of the data. Panchenko A.V., Kolesnik Yu.A. – drafting of the manuscript. All the authors conducted critical revision of the manuscript, made comments, and approved the final version of the manuscript for publication.

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Precision medicine in oncology: role and prospects of mass spectrometry

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ABSTRACT

The aim of this review was to analyze the accumulated data on the use of mass spectrometry in diagnosing, treating, and prognosing cancer from the perspective of precision medicine.

Currently, universally accepted methods for early cancer diagnosis are not available, primarily due to low molecular specificity of pathological changes at early stages of cancer development. Additionally, the existing diagnostic modalities are notably limited in sensitivity. However, early detection is imperative for selection of the most suitable cancer treatment strategy and its successful implementation.

In the realm of oncology, mass spectrometry approaches show great potential for advancement and utilization. Mass spectrometry is becoming an indispensable tool in basic and applied research due to its sensitivity, specificity, and accuracy. It allows for efficient analysis of complex biological compounds, even at low concentrations. Moreover, contemporary mass spectrometry technology is capable of automating the analysis, thereby facilitating its diverse clinical applications in diagnosis, drug therapy selection, and even potential assistance to surgical oncologists in the operating room.

Considering all these characteristics and advantages, mass spectrometry methods for the analysis of biological samples can be defined as some of the most promising and dynamically developing tools in precision medicine, as they are capable of providing clinically valuable information based on omics technologies, taking into account personal characteristics of the patient.

Over the next decade, introduction of mass spectrometry-based methods into clinical practice based on the principles of precision medicine is expected to optimize selection of personalized treatment strategies for cancer patients and provide significant economic benefits by reducing morbidity, disability, and mortality.

This comprehensive review presents the analysis of 65 scientific publications, highlighting the results of clinical and experimental studies utilizing mass spectrometry methods for diagnosing cancer, investigating the underlying mechanisms of disease development, and evaluating the efficacy of therapeutic interventions. The review encompasses original articles published from January 1, 2018 to November 30, 2023.

The majority of studies back the potential of mass spectrometry as a valuable tool for cancer diagnosis and treatment monitoring. Broadening application of mass spectrometry techniques in the field of oncology holds significant promise and represents a relevant area for future research.

Keywords: mass spectrometric study, mass spectrometry, molecular profiling, cancer, tumor process, carcinogenesis, low-molecular-weight metabolites

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Прецизионная медицина в онкологии: роль и перспективы масс-спектрометрии

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РЕЗЮМЕ

Цель исследования – анализ накопленных данных о применении методов масс-спектрометрии в диагностике, лечении и прогнозировании течения онкологических заболеваний с позиций прецизионной медицины.

На сегодняшний день не существует общепризнанных методов ранней диагностики рака, так как патологические изменения на начальных стадиях развития обладают низкой молекулярной специфичностью, а существующие методы известны своей ограниченной чувствительностью. Однако именно возможность как можно более ранней диагностики является важным, если не определяющим, фактором адекватного выбора стратегии терапии онкологических заболеваний и ее успешного применения.

В этом контексте высокотехнологические подходы к профилированию молекулярного ландшафта тканей, использующие в своей основе метод масс-спектрометрии, имеют большой потенциал к развитию и применению в области онкологии. Современная масс-спектрометрия становится незаменимым инструментом в проведении фундаментальных и прикладных исследований за счет своей чувствительности, специфичности и точности, обеспечивающим эффективный анализ сложных биологических соединений даже в незначительных концентрациях. Важным свойством современных масс-спектрометрических технологий является возможность автоматизации аналитического процесса, что открывает перспективы широкого внедрения в клиническую практику на этапах постановки диагноза, выбора стратегии лекарственной терапии и даже сопровождения работы хирурга-онколога в операционной.

С учетом всех приведенных выше характеристик и преимуществ масс-спектрометрические методы анализа биологических образцов можно определить как один из наиболее перспективных и динамически развивающихся инструментов прецизионной медицины вследствие их способности на базе омиксных технологий предоставить клинически ценную информацию с учетом персональных особенностей пациента.

В ближайшее десятилетие можно ожидать, что внедрение методов, основанных на технологии масс-спектрометрии, в клиническую практику, стоящую на принципах прецизионной медицины, приведет к оптимизации процесса определения индивидуальных стратегий лечения онкологических больных и принесет значительный экономический эффект за счет снижения заболеваемости, инвалидности и смертности.

В настоящей обзорной статье представлен анализ 65 научных публикаций, посвященных результатам клинических и экспериментальных исследований, в которых методы масс-спектрометрического анализа применялись для диагностики онкологических заболеваний, выяснения механизмов их развития и оценки эффективности терапевтических воздействий. Обзор включает оригинальные статьи, опубликованные в период с 1 января 2018 г. по 30 ноября 2023 г.

Большинство исследований подтверждают, что масс-спектрометрия является перспективным инструментом в онкологической диагностике и мониторинге эффективности лечения. Расширение использования методов масс-спектрометрии в онкологии имеет большой потенциал и является актуальной задачей для дальнейших исследований.

Ключевые слова: масс-спектрометрическое исследование, масс-спектрометрия, молекулярное профилирование, рак, опухолевой процесс, канцерогенез, низкомолекулярные метаболиты

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

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INTRODUCTION

World Health Organization (WHO) mortality data up to 2023 report that cancer is one of the leading causes of death around the world. In 2020, cancer caused the death of almost 10 million people, which means that one in six people died from cancer. The most common causes of cancer death in 2020 were lung cancer, colon and rectal cancer, liver cancer, stomach cancer, and breast cancer [1]. In countries where healthcare systems are effective, survival rates for many forms of cancer are increasing due to widespread availability of early diagnosis, quality treatment, and care for cancer patients [2]. Every year in the world, about 10 million new cancer diagnoses are made, which accounts for about 30% of the world population [3].

As early diagnosis improves disease prognosis, the discovery of sensitive biomarkers associated with carcinogenesis through precision medicine has become a priority in cancer research. The use of sensitive methods and the implementation of specific biomarkers for the treatment of cancer into clinical practice will allow early therapy to improve survival and preserve the quality of life of patients.

An innovative approach in healthcare is to understand the unique characteristics of each patient and apply this information to diagnosis, prevention, and treatment. Precision medicine makes it possible to more accurately determine the risk of developing diseases, select the most effective diagnostic methods, and provide personalized treatment recommendations. Precision medicine has the potential to significantly improve health outcomes, reduce unwanted side effects, and optimize healthcare costs. However, its widespread implementation requires joint efforts in the field of scientific research, development of new technologies, and ethical issues related to the use of personal patient data.

The concept of precision medicine is aimed at effective diagnosis and prescription of drugs taking into account the genotype. Metabolomics allows to combine the phenotypic characteristics and genotype of a particular person. Technologies for studying the molecular landscape using chromatography – mass spectrometry are used to search for molecular markers that may be associated with the development of tumors and their growth, which allows for differential diagnosis and verification of tumor diseases, predicting the effectiveness of proposed therapeutic strategies and monitoring the chosen treatment.

Oncogenesis is accompanied by global changes in the metabolic state, affecting both tumor tissues and the micro- and macroenvironment [1]. Advances in metabolomics make it possible to measure a wide range of cellular metabolites, providing an approach to identifying specific changes associated with tumor transformation of cells. Compared to genomic and proteomic changes, metabolic changes can be directly observed in relation to tumor cell states and are therefore a promising source of biomarkers for identifying cancer.

Differences in metabolomic expression can be used to monitor disease progression and search for promising therapeutic approaches. Empirical switching between drugs in search of the desired one with a satisfactory therapeutic response and the lowest toxicity profile is not ideal clinical practice. Thus, the search for biomarkers that provide an insight into the pathophysiology of the disease should help in personalizing diagnosis and stratifying treatment. In addition, mass spectrometry-based metabolomics has high potential for detecting molecular signatures of human diseases, and, ideally, the detected biomarkers

can be validated for use in routine clinical practice. The value of using high-throughput technologies, such as metabolomics, is the ability to develop predictive panels of biomarkers that can be used to identify patients who are less likely to benefit from therapy because they are at risk of developing adverse complications.

Mass spectrometry-based metabolomics provides an insight into molecular events occurring in a patient's cancerous tumor and in normal tissues. Metabolic reprogramming is considered as a hallmark of cancer that contributes to disease progression [4]. The differential metabolic demands of rapidly proliferating malignant cells compared to their non-transformed counterparts suggest that targeting metabolism may be a potential strategy for developing new cancer treatments [4]. A key step in developing new therapeutic approaches that exploit metabolic vulnerabilities is identifying metabolic changes that are relevant to a specific malignancy.

Changes in metabolites during tumorigenesis and standardization of data analysis have shown that mass spectrometry can be an effective tool for use in cancer epidemiology and translational research.

The aim of this review was to analyze the accumulated clinical data on the use of mass spectrometry in diagnosing, treating, and prognosing cancer from the perspective of precision medicine.

METHODS

The analysis of scientific publications of clinical trials and experimental studies was carried out in the PubMed electronic search system. The review includes original articles published from January 1, 2018 to November 30, 2023.

The query for searching for English-language publications included the words: mass spectrometry and (((cancer) or (tumors)) and (molecular profiling)). We identified 1,995 publications in English. At the first stage, articles were selected whose titles mentioned mass spectrometry methods in oncology, while review-type and duplicate publications were excluded. At the second stage, the abstracts of the publications were analyzed, and works where studies were performed on cell cultures or animal models were excluded. At the third stage, articles with full-text access were selected, resulting in a detailed analysis of 65 publications containing data on modern original research in the field of oncogenesis and molecular profiling. Description of the studies (type of biological material, methodological approach, study characteristics) is presented in Table.

RESULTS

Following the analysis, we can note a significant spread of molecular profiling methods in clinical practice. Research conducted in this area concerns various types of cancer, such as colorectal cancer, non-small cell lung cancer, gastric adenocarcinoma, prostate cancer, breast cancer, etc.

The results of the literature review indicate a significant contribution of molecular profiling methods to the study of cancer and their clinical application. In the discussion section, we reviewed several of the most interesting articles from those devoted to the use of mass spectrometry methods in oncology. The articles we selected are only part of the extensive work of researchers in this field and are a valuable contribution to the development of oncology and practical medicine.

Using mass spectrometry to identify biomarkers of tumorigenesis

During tumorigenesis, extensive changes in the metabolic state occur, which affect not only tumor tissues, but also their micro- and macroenvironment. Advances in metabolomics make it possible to measure a wide range of metabolites, allowing for the identification of specific metabolic changes associated with tumor processes. Cancer is a multifactorial disease, and understanding its basis requires not only an analysis of genetic predisposition, but also phenotypic characteristics of the body.

In recent decades, medical research has actively studied non-invasive cancer screening methods based on the analysis of biological material, with special attention paid to identifying early stages of the disease. Some types of cancer, such as pancreatic cancer, do not show specific symptoms, making it difficult to diagnose early stages using conventional screening methods [51]. Pancreatic ductal adenocarcinoma (PDAC), which accounts for 90% of pancreatic cancers, is most often diagnosed at an advanced stage, resulting in the worst 5-year survival rate (7%) among all cancers. An international team of researchers from the Czech Republic, Germany, and Singapore have developed a lipidomic profiling approach to detect pancreatic cancer. The sensitivity and specificity of the method are more than 90%, which is superior to the

Table

Data on modern original research in the field of oncogenesis and molecular profiling

Title	Authors	Year, country	Methodological approach	No.
Metabolic biomarker signature to differentiate pancreatic ductal adenocarcinoma from chronic pancreatitis	Mayerle J., Kalthoff H., Reszka R. et al.	2018, Germany	Biomarkers for the differential diagnosis of pancreatic adenocarcinoma and chronic pancreatitis were identified using gas and liquid chromatography – mass spectrometry in serum and blood plasma	[5]
Discrimination of papillary thyroid cancer from non-cancerous thyroid tissue based on lipid profiling by mass spectrometry imaging	Wojakowska A., Cole L.M., Chekan M. et al.	2018, Poland	Using high-resolution MALDI-Q-Ion Mobility-TOF-MS, a method has been developed for the differential diagnosis of papillary thyroid carcinoma based on the lipid profile of histological samples fixed in formalin	[6]
Identification of Potential Biomarkers and Metabolic Profiling of Serum in Ovarian Cancer Patients Using UPLC/Q-TOF MS	Yang W., Mu T., Jiang J. et al.	2018, China	Using ultraperformance liquid chromatography and quadrupole mass spectrometry with positive electrospray ionization, biomarkers of ovarian cancer were identified in blood serum, which can be used to diagnose the disease	[7]
A quantitative multimodal metabolomic assay for colorectal cancer	Farshidfar F., Kopciuk K.A., Hilsden R. et al.	2018, Canada	Using a multimodal approach using gas chromatography-mass spectrometry, a metabolomic profile was identified in blood serum that distinguishes colorectal cancer from the control group, including 48 metabolites	[8]
Molecular profiling of lung cancer specimens and liquid biopsies using MALDI-TOF mass spectrometry	Bonaparte E., Pesenti C., Fontana L. et al.	2018, Italy	Using mass spectrometry, which allows for multiplex genotyping, a panel capable of identifying the most common mutations in non-small cell lung cancer has been proposed	[9]
Comparing intestinal versus diffuse gastric cancer using a PEFF-oriented proteomic pipeline	Wippel H.H., Santos M.D.M., Clasen M.A. et al.	2018, Brazil	A method for differential diagnosis of diffuse and interstitial gastric cancer has been developed, based on comparison of proteomic profiles obtained by isobaric labeling of peptides, 10-step fractionation and reversed-phase nanochromatography in combination with mass spectrometry	[10]
Metabolomic prediction of treatment outcome in pancreatic ductal adenocarcinoma patients receiving gemcitabine	Phua L.C., Goh S., Tai D.W.M. et al.	2018, Singapore	Using gas chromatography / time of flight mass spectrometry (GC/TOFMS) for metabolomic profiling of histological samples, metabolic biomarkers have been proposed that predict resistance to gemcitabine in chemotherapy for pancreatic ductal adenocarcinoma	[11]
Proteomic Characterization of Prostate Cancer to Distinguish Nonmetastasizing and Metastasizing Primary Tumors and Lymph Node Metastases	Müller A.K., Föll M., Heckelmann B. et al.	2018, Germany	Using liquid chromatography with tandem mass spectrometry and subsequent quantitative determination, a proteomic signature of primary prostate cancer was revealed in which metastasis to the lymph nodes occurs	[12]
Designation of fingerprint glycopeptides for targeted glycoproteomic analysis of serum haptoglobin: insights into gastric cancer biomarker discovery	Lee J., Hua S., Lee S.H. et al.	2018, Korea	Biomarkers of gastric cancer (glycopeptides) were identified in blood serum using mass spectrometry. An analytical platform with a targeted glycoproteomic approach has been created for the detection of gastric cancer biomarkers in blood serum, including 3 glycopeptides that can be used to diagnose the disease	[13]
Expression of small leucine-rich extracellular matrix proteoglycans biglycan and lumican reveals oral lichen planus malignant potential	Lončar-Brzak B., Klobučar M., Veliki-Dalić I. et al.	2018, Croatia	Using the method of global protein profiling based on liquid chromatography with mass spectrometry, a method for the differential diagnosis of lichenoid disease and squamous cell carcinoma of the oral cavity has been developed. The small leucine-rich proteoglycans biglycan and lumican have been identified as important biomarkers of oral squamous cell carcinoma	[14]
Differential diagnosis between hepatocellular carcinoma and cirrhosis by serum amino acids and acylcarnitines	Zhang Y., Ding N., Cao Y. et al.	2018, China	A method for the differential diagnosis of hepatocarcinoma and liver cirrhosis has been proposed based on determining the profile of amino acids and acylcarnitines using mass spectrometry of whole blood samples dried on filter paper	[15]

Clinical Significance of Extracellular Vesicles in Plasma from Glioblastoma Patients	Osti D., Del Bene M., Rappa G. et al.	2019, USA, Italy	An increase in the level of extracellular vesicles in the blood plasma can help in the clinical diagnosis of glioblastoma: their decrease after resection, increase during relapse, as well as their proteomic profiling using mass spectrometry provide insight into the molecular profile of the tumor and can serve as a prognostic criterion for the response to therapy	[16]
Breast cancer detection using targeted plasma metabolomics	Jasbi P., Wang D., Cheng S.L. et al.	2019, USA, China	Using liquid chromatography with tandem mass spectrometry, a new panel containing 6 metabolites of potential biomarkers for the diagnosis of breast cancer was created	[17]
The decrease of some serum free amino acids can predict breast cancer diagnosis and progression	Eniu D.T., Romanciuc F., Moraru C. et al.	2019, Romania	Ultra-high performance liquid chromatography combined with mass spectrometry showed a significant decrease in the concentrations of arginine, alanine, isoleucine, tyrosine, and tryptophan in the blood serum of patients with confirmed breast cancer	[18]
Glycerophospholipids pathways and chromosomal instability in gastric cancer: Global lipidomics analysis	Hung C.Y., Yeh T.S., Tsai C.K. et al.	2019, Taiwan	Using liquid chromatography – mass spectrometry, it was found that chromosomal instability of gastric cancer is associated with changes in the lipid profile of tumors toward an increase in glycerolipids and glycerophospholipids	[19]
Metabolomics-Based Biosignatures of Prostate Cancer in Patients Following Radiotherapy	Nalbantoglu S., Abu-Asab M., Suy S. et al.	2019, USA, Turkey	Radiation metabolomics has been applied to search for metabolomic biomarkers of prostate cancer and tumor response to radiotherapy. The metabolome of blood serum of patients who underwent radiation therapy revealed predominance of aberrations in the metabolic pathways of nitrogen, pyrimidines, purines, porphyrins, and glycerophospholipids	[20]
Proteomics of Melanoma Response to Immunotherapy Reveals Mitochondrial Dependence	Harel M., Ortenberg R., Varanasi S.K. et al.	2019, USA, Israel	Proteomic analysis of melanoma samples using high-resolution liquid chromatography – mass spectrometry revealed a relationship between the metabolic state of melanoma and response to immunotherapy, which may form the basis for therapy adjustments	[21]
Reliable identification of prostate cancer using mass spectrometry metabolomic imaging in needle core biopsies	Morse N., Jamaspishvili T., Simon D. et al.	2019, Canada	Multidimensional metabolomic classifier for prostate cancer with potential for clinical application was developed	[22]
Proteomic signatures of 16 major types of human cancer reveal universal and cancer-type-specific proteins for the identification of potential therapeutic targets	Zhou Y., Lih T. M., Pan J. et al.	2020, USA	Using liquid chromatography with tandem mass spectrometry, the proteomic landscape of 16 major cancer types was presented, and universally expressed proteins specific to a particular tissue and cancer type were identified	[23]
Untargeted Metabolomics and Polyamine Profiling in Serum before and after Surgery in Colorectal Cancer Patients	Lee Y. R., An K. Y., Jeon J. et al.	2020, Korea	Liquid chromatography – mass spectrometry of blood serum in patients with colorectal cancer before and after surgery revealed differences in the metabolism of sphingolipids, arginine, proline, and steroid biosynthesis.	[24]
Metabolic Alterations Related to Glioma Grading Based on Metabolomics and Lipidomics Analyses	Yu D., Xuan Q., Zhang C. et al.	2020, China	Using gas chromatography – mass spectrometry, differential metabolites between various types of gliomas and paratumor tissues were studied. It was found that in high-grade gliomas the content of short-chain acylcarnitines is increased, and lysophosphatidylethanolamines are decreased	[25]
Stromal vapors for real-time molecular guidance of breast-conserving surgery	Vaysse P.M., Kooreman L.F., Engelen S.M. et al.	2020, Netherlands	Using mass spectrometry with rapid evaporative ionization, an intraoperative diagnostic method has been developed for rapid analysis of the border of a breast cancer tumor and healthy tissue using *in vivo* and real-time analysis based on electrosurgical vapors with a metabolomic profile	[26]
Histo-molecular differentiation of renal cancer subtypes by mass spectrometry imaging and rapid proteome profiling of formalin-fixed paraffin-embedded tumor tissue sections	Möginger U., Marcussen N., Jensen O.N. et al.	2020, Denmark	The combination of MALDI-MS imaging (MSI) and rapid LC-MS/MS-based microproteomics (15 min / sample) to analyze tissue sections can identify molecular features and correctly classify 100% of patients with renal oncocytoma, clear cell renal cell carcinoma, and chromophobe renal cell carcinoma	[27]

Table (continued)

Title	Authors	Year, country	Methodological approach	No.
Ion mobility mass spectrometry of human melanoma gangliosides	Sarbu M., Clemmer D.E., Zamfir A.D. et al.	2020, Romania	The combination of mass spectrometry with ion mobility separation revealed high frequency of gangliosides GD3 and GM3 as well as de-N-acetyl GM3 (d-GM3) and de-N-acetyl GD3 (d-GD3) when profiling them in human melanoma, which complements the existing list of biomarkers associated with this type of cancer	[28]
Identification of plasma lipid species as promising diagnostic markers for prostate cancer	Chen X., Zhu Y., Jijiwa M. et al.	2020, China, USA	Plasma lipid profiling using liquid chromatography electrospray ionization and tandem mass spectrometry identified a five-lipid panel of potential biomarkers to distinguish prostate cancer from benign prostatic hyperplasia. A combination of lipid biomarkers provides a new diagnostic strategy for patients with prostate cancer	[29]
Serum lipidomic biomarkers for non-small cell lung cancer in nonsmoking female patients	Noreldeen H.A., Du L., Li W. et al.	2020, Egypt, China	Untargeted lipidomic profiling of serum, based on ultra-high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry, showed changes in the lipid profile in women with non-small cell lung cancer. Levels of unsaturated fatty acids decreased and those of saturated fatty acids and lysophosphatidylethanolamines increased, indicating changes in the metabolism of fatty acids and phosphatidylethanolamines. The developed serum combination lipid biomarker can be used for early diagnosis of non-small cell lung cancer	[30]
A serum lipidomic strategy revealed potential lipid biomarkers for early-stage cervical cancer	Cheng F., Wen Z., Feng X. et al.	2020, China	A panel of lipid biomarkers, including phosphatidylcholine and phosphatidylethanolamine, was developed by ultra-high-pressure liquid chromatography with quadrupole time-of-flight tandem mass spectrometry for the effective diagnosis of cervical cancer and squamous intraepithelial lesions	[31]
Liquid Chromatography-Mass Spectrometry-Based Tissue Metabolic Profiling Reveals Major Metabolic Pathway Alterations and Potential Biomarkers of Lung Cancer	You L., Fan Y., Liu X. et al.	2020, China	Untargeted metabolomics analysis based on liquid chromatography – mass spectrometry of lung carcinoma tissue and distal healthy lung tissue revealed an increase in lysophospholipids and a decrease in the content of 3-phosphoglyceric acid, phosphoenolpyruvate, 6-phosphogluconate and citrate in tumor tissue. It has been shown that during carcinogenesis, energy, purine, amino acid, lipid, and glutathione metabolism is disrupted.	[32]
Classification of thyroid tumors based on mass spectrometry imaging of tissue microarrays; a single-pixel approach	Kurczyk A., Gawin M., Chekan M. et al.	2020, Poland	The matrix laser desorption ionization method in the analysis of tissue microchips made it possible to identify high molecular similarities between different types of malignant neoplasms of the thyroid gland. Tumors with follicular morphology, such as adenoma, follicular cancer, and the follicular variant of papillary cancer, turned out to be especially similar in molecular structure	[33]
Study on the Diagnosis of Gastric Cancer by Magnetic Beads Extraction and Mass Spectrometry	Zhu N., Xing X., Cao L. et al.	2020, China	Using matrix-assisted laser desorption / ionization time-of-flight mass spectrometry, it was found that the expression of two peptide peaks with molecular weights of 2,863 Da and 2,953 Da was significantly increased, and the expression of two peptide peaks with molecular weights of 1,945 Da and 2,082 Da was decreased in the serum of patients with stomach cancer	[34]
Rapid estimation of tumor cell percentage in brain tissue biopsy samples using inline cartridge extraction mass spectrometry	Pekov S. I., Bormotov D.S., Nikitin P.V. et al.	2021, Russia	A study of 58 glial tumors was conducted to assess the percentage of tumor cells using the mass spectrometry method, which showed the possibility of automating routine tissue screening and its implementation in clinical practice during surgical interventions	[35]

Large-scale and high-resolution mass spectrometry-based proteomics profiling defines molecular subtypes of esophageal cancer for therapeutic targeting	Liu W., Xie L., He Y. H. et al.	2021, China	The study performed a proteomic analysis of 124 paired esophageal cancer tumors and corresponding adjacent non-tumor tissues, which identified two subtypes of esophageal cancer that are associated with patient survival and predicted potential drugs for one of the molecular subtypes	[36]
Early Breast Cancer Detection Using Untargeted and Targeted Metabolomics	Wei Y., Jasbi P., Shi X. et al.	2021, USA	In the current study, plasma samples from breast cancer patients and healthy controls were analyzed by untargeted liquid chromatography and quadrupole time-of-flight mass spectrometry, identifying 33 altered metabolites that provided accurate classification of early-stage breast cancer	[37]
The Colorectal Cancer Lipidome: Identification of a Robust Tumor-Specific Lipid Species Signature	Ecker J., Benedetti E., Kindt AS. et al.	2021, Germany	Using direct infusion electrospray ionization techniques coupled with tandem mass spectrometry and high-resolution mass spectrometry, significant differences in lipid composition were found between tumor and normal tissue in colorectal cancer. Glycerophospholipids showed a wide range of variation between patients, while the quantitative composition of glycero- and sphingolipids was more stable. There was also a significant increase in lipogenic enzyme activity and an association of triglyceride metabolic profile with postoperative disease-free survival and lymphovascular invasion in colorectal cancer	[38]
Proteomic profiling identifies signatures associated with progression of precancerous gastric lesions and risk of early gastric cancer	Li X., Zheng N. R., Wang L.H. et al.	2021, China	Using liquid chromatography and tandem mass spectrometry of blood serum, the proteomic landscape of precancerous lesions of the stomach and gastric cancer was determined, proteomic characteristics were determined, including proteomic subtypes and individual proteins associated with the progression of precancerous lesions of the stomach and the risk of early development of this disease	[39]
Proteomic profiling of soft tissue sarcomas with SWATH mass spectrometry	Milighetti M., Krasny L., Lee A. T. et al.	2021, Great Britain	Mass spectrometry techniques have been applied to comprehensive proteomic analysis of different soft tissue sarcoma subtypes to identify unique proteomic signatures, identify biological processes and key protein networks within histological tumor subtypes, and identify potential candidate proteins associated with predicting patient outcomes	[40]
Mass-spectrometry-based proteomic correlates of grade and stage reveal pathways and kinases associated with aggressive human cancers	Monsivais D., Vasquez Y.M., Chen F. et al.	2021, USA	Mass spectrometry was used to determine differential patterns of protein expression associated with the grade or stage of development of seven types of cancer – invasive breast carcinoma, colon adenocarcinoma, lung adenocarcinoma, clear cell renal cell carcinoma, serous ovarian tumor, uterine corpus carcinoma, and childhood glioma. The study showed that each cancer type had a proteomic signature that was different from those of other cancer types. Differentially expressed proteins and mRNAs were identified for late cancer development for each of the seven tumor types	[41]
Comprehensive Metabolomics and Lipidomics Profiling of Prostate Cancer Tissue Reveals Metabolic Dysregulations Associated with Disease Development	Lima A.R., Carvalho M., Aveiro S.S. et al.	2021, Portugal	Untargeted mass spectrometry and nuclear magnetic resonance techniques have been applied to prostate cancer research. The results revealed significant changes in the levels of 26 metabolites and 21 types of phospholipids in prostate cancer tissue, indicating dysregulation of 13 metabolic pathways associated with cancer development	[42]
Interim clinical trial analysis of intraoperative mass spectrometry for breast cancer surgery	Basu S.S., Stopka S.A., Abdelmoula W.M. et al.	2021, USA	Ambient ionization mass spectrometry was used to rapidly analyze the lipid profile of invasive breast carcinoma for potential application in breast-conserving surgery	[43]
Imaging Mass Spectrometry-Based Proteomic Analysis to Differentiate Melanocytic Nevi and Malignant Melanoma	Casadonte R., Kriegsmann M., Kriegsmann K. et al.	2021, Germany	Imaging mass spectrometry determined differences in the proteomic profile between malignant melanomas and melanocytic benign nevi with an overall accuracy of > 98%	[44]

Table (continued)

Title	Authors	Year, country	Methodological approach	No.
Intraoperative Mass Spectrometry Platform for IDH Mutation Status Prediction, Glioma Diagnosis, and Estimation of Tumor Cell Infiltration	Brown H.M., Alfaro C.M., Pirro V. et al.	2021, USA	To determine the extent of surgical intervention and increase the degree of safe resection, assessing the infiltration of tumor cells using desorption mass spectrometry with electrospray ionization, the isocitrate dehydrogenase mutation status was determined intraoperatively in patients with glioma	4527]
Metabolomic Profiling of Blood-Derived Microvesicles in Breast Cancer Patients	Buentzel J., Klemp H.G., Kraetzner R. et al.	2021, Germany	Using mass spectrometry and targeted metabolomic profiling of blood-derived microvesicles, it was shown that a combination of eight metabolites distinguishes breast cancer patients from healthy controls, there are differences between the molecular subtypes of breast cancer, and microvesicle biomarkers are a prognostic factor for overall survival	[46]
Lipidomic Signatures for Colorectal Cancer Diagnosis and Progression Using UPLC-QTOF-ESI+MS	Răchieriu C., Eniu D.T., Moiş E. et al.	2021, Romania	Using high-performance liquid chromatography – mass spectrometry, it was shown that several subclasses of lipids, including phosphatidylglycerols-phosphatidylethanolamines and phosphatidic acids, fatty acids and sterol esters, as well as ceramides, can be considered as specific markers for the development of colorectal cancer, dependent on lipogenesis and lipolysis	[47]
Global metabolomics profiling of colorectal cancer in Malaysian patients	Hashim N.A.A., Ab-Rahim S., Ngah W.Z.W. et al.	2021, Malaisia	High performance liquid chromatography – time of flight mass spectrometry identified 11 differential metabolites in blood serum, the levels of which were significantly different in patients with colorectal cancer. Using this panel allows to distinguish colorectal cancer with 80% accuracy	[48]
Lipidomic Profiling of Clinical Prostate Cancer Reveals Targetable Alterations in Membrane Lipid Composition	Butler L.M., Mah C.Y., Machiels J. et al.	2021, Australia	Using chromatography coupled with tandem mass spectrometry, significant differences in lipid composition were detected and spatially visualized in prostate cancer tumors compared to benign samples. The tumors were characterized by a higher proportion of monounsaturated lipids and elongated fatty acid chains in phosphatidylinositol and phosphatidylserine lipids	[49]
Integrated analysis of the faecal metagenome and serum metabolome reveals the role of gut microbiome-associated metabolites in the detection of colorectal cancer and adenoma	Chen F., Dai X. Zhou C.C. et al.	2022, China	Using mass spectrometry in combination with ultra-performance liquid chromatography, it was shown that reprogramming of the intestinal microbiome in patients with colorectal cancer is associated with changes in the serum metabolome. The model based on a panel of 8 gut microbiome-associated serum metabolites predicts colorectal cancer and colon adenomas with 84% and 93% accuracy, respectively	[50]
Lipidomic profiling of human serum enables detection of pancreatic cancer	Wolrab D., Jirásko R., Cífková E.et al.	2022, Czech Republic, Germany, Singapore, etc.	A three-phase biomarker study using comprehensive mass spectrometric determination of a wide range of serum lipids revealed statistically significant differences in long-chain sphingomyelins, ceramides, and (lyso)phosphatidylcholines between patients with pancreatic cancer and healthy controls	[51]
Proteogenomic analysis of lung adenocarcinoma reveals tumor heterogeneity, survival determinants, and therapeutically relevant pathways	Soltis A. R., Bateman N.W., Liu J. et al.	2022, USA	The use of molecular profiling technologies in the study of lung adenocarcinoma has identified three distinct cancer subtypes with unique characteristics, such as expression levels, mutations, proteomic regulatory networks, and therapeutic vulnerabilities	[52]

Profiling of Urine Carbonyl Metabolic Fingerprints in Bladder Cancer Based on Ambient Ionization Mass Spectrometry	Li Y., Jiang L., Wang, Z. et al.	2022, China	Metabolic fingerprint profiling of carbonyl compounds in bladder cancer based on the N,N-dimethylethylenediamine desorption, separation, and ionization mass spectrometry platform identified 9 potential biomarkers for early non-invasive diagnosis	[53]
N-Glycan and Glycopeptide Serum Biomarkers in Philippine Lung Cancer Patients Identified Using Liquid Chromatography–Tandem Mass Spectrometry	Alvarez M.R.S., Zhou Q., Tena J. et al.	2022, Philippines	Using ultra-high performance liquid chromatography coupled with triple quadrupole mass spectrometry, specifically expressed glycoproteins were identified in the serum of lung cancer patients	[54]
Integrating age, BMI, and serum N-glycans detected by MALDI mass spectrometry to classify suspicious mammogram findings as benign lesions or breast cancer	Blaschke C.R., Hill E.G., Mehta A.S. et al.	2022, USA	Using matrix-assisted laser desorption / ionization, serum N-glycans were identified that have diagnostic potential for the differential diagnosis of benign tumors and invasive breast cancer	[55]
Deep mining and quantification of oxidized cholesteryl esters discovers potential biomarkers involved in breast cancer by liquid chromatography-mass spectrometry	Wang X., Li H., Zou X., Yan X. et al.	2022, China	Using quadrupole time-of-flight mass spectrometry combined with reversed-phase liquid chromatography, an increased content of oxidized cholesteryl ester was determined in serum samples from patients with breast cancer	[56]
Identification of prediagnostic metabolites associated with prostate cancer risk by untargeted mass spectrometry-based metabolomics	Östman J.R., Pinto R.C., Ebbels T.M. et al.	2022, Sweden, Great Britain	Untargeted metabolomic profiling based on high-resolution mass spectrometry of blood plasma has identified a number of phospholipids and some free fatty acids that are positively associated with the risk of developing common and non-aggressive prostate cancer	[57]
MALDI Mass Spectrometry Imaging highlights specific metabolome and lipidome profiles in salivary gland tumor tissues	Sommella E., Salviati E., Caponigro V. et al.	2022, Italy	Laser desorption / ionization mass spectrometry imaging was used to identify lipid and metabolic markers of parotid neoplasms: glycerophospholipids, glutamate metabolism, and nucleotides were markedly increased in tumor tissues, while sphingomyelins and triacylglycerols were decreased	[58]
Coated Blade Spray-Mass Spectrometry as a New Approach for the Rapid Characterization of Brain Tumors	Bogusiewicz J., Gaca-Tabaszewska M., Olszówka, D. et al.	2022, Poland, Canada	Coated blade spray mass spectrometry coupled with high-resolution mass spectrometry has developed a method for the differential diagnosis of meningioma and glioma based on differences in the lipid profile of surgical biopsies	[59]
Metabolomic profile of prostate cancer-specific survival among 1812 Finnish men	Huang J., Zhao B., Weinstein S. J. et al.	2022, China, USA	A prospective, untargeted metabolomic analysis identified prediagnostic serum metabolites associated with the molecular basis of prostate cancer progression and patient survival	[60]
Blood plasma metabolome profiling at different stages of renal cell carcinoma	Maslov D.L., Trifonova O.P., Lichtenberg S. et al.	2022, USA, Russia	Mass spectrometry of blood plasma revealed an association between changes in the levels of certain amino acids and the progression of kidney cancer	[61]
Molecular pathological diagnosis of thyroid tumors using spatially resolved metabolomics	Huang L., Mao X., Sun C. et al.	2022, China	Using metabolomic analysis with spatial resolution, a molecular diagnostic method has been developed to distinguish benign adenomas and malignant papillary carcinomas of the thyroid gland	[62]
Proteomic signatures of infiltrative gastric cancer by proteomic and bioinformatic analysis	Zhang L.H., Zhuo H.Q., Hou J.J. et al.	2022, China	Using high-performance liquid chromatography and tandem mass spectrometry, 20 differentially expressed proteins were identified that can be used as prognostic markers in infiltrating gastric cancer	[63]
Identifying cancer cell-secreted proteins that activate cancer-associated fibroblasts as prognostic factors for patients with pancreatic cancer	Luo Q., Liu J., Fu Q. et al.	2022, China	Liquid chromatography – tandem mass spectrometry was used to identify 6 proteins secreted by pancreatic cancer cells that promote cancer cell migration and invasion to evaluate the prognosis of 3-year postoperative survival	[64]

Table (continued)

Title	Authors	Year, country	Methodological approach	No.
Salivary Lipids of Patients with Non-Small Cell Lung Cancer Show Perturbation with Respect to Plasma	Hwang B.Y., Seo J.W., Muftuoglu C. et al.	2023, Turkey	Using nanoflow ultra-high performance liquid chromatography – electrospray ionization – tandem mass spectrometry, 27 salivary lipids and 10 plasma lipids were isolated as candidate markers for non-small cell lung cancer. The study showed that changes in the distribution of salivary lipid profile may be more informative markers than changes in blood plasma	[65]
Data-Independent Acquisition Mass Spectrometry Analysis of FFPE Rectal Cancer Samples Offers In-Depth Proteomics Characterization of the Response to Neoadjuvant Chemoradiotherapy	Stanojevic A., Samiotaki M., Lygirou V. et al.	2023, Serbia, Greece, the Netherlands, Spain	Using mass spectrometry, proteins were isolated in biopsy samples of rectal cancer tumors – prognostic markers of a favorable outcome of non-adjuvant chemoradiation therapy for selecting the optimal volume of surgical intervention and choosing the optimal treatment strategy	[66]
Serum Proteomic Profiles of Patients with High and Low Risk of Endometrial Cancer Recurrence	Pietkiewicz D., Zaborowski M.P., Jaz K. et al.	2023, Poland	Using matrix laser desorption / ionization – time-of-flight mass spectrometry of blood serum, protein biomarkers were isolated to stratify patients with low and high risks of developing relapses for more clinically based treatment and intensification of first-line therapy	[67]
Kinase Inhibitor Pulldown Assay Identifies a Chemotherapy Response Signature in Triple-negative Breast Cancer Based on Purine-binding Proteins	Wang J., Saltzman A.B., Jaehnig E. et al.	2023, USA	Using chromatography – mass spectrometry, an approach based on the quantitative determination of kinase inhibitors has been developed to predict the effectiveness of non-adjuvant chemotherapy for triple-negative breast cancer	[68]
Lung adenocarcinoma and squamous cell carcinoma difficult for immunohistochemical diagnosis can be distinguished by lipid profile	Yamashita T., Takanashi Y., Uebayashi A. et al.	2023, Japan	Using liquid chromatography and tandem mass spectrometry, a method has been developed for the differential diagnosis of lung adenocarcinoma and squamous cell carcinoma of the lung by analyzing the lipid profile of a biopsy specimen	[69]
Proteomic and Metabolomic Analysis of Bone Marrow and Plasma from Patients with Extramedullary Multiple Myeloma Identifies Distinct Protein and Metabolite Signatures	Dunphy K., Bazou D., Henry M. et al.	2023, Israel, Finland	Using chromatography – mass spectrometry methods, the proteome of mononuclear cells of bone marrow cells and blood plasma was studied, and a method was developed for the differential diagnosis of multiple myeloma and extramedullary multiple myeloma by analyzing the metabolomic profile of blood plasma	[70]

existing marker glycoprotein CA 19-9, especially at an early stage.

A colorectal cancer study [50] developed a diagnostic panel including eight serum metabolites associated with the gut microbiome to predict colorectal cancer and colorectal adenoma, which showed high performance and specificity values in targeted and untargeted metabolomic analyses.

A study on bladder cancer [53] considered the use of mass spectrometry of urine to search for metabolites containing carbonyl groups for diagnosis. The work identified nine potential biomarkers that can be used as a non-invasive and less expensive alternative to traditional cystoscopy.

Mass spectrometry-based techniques and approaches can be an invaluable tool in the operating room as they allow for a rapid analysis of biological materials. Differentiation of cancerous and healthy tissues, as well as tumor classification based on its histologic and molecular features using rapid evaporative ionization mass spectrometry (REIMS) is shown in the article [26]. This method is based on the analysis of aerosols generated when tissue is cut with an electrocautery blade or other instruments and then sent to a mass spectrometer. This tool, also known as the smart knife (iKnife), has been widely used in the surgical differentiation of healthy and cancerous tissues [59].

The possibility of using ambient mass spectrometry to assess the percentage of tumor cells in the tissues of glial brain tumors is presented in the article [35]: a quick and accurate assessment of the percentage of tumor cells in clinical practice and neurosurgery plays an important role for diagnosis and surgical intervention. The researchers trained the system to estimate the percentage of tumor cells with an accuracy of about 90% using a cassette embedding method, which allows for samples to be analyzed without preliminary preparation and machine learning methods. The use of such techniques can automate routine tissue screening and tumor cell percentage assessment, speeding up estimation of tumor cell percentage during neurosurgery.

In cancer research, mass spectrometry-based proteomic approaches are used to initially identify potential new prognostic, diagnostic or therapeutic markers, opening new opportunities for subsequent research. The ability to conduct proteomic studies on formalin-fixed and paraffin-embedded tissues [10, 12, 23] provided an opportunity for retrospective proteomic studies on selective cohorts with long-term follow-up.

Biological materials obtained during autopsies of patients with cancer are of great interest for research because they provide an adequate assessment of the quality of clinical diagnosis and contribute to the development of optimized approaches to therapy [71]. Implementation into clinical practice of advanced methods for assessing protein expression and conducting advanced translational multi-omics studies allows for the most comprehensive understanding of the pathogenetic processes in cancers associated with changes in the molecular, metabolic, and genetic landscapes [72].

Using mass spectrometry to determine the treatment strategy and evaluate therapy effectiveness

Patients with cancer undergo clinical staging, which influences the choice of optimal treatment and prognosis of the disease. Treatment options may include various combinations: follow-up, surgery, and / or radiation therapy. Thus, initial risk stratification in patients with carcinogenesis is important to achieve optimal therapeutic results and maintain the quality of life. Predictive biomarkers of a risk of complications or late effects of treatment are needed for clinical decisions on treatment selection.

A proteomic study of 116 melanoma tumors found that the response to immunotherapy was associated with enriched mitochondrial lipid metabolism. High levels of mitochondrial metabolism resulted in increased antigen presentation and interferon signaling. In addition, knockout of genes associated with beta oxidation reduced the sensitivity of melanoma to T cell killing. These results indicate an important role of mitochondrial lipid metabolism in response to immunotherapy and may have implications for the development of new treatment strategies for melanoma.

The results of a study by scientists from the USA and Turkey indicate an association of metabolic pathways of purines, pyrimidines, nitrogen, and porphyrins with radiosensitivity or radioresistance [20]. The work involved metabolomic profiling of patients with prostate cancer who underwent radiation therapy, which is important for identifying their new metabolomic status and assessing the consequences of radiation. It was shown that bilirubin can be used

as a reference value for prostate cancer patients receiving radiotherapy, as its level may be associated with treatment-induced liver toxicity. The change in the porphyrin pathway and its associations with bilirubin and phosphoric acid during radiation therapy are of particular interest. This may have practical implications in selecting optimal treatment strategies and monitoring the effectiveness of radiation therapy in patients with this type of cancer.

A study [16] assessing the role of patient plasma extracellular vesicles for early diagnosis of glioblastoma and as a prognostic tool for optimal clinical management showed that extracellular vesicle concentrations were increased in plasma of glioblastoma patients compared to healthy controls. After tumor resection, a decrease in the number of extracellular vesicles to the level of healthy people was observed. During relapse, an increase in the number of extracellular vesicles in plasma was revealed.

A team of scientists using high-resolution mass spectrometry-based proteomic profiling identified molecular subtypes of esophageal cancer [36]. The study performed a proteomic analysis of 124 paired esophageal cancer tumors and corresponding adjacent healthy tissues. The proteomic analysis revealed a catalog of proteins, phosphosites, and pathways that are dysregulated in esophageal cancer. Proteomic molecular subtyping identified two cancer subtypes associated with patient survival. In addition, several promising drugs specific to the malignant subtype of esophageal cancer were predicted and tested, which may open new treatment prospects.

An interesting approach, including the use of proteomic methods in patients with prostate cancer, is the combined use of mass spectrometry and immunohistochemistry methods aimed at differential separation of non-metastatic and metastatic primary tumors and detection of metastases in lymph nodes [12]. One of the serious problems that specialists faced when assessing the risk and prognosis of prostate cancer was the inability to use standard prognostic schemes (prostate-specific antigen (PSA) concentration, Gleason / ISUP score, etc.) for accurate prediction of the biological course and metastatic potential of the tumor process [12]. The approach proposed by the authors [12] made it possible to compare the proteomic composition of tissues affected by various prostate tumors (non-metastatic primary, metastatic primary, secondary) with each other, with healthy prostate tissue or with benign hyperplasia. The results showed that zones of prostate cancer have measurable and significantly different biological markers compared to healthy tissue and also demonstrated several proteins that are likely to be involved in the tumorigenesis of differentially various tumors [12].

Using mass spectrometry in basic cancer research

Mass spectrometry-based methods are also used in basic research to search for molecular mechanisms of oncogenesis. Using this powerful tool in tandem with proteomic analysis enables to study protein characteristics on a large scale, including their expression levels, specific post-translational modifications, and protein – protein interactions. All this allows for the most complete understanding of the factors initiating carcinogenesis and cellular metabolism in oncology at the protein level. Quantitative proteomics makes significant contributions to the fundamental understanding of the molecular pathogenesis of cancer, providing researchers with comprehensive information about protein interactions and signaling pathways, altered metabolites and gene regulation in cancer cells. The above is also true for lipidomic, metabolomic, and glycomic analysis methods.

Indeed, many studies published in recent years provide important insights into cancer biology. Thus, recent work has substantiated the relationship between the occurrence and progression of colorectal cancer and the specific effects of the lipid environment [50]. It is important to note that the authors took into account the already known relationship between colorectal cancer, diet, metabolic disorders, and gut microbiome status.

When studying brain tumors, it was shown that primary glioblastomas, as well as those developed from astrocytomas, are enriched with mono- and diunsaturated phosphatidylcholines, while the content of saturated and polyunsaturated phosphatidylcholines and phosphatidylethanolamines decreases. These changes are apparently associated with the availability of polyunsaturated fatty acids and the activation of de novo lipid synthesis and beta-oxidation pathways under anaerobic conditions in the tumor center [75].

Other studies have carried out a detailed study of the significance of lipid markers that are valid

for subtyping non-small cell lung cancer regardless of tissue morphology and immunohistochemical markers [69], the role of aberrant glycosylation in tumorigenesis [54], and the contribution of low-molecular-weight metabolites (initiators, intermediates and products of various biochemical reactions) to the development of tumors [61]. Although all of the above advances relate primarily to the basic cancer research, in the future, these and other discoveries will allow for the identification of new cancer biomarkers and lead to the development of new therapeutic strategies.

Summarizing the results of modern research aimed at studying the possibility of using mass spectrometry for diagnosing and predicting the course of cancer from the perspective of precision medicine, we can say that the use of omics research methods based on mass spectrometry in oncology is of great fundamental and practical importance. Metabolomics studies are aimed at identifying and quantitative (or semi-quantitative) assessment of metabolites of different classes, understanding their nature and mechanisms of formation. Understanding the molecular biology of tumors is important for the discovery of new biomarkers of the tumor process, as well as for early detection or subtyping of the tumor. Expanding the use of mass spectrometry-based methods in oncology will help increase the clinical value of biomarkers and find specific metabolites for various cancers, which is a key point in the search for new therapeutic targets, and will contribute to the development of new pathogenetically grounded preventive measures and recommendations for the clinical management of cancer patients.

CONCLUSION

Cancers are some of the most serious problems of modern healthcare [76, 77]. Despite some progress in the diagnosis and development of approaches to treatment and prognosis of cancer in recent years, there is still an urgent need for the discovery of new cancer biomarkers. Efforts in this direction have been made using various approaches, including new high-tech and knowledge-intensive methods, such as genomics, transcriptomics, metabolomics, and proteomics [78].

Identification of molecules of interest and total screening of the molecular landscape in these methods is carried out using mass spectrometry, which has come a long way in recent decades, and today is a powerful technology thanks to the unprecedented level of sensitivity and specificity of the analysis, which is used in clinical practice for analyzing biological samples.

While nucleic acid-based methods of analysis have the advantage of amplification potential, but lack the “effector” component, omics technologies and proteomics, in particular, are considered to be more relevant in the context of application in oncology, since the proteome is directly involved in the implementation of biological effects [79]. Detection of quantitative and / or qualitative modifications of proteins characterized by low abundance in biological samples requires the use of highly sensitive and specific analytical methods. Liquid chromatography in tandem with mass spectrometry is the most widely used method for comprehensive protein identification and quantification.

The use of mass spectrometry in oncology research is not limited to the proteomic analysis and affects other areas of omics research (lipidomics [80], metabolomics [81], glycomics [82]).

Following the literature review, it was found that many publications of recent years describe various approaches and technologies for using mass spectrometry for analyzing biological samples. Mass spectrometry was initially used in clinical toxicology laboratories for specialized identification of target compounds (1950s) [78]. However, already in 1957, the development and use of a portable clinical mass spectrometer for direct, continuous quantitative analysis of four gases in patient’s exhaled breath was reported in assessing the function of external respiration [83].

With the development of new ionization sources and mass analyzers in the 1990s, mass spectrometry has made a transition from a complex analytical tool used almost exclusively by experienced scientists in research laboratories to a more robust and versatile technology suitable for a wide range of applications, including clinical ones [84, 85]. Finally, in recent years, mass spectrometry in combination with gas chromatography and liquid chromatography has become a routine clinical laboratory technology that can provide critical information about clinical samples [86]. The development of electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) technologies has revolutionized the use of mass spectrometry, allowing researchers to study larger biological

molecules, such as glycoconjugates, proteins, and nucleic acids [87, 88]. The development of ESI has greatly facilitated the use of mass spectrometry in clinical analyzes of biological fluids, such as blood and urine [87], while the development of MALDI has led to advances in molecular imaging technologies for biological tissue sections for the diagnosis of cancer [88].

Ambient mass spectrometry-based methods, which do not involve chromatographic separation, are extremely promising due to their rapidity [89], while desorption electrospray ionization mass spectrometry (DESI-MS) allows for rapid imaging of biological tissues [90, 91].

The discovery of new cancer biomarkers has changed current practice in oncology [92, 93]. Tumor genome and transcriptome profiling are now established tools for the discovery of new biomarkers, but changes in proteome expression are more likely to reflect changes in tumor pathophysiology. Mass spectrometry is a powerful method that allows to carry out proteomic studies in personalized medicine, and the overlay of proteomic data on the results of genomic and transcriptomic studies allows us to move into the new field of proteogenomics, which demonstrates a growing potential in understanding cancer biology [94]. All this makes clinical proteomics and proteogenomics some of the most promising areas of molecular clinical research, including large-scale study of proteins, their expression, functions and structure, as well as application of the results obtained for the development of approaches to diagnosis, treatment, and monitoring of the effectiveness of cancer therapy. The implementation of all these methods is impossible without the use of mass spectrometry.

In the meantime, metabolomics is also one of the most promising omics technologies, which comprehensively analyzes low-molecular-weight molecules (metabolites) in biological systems [95, 96]. Since metabolites are initiators, intermediates, and products of various biochemical reactions, significant metabolic changes most accurately reflect the physiological and pathological processes occurring in the human body, including the occurrence and development of cancer [97, 98]. Numerous studies have demonstrated the ability of mass spectrometry-based approaches for detecting the results of metabolomic profiling for cancer diagnosis to predict the effectiveness of proposed therapeutic strategies and quickly monitor the progress of treatment [61, 99].

Finally, the latest mass spectrometry-based methods can be an invaluable tool in the operating room, as they can quickly analyze biological materials while the oncologist is working, which will allow for adjustment of the surgical strategy [59]. Indeed, the ability to accurately differentiate between cancerous and healthy tissue within a short time, as well as to classify a tumor based on its histologic and molecular features is an extremely valuable tool when performing operations with cancer resection [101]. The most prominent representative of such approaches to the use of mass spectrometry in oncology is REIMS based on the analysis of aerosols generated when the tissue is cut with an electrocautery blade or another instrument and then placed in a mass spectrometer [102]. This tool, also known as the smart knife (iKnife), has been widely used in the surgical differentiation of healthy and cancerous tissues. [103]. The iKnife is not the only technology for rapid analysis of such small volumes of biological samples. Alternative methods include paper spray (PS) ionization and probe electrospray ionization (PESI), which involve ionization of selected analytes by applying high voltage to the probe, installed directly into the mass spectrometer [73, 74]. In the CBS-MS approach, analytes are extracted from a biological matrix using a specially designed sword-shaped probe coated with immobilized sorption particles. After extraction, the probe is inserted into the mass spectrometer interface, where a drop of desorption solvent is placed on the surface of the blade to release analytes, and high voltage is applied to ensure ionization and analysis of the extracted substances [104]. All of the described approaches, in addition to high accuracy and rapidity of the results obtained, allow for the standardization of diagnostic procedures, which is necessary for the use of these methods for precision medicine in clinical practice.

Therefore, mass spectrometry-based technologies are rapidly moving from laboratory to clinical use in the direct analysis of biological samples of tissues and body fluids. At the same time, there is parallel search for new ways of using them, which can have a significant positive impact on the development of approaches to the study of the pathogenetic features of carcinogenesis at the molecular level, as well as to the diagnosis and treatment of cancer

patients. It can be expected that in the next decade, mass spectrometry and technologies based on direct analysis of tissue samples will continue to be implemented into clinical practice and, after approval by regulatory authorities, will become routine methods in oncology.

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Functional analysis of a new splicing mutation in the *MYBPC3* gene in hypertrophic cardiomyopathy

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ABSTRACT

Aim. To study the pathogenic effect in the *MYBPC3* splice-site variant in the patient with hypertrophic cardiomyopathy.

Materials and methods. The study was conducted using a DNA sample obtained from a patient with hypertrophic cardiomyopathy, in whom a previously undescribed variant was identified in the splice donor site of intron 21. The methods used included constructing and cloning of minigenes (vector pSpl3-Flu2-TKdel) and transfection of a human cell culture (HEK293T), followed by isolation of mRNA, production of cDNA, PCR of the minigene region containing the analyzed fragment, agarose gel electrophoresis, and Sanger sequencing.

Results. The chr11:47339649-A-C (hg38) variant, disrupting the splice donor site in intron 21 (NM_000256.3: c.2067+2T>G), was identified in the 23-year-old patient with obstructive hypertrophic cardiomyopathy. To directly analyze the effect of this variant on splicing, a vector containing exon 21, intron 21, exon 22, and partially introns 20 and 22 of the *MYBPC3* gene was obtained. A comparison of mRNAs from the minigenes containing / not containing the variant showed that the chr11:47339649-A-C substitution led to exon 21 and exon 22 skipping during splicing.

Conclusion. The study established the functional significance of the previously undescribed variant c.2067+2T>G in the *MYBPC3* gene, resulting in disruption of the mRNA splicing mechanism in the patient with hypertrophic cardiomyopathy. This variant can be classified as pathogenic.

Keywords: hypertrophic cardiomyopathy, *MYBPC3*, minigene, splicing

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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Функциональный анализ новой мутации сплайсинга c.2067+2T>G в гене *MYBPC3* при гипертрофической кардиомиопатии

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РЕЗЮМЕ

Цель – исследование патогенного эффекта варианта в сайте сплайсинга *MYBPC3* у пациента с гипертрофической кардиомиопатией.

Материалы и методы. Исследование проведено с использованием образца ДНК пациентки с гипертрофической кардиомиопатией, у которой был выявлен ранее не описанный вариант в донорном сайте сплайсинга интрона 21. Применены методы конструирования и клонирования мини-генов (вектор pSpl3-Flu2-TKdel), трансфекции культуры клеток человека (HEK293T), с последующим выделением мРНК, получением кДНК, ПЦР участка мини-гена, содержащего анализируемый фрагмент, электрофореза в агарозном геле, секвенирования по Сэнгеру.

Результаты. Вариант chr11:47339649-A-C (hg38), нарушающий донорный сайт сплайсинга в интроне 21 (NM_000256.3: c.2067+2T>G), был выявлен у пациентки 23 лет с обструктивной формой гипертрофической кардиомиопатии. Для прямого анализа влияния этого варианта на сплайсинг был получен вектор, содержащий экзон 21, интрон 21, экзон 22, частично интроны 20 и 22 *MYBPC3*. Сравнение мРНК, полученных для мини-генов, содержащих или несодержащих исследуемый вариант, показало, что замена chr11:47339649-A-C приводит к пропуску экзонов 21 и 22 в процессе сплайсинга.

Заключение. В результате исследования установлена функциональная значимость ранее не описанного варианта c.2067+2T>G в гене *MYBPC3*, приводящего к нарушению механизма сплайсинга мРНК у пациента с гипертрофической кардиомиопатией. Данный вариант может быть классифицирован как патогенный.

Ключевые слова: гипертрофическая кардиомиопатия, *MYBPC3*, мини-гены, сплайсинг

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

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INTRODUCTION

Hypertrophic cardiomyopathy is the most common hereditary cardiovascular disease with the prevalence of 1:500 in the population [1], and even 1:200, according to some data [2]. The disease is characterized by left ventricular hypertrophy, diastolic dysfunction, arrhythmias, and sudden cardiac death. Mutations in the sarcomeric protein genes are primarily distinguished among the causes underlying the development of the disease [3].

The most common causes of the disease are pathogenic variants found in the myosin binding protein C (*MYBPC3*) gene [4]. It is worth noting that more than 60% of the total number of variants of this gene are nonsense mutations and splicing mutations, leading to the nonsense-mediated degradation of truncated transcripts [5].

In recent years, both the number of identified genetic variants and the number of bioinformatics tools for predicting their pathogenicity have been steadily growing. However, despite all the progress in the development of algorithms for predicting the effect of variants *in silico*, functional analysis with modeling of the effect at the transcriptional or post-transcriptional level remains the classic confirmation of the pathogenicity of a variant. According to the guidelines for the interpretation of DNA sequence variants [6], functional studies confirming the influence of a genetic variant either on the protein structure and function or on the mRNA structure are some of the decisive criteria for assessing the pathogenicity of a variant (PS category criterion – Pathogenic strong).

In cases when it is not possible to obtain mRNA from the affected tissue of the patient for reverse transcription polymerase chain reaction (RT-PCR), a minigene system can be used for functional analysis in order to study the effect of identified genetic variants on splicing. The minigene method allows for studying the effect of the variant using genomic DNA as the starting material. This approach is very convenient for studying putative splicing mutations. A significant advantage of this method is the ability to analyze both the variant and the wild-type sequence simultaneously on an identical cell line. This feature makes it possible to exclude the influence of the *in vitro* experiment on the events occurring *in vivo*. In addition, the use of this approach allows for interpreting the influence of the variant on the splicing process itself [7, 8].

During the study of sarcomeric protein genes in patients with HCM, we identified a previously undescribed variant at the canonical splice site in the *MYBPC3* gene, which was assessed by the online resource VarSome [9] as "potentially pathogenic". The variant was a chr11:47339649-A-C (hg38) substitution disrupting the splice donor site in exon 21 (NM_000256.3: c.2067+2T>G). The variant was identified in a 23-year-old female patient with signs of left ventricular outflow tract obstruction and left ventricular myocardium mass index of 144.9 g / m^2 following the results of an echocardiographic examination. The patient's family history is not available. Since the c.2067+2T>G variant has not been previously described in the literature, the aim of the study was to perform a functional analysis using a minigene construct to confirm its effect on mRNA splicing.

MATERIALS AND METHODS

For amplification and subsequent cloning in the vector, primers with vector linker sequences at the ends were selected for a genomic fragment containing exons 21 and 22 and flanking intronic regions (with at least 100 base pairs) in the *MYBPC3* gene (Fig. 1).

F:5'-accagaattctggagctcgagTGACCTGAATATT ACAAGCCTCCC-3' and R:5'-attaaggagtgtattaagctt AGCACACTTCACAGAGACCC-3'.

With these primers, the specified region was amplified from the patient's genomic DNA using the Q5® High-Fidelity 2X Master Mix kit (New England Biolabs, USA), with the PCR conditions described further. Step 1: denaturation at 98 °C for 30 sec. Step 2 (35 cycles): denaturation at 98 °C for 10 sec, primer annealing at 60 °C for 15 sec, elongation at 72 °C for 30 sec. Step 3: final elongation at 72 °C for 2 minutes.

The pSpl3-Flu2-TKdel vector used was digested by XhoI and HindIII restriction enzymes (New England Biolabs, USA). The PCR product and the restricted plasmid fragment were purified using the CleanUp kit (Evrogen, Russia). Then the PCR product with a total length of 946 bp was cloned into the vector using the Gibson Assembly® Cloning Kit (New England Biolabs, USA), as described earlier [10]. The resulting recombinant vectors were introduced into NEB® 5-alpha Competent *E. coli* cell culture by chemical transformation, according to the manufacturer's protocol (New England Biolabs, USA).

The selection of colonies containing recombinant vectors was carried out by seeding the culture onto Petri dishes containing solid LB medium with kanamycin (selection marker, 50 μg / ml), followed by reseeding of the grown colonies into liquid LB medium. Plasmid DNA was isolated from an overnight culture of NEB® 5-alpha Competent *E. coli*. Testing for the presence of an insert containing or not containing the c.2067+2T>G variant was carried out using Sanger sequencing.

HEK293 FT cells (6×10^5 cells) were seeded in a 6-well plate in the DMEM medium (PanEco, Russia) with 10% FBS (Capricorn Scientific, Germany) at 37 °C in an atmosphere of 5% CO_2. Transfection of the plasmids carrying the minigene constructs, as well as empty plasmids, was carried out in 6-well

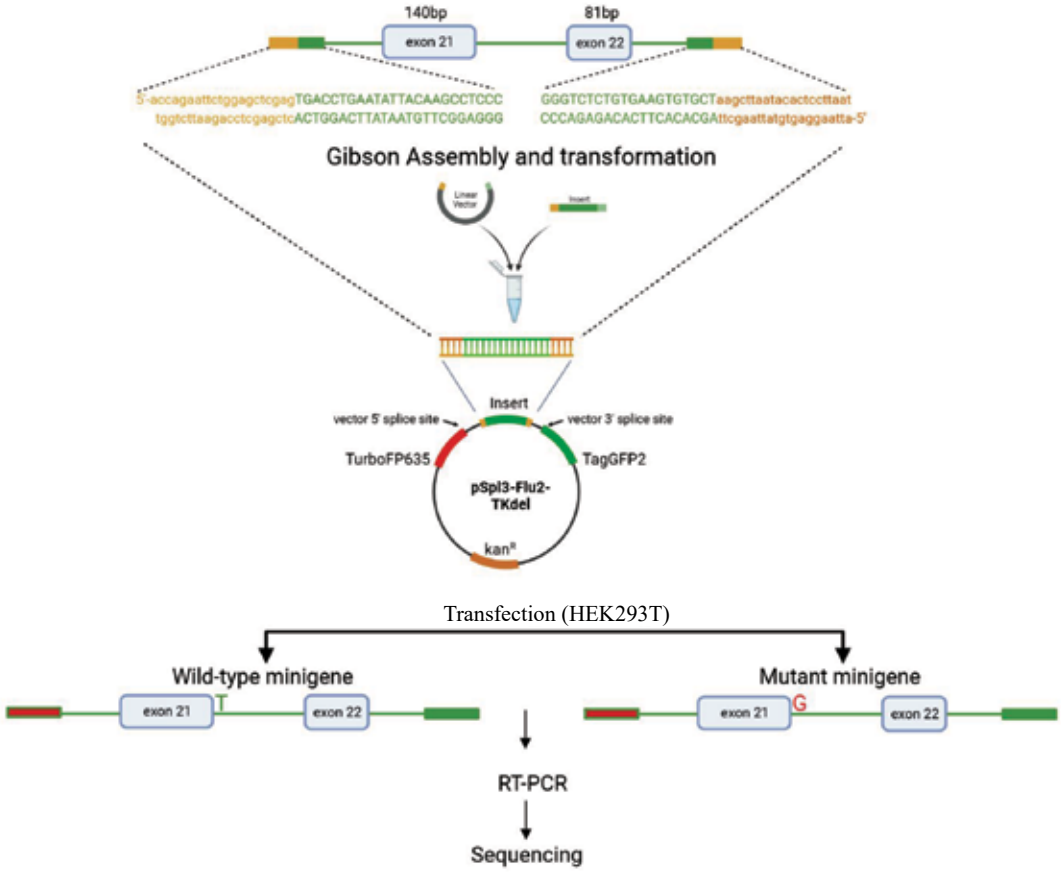

Fig. 1. Schematic representation of the pSpl3-Flu2-TKdel vector and minigene assay protocol. Orange rectangles correspond to the vector intronic sequences limiting the insertion. Red and green rectangles correspond to exonic sequences of the vector. Gray rectangles are the exons of the *MYBPC3* gene, and a thin green line designates intronic sequences.

plates using the GenJect-39 reagent (Molecta, Russia) according to the manufacturer's protocol. Forty-eight hours after transfection, the cells were harvested for total RNA extraction using the Lyra kit (Biolabmix, Russia). The resulting RNA samples were used for reverse transcription followed by PCR with primers flanking the minigene construct. PCR products were visualized using a 1.5% agarose gel with ethidium bromide.

RESULTS AND DISCUSSION

As a result of the study, we constructed the pSpl3-Flu2-TKdel vector containing a fragment of the *MYBPC3* gene limited by introns 20 and 22 (chr11:47360694-47361598, hg38) (Fig. 1). After assemblying the construct and transforming it into *E. coli*, we screened colonies containing the wild-type insert and the potentially pathogenic variant (Fig. 2, *a*). Next, the isolated plasmids were transfected into HEK293T cell culture. Two days later, total RNA was isolated and reverse transcription was performed with DNase treatment. Next, PCR was carried out with primers selected for the flanking regions of the vector encoding fluorescent proteins (TurboFP365 and TagGFP2).

Electrophoresis of PCR fragments obtained using cDNA as a template showed that in the presence of the c.2067+2T>G variant, the length of the PCR product was 140 bp less than in the case of the reference sequence of this region. Moreover, in the case of the wild type, the predominant presence of a transcript containing only exon 21 and a small amount of a transcript containing exons 21 and 22 was observed (Fig. 2, *b, c*). Sequencing of these products showed that in the case of the c.2067+2T>G variant, both exons 21 and 22 are removed from the mRNA, and the two transcripts observed in the case of the c.2067+2T allele contain either both exons or only exon 21 (Fig. 2, *c*).

It is known that the accuracy and efficiency of splicing are influenced by many factors, including the efficiency of splice site recognition, masking of splice sites and branch points by RNA secondary structures, intron – exon gene architecture, exonic and intronic silencers, and enhancers of splicing [11].

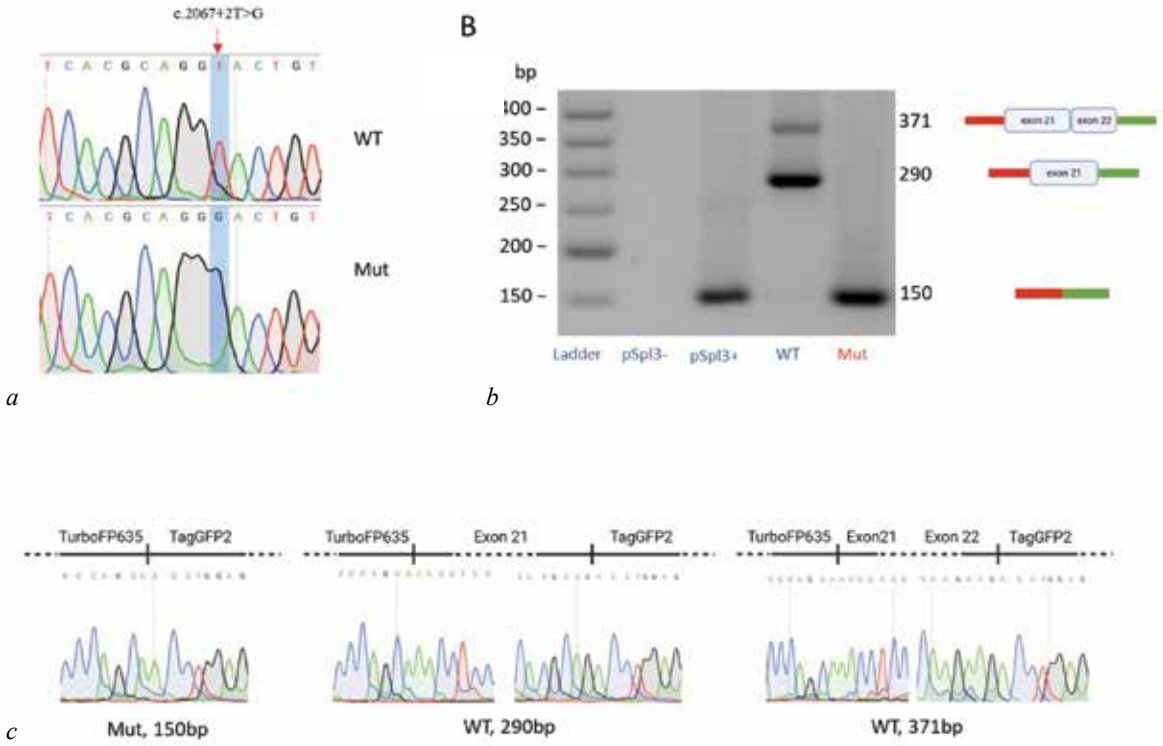

Fig. 2. Analysis of the c.2067+2T>G variant of the *MYBPC3* gene: *a)* results of plasmid sequencing for the presence of mutant and wild-type variants; *b)* results of electrophoretic separation of RT-PCR products in HEK293T cell lines: cell line without plasmids (pSpl3-), cell line with an empty plasmid (pSpl3+), cell line with plasmid containing the wild type (WT), cell line with a plasmid containing a mutant variant (Mut); red and green rectangles correspond to exonic sequences of the vector; the gray rectangle represents exons of the *MYBPC3* gene; *c)* sequencing results of RT-PCR products obtained from lines containing the mutant and wild-type variants.

Given that introns 20 and 22 are not fully included in the minigene, it can be assumed that splicing enhancers may be located in these regions, affecting the efficiency of excision of introns 21 and 22 and the retention of exon 22 in the transcript.

Thus, the experiment showed that the studied variant leads to the loss of the donor splicing site and skipping of the entire exon 21. This fact makes it possible to classify the c.2067+2T>G variant as a pathogenic variant underlying the development of hypertrophic cardiomyopathy and to identify it as the cause of HCM in this patient.

RNA splicing is the post-transcriptional process of removing non-coding intronic sequences from the original transcripts and joining exons to create a messenger RNA (mRNA). A significant number of pathogenic variants in the *MYBPC3* gene lead to a frameshift and subsequent gain of premature stop codon in the mRNA, or to the splicing alterations and, as a consequence, to skipping of individual exons (and also, in some cases, to a frameshift). It is also reported that variants localized in gene exons can alter exonic splicing enhancers and also lead to a disruption of this process [12].

The resulting truncated mRNA undergoes nonsense-mediated decay, leading to haploinsufficiency (insufficient amount of protein synthesized from one allele) as the mechanism of action of pathogenic variants in this gene in the development of HCM [13]. However, it has recently been shown that induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) with LoF mutations in the *MYBPC3* gene do not always demonstrate a decrease in myosin binding protein C (MyBP-C) [14]. Similarly, another study showed that iPSC-CMs containing mutations resulting in a premature stop codon in *MYBPC3* exhibit abnormal calcium signaling and molecular dysregulation even with normal amounts of MyBP-C, leading to the activation of the nonsense-mediated decay pathway and ultimately to the development of the HCM phenotype [15]. Thus, the very fact of activation of this pathway triggers the pathogenetic mechanism of disease development for *MYBPC3* variants.

CONCLUSION

Variations in canonical splice sites almost always result in splicing errors. However, this disruption type must be confirmed by studying the mRNA sequence, since the structure of mRNA cannot yet be accurately predicted *in silico* using bioinformatics methods [16–18]. It should be noted that although some genetic effects are tissue specific, cis-regulatory effects on splicing are typically present in a variety of tissues and cell types [19]. Thus, the effects that a pathogenic splice variant may have in one tissue are likely to be very similar to those in other tissues. Therefore, in *vitro* cell line studies represent well the in *vivo* situation. Our results showed that the c.2067+2T>G variant at the donor splice site in intron 21 leads to skipping of exon 21, and moreover, to skipping of exon 22, at least when using this minigene construct. Thus, the results of the study prove the pathogenic effect of the chr11:47339649-A-C (NM_000256.3: c.2067+2T>G) variant.

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Features of cystic fibrosis development in a patient with coinfection by *Mycobacterium abscessus* and *Mycobacterium tuberculosis* (clinical case report)

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ABSTRACT

The article presents a clinical case describing a favorable clinical outcome of mycobacterial infection and pulmonary tuberculosis caused by coinfection of *M. abscessus* and *M. tuberculosis* in a patient with pulmonary manifestations of cystic fibrosis one year after delivery. This outcome was achieved due to timely diagnosis and treatment of pulmonary tuberculosis and non-tuberculous mycobacterial infection in the patient with cystic fibrosis. Due to the development of molecular identification of mycobacteria species in the Tomsk region, mycobacterial lung disease was verified, which was challenging in the recent past. Previously, all cases with microscopic examination results positive for mycobacteria were classified as tuberculosis.

Keywords: cystic fibrosis, nontuberculous mycobacteria, M. abscessus, pulmonary tuberculosis, M. tuberculosis, coinfection

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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Особенности течения муковисцидоза при сочетании *Mycobacterium abscessus* и *Mycobacterium tuberculosis* (клиническое наблюдение)

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РЕЗЮМЕ

Представлено клиническое наблюдение, описывающее случай благоприятного клинического течения микобактериоза и туберкулеза легких, вызванный микст-инфекцией *M. abscessus* и *M. tuberculosis* у пациентки с легочными проявлениями кистозного фиброза (муковисцидоза) через 1 год после родоразрешения. Данный результат лечения был достигнут с помощью своевременной диагностики, начатому лечению туберкулеза и микобактериоза легких у пациентки с кистозным фиброзом (муковисцидозом). Благодаря развитию в Томской области микробиологической видовой идентификации микобактерий, был верифицирован микобактериоз легких, что еще в недавнем прошлом было проблематично, и все случаи с положительной микроскопией были отнесены к туберкулезу.

Ключевые слова: кистозный фиброз (муковисцидоз), нетуберкулезные микобактерии, *M. abscessus,* туберкулез легких, *M. tuberculosis*, микст-инфекция

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

In 1953, the extremely rare opportunistic pathogenic microorganism *Mycobacterium abscessus* that is fast-growing non-tuberculous mycobacteria (NTMB), whose natural environment is soil and water, was isolated from a soft tissue abscess of a patient after a knee injury [1, 2]. Due to the presence of identical biochemical properties, until 1992 they were combined into one species with *Mycobacterium chelonae*, which causes mycobacterial infection of soft tissues in a fish. Subsequently, after genomic analysis, the *M. abscessus* species was divided into three subspecies combined into the MABSc complex: *M. abscessus subsp. abscessus, M. abscessus subsp. massiliense, M. abscessus subsp. bolletii*. These microorganisms have a single sequence of the 16S rRNA gene, but there are differences in specific genes, in particular, those of great clinical importance for resistance to macrolides (*erm41* gene) [3].

Currently, according to foreign sources, NTMB of the MABSc complex are the second most common in patients with various chronic lung diseases after representatives of the *M. avium* complex and the first among all fast-growing mycobacteria [4]. The results of studies conducted in our country similarly show that in patients undergoing differential diagnosis in specialized anti-tuberculosis institutions, *M. abscessus* is most often isolated from fast-growing mycobacteria [6–8]. At the same time, the intraspecies differentiation of *M. abscessus* shows that the subspecies *M. abscessus subsp. abscessus* dominates in Russia (up to 70%), the subspecies *M. abscessus subsp. massiliense* is in the second place in terms of detection frequency (27%), and *M. abscessus subsp. bolletii* is not actually found [9]. In clinical practice, patients undergoing immunosuppressive therapy are mainly at high risk of MABSc infection: recipients of parenchymal organ transplants and patients receiving glucocorticoid and cytostatic therapy for various reasons [2, 10–12].

CLINICAL CASE

We present a clinical case of coexisting mycobacterial infection and pulmonary tuberculosis in a patient with cystic fibrosis. Actually, we narrate a further medical fate of a woman who had complex clinical features of cystic fibrosis during pregnancy and childbirth, described earlier [13].

Medical history. A female patient born in 1997 with a family history of cystic fibrosis (her sister is also ill) had symptoms of the underlying disease from the age of 1.5 months, the diagnosis was verified at almost 5 years old (sweat chlorides – 119 mEq / l). At the

age of 14, lower lobar thoracoscopic lobectomy was performed for bronchiectasis in S 8–10 on the right. In 2017, the patient had chronic vasomotor rhinitis at the vasodilation stage and underwent another surgical intervention – submucosal vasotomy of the lower nasal concha. The patient had one pregnancy and one childbirth. She lives in a comfortable apartment with her parents, husband, and one-year-old child. The patient claims she does not smoke, drink alcohol or use drugs.

The patient is constantly followed up at the Research Institute of Medical Genetics with the diagnosis: cystic fibrosis, mixed form (pulmonary intestinal), moderate severity, continuously recurrent course. Compound heterozygous for Dele 2,3/E92,K. The patient had chronic pancreatic insufficiency, severe course. According to the underlying disease, the patient was granted the status of category 3 disability indefinitely. The patient receives basic therapy: pulmozyme (tigerase) 1 ampoule per day through a nebulizer; bramitob 300 mg 2 times a day through a nebulizer (a cycle of 28 days followed by a break of 28 days) or in a similar scheme colistin 80 mg 2 times a day; creon 25,000 units (based on 6,000 units / kg of body weight) 10 capsules per day with meals; ursofalk 250 mg, 5 tablets per day (3 tablets at lunch after meals and 2 tablets at night); periodically – bronchitol 400 mg 2 times a day. Twice a year, she had scheduled hospitalizations in pulmonology departments, if necessary, with an exacerbation of bronchopulmonary infection, she was hospitalized urgently.

Starting from 20.05.2021, the patient had another scheduled hospitalization in the Pulmonology Department of the City Clinical Hospital No. 3. of Tomsk. She was worried about a constant cough with the release of a small amount of yellow sputum, an increase in body temperature to 38.0 °C. On auscultation, wet wheezing was noted in the lungs, moderate leukocytosis (11.8×10^9 / l) was observed in the CBC, *Staphylococcus aureus* was cultured from the sputum, with sensitivity to meropenem, ertapenem, and imipenem. When the patient was receiving antibacterial therapy, negative changes were established in the form of an increase in symptoms of intoxication and leukocytosis. Lung X-ray upon admission detected focal infiltrative changes in the pulmonary parenchyma around bronchiectasis that were also without significant changes. According to the examination algorithms for patients with chronic pulmonary diseases accepted in clinical practice, the patient's sputum was redirected to Tomsk Phthisiopulmonology Center (TPMC) for testing for tuberculosis by molecular genetic methods. MBTs with preserved sensitivity to rifampicin were detected by the GeneXpert method. On 28.05.2021, for further examination and treatment, she was transferred to the TPMCn to the department for patients with pulmonary tuberculosis.

Upon admission, the condition was of moderate severity, with shortness of breath during physical exertion and cough with mucopurulent sputum discharge. The patient's indicators were as follows: blood pressure 94/63 mm Hg, pulse 118 per minute, body temperature 36.4 °C, saturation 99%. The patient was conscious and alert. The skin was pale, with normal humidity. The body build was hyposthenic, the subcutaneous fat layer was poorly developed. The mucous membranes were normal and pink. Peripheral lymph nodes were not palpable. The heart sounds were clear and rhythmic. The chest was of the correct shape, breathing was harsh with multiple moist fine bubbling rales, respiratory rate was 18 per minute. The abdomen was soft and non-tender upon palpation. The liver was not enlarged. The blistering symptoms were negative. The spleen and kidneys were not palpable. There was no peripheral edema. The bowel movement occurred 2 times, it was liquid without pathological impurities of the usual color, diuresis was normal.

Data from laboratory and instrumental examinations upon admission were as follows. Complete blood count: hemoglobin – 107 g / l, erythrocytes – 4.64×10^{12} / l, leukocytes – 5.09×10^9/ l, neutrophils 30%, lymphocytes 62%, monocytes 8%. Erythrocyte sedimentation rate was 5 mm / h. Blood biochemistry: total protein 72.5 g / l; albumin 40.4 g / l; alanine aminotransferase – 7.1 U / l; aspartate aminotransferase – 17.8 U / l; creatinine – 75.8 mmol / l; urea – 2.9 mmol / l; fasting blood glucose – 5.1 mmol / l; amylase 35.8 U / l; alkaline phosphatase 262.6 U / l. Blood tests for HIV, hepatitis B and C were negative. General urine analysis: light yellow color; relative density 1,015; pH 6; protein, glucose – negative; epithelium 1–2; leukocytes 2–4; erythrocytes 1–2. Spirometry: forced vital capacity (FVC) – 3.53 (70.24%); forced expiratory volume in 1 second (FEV1) – 2.87 (67.79%); FEV1 / FVC – 79.4 (96%). Conclusion: moderate type I obstructive impairment of lung ventilation function. ECG: the position of the electrical axis of the heart was vertical, the rhythm was sinus, the heart rate was 112 beats per minute.

Sputum test results from 31.05.2021: by luminescent microscopy – acid-resistant microorganisms (ARM) (+++). PCR detected MTB DNA (sensitivity to isonazide and rifampicin was preserved), MTB growth was detected using microbial culture on liquid nutrient media (BACTEC MGIT 960), sensitivity to first–line drugs was preserved. Sputum culture on dense media – continuous MTB growth (+++), sensitivity to isoniazid, rifampicin, streptomycin, ethambutol, kanamycin (HRSE Km, respectively) was preserved. When cultured on liquid and then on dense media, NTMB was found, which in two samples were identified as *M. abscessus subsp. abscessus.* Typing was performed at the Novosibirsk Tuberculosis Research Institute using DNA hybridization on strips/time-of-flight mass spectrometry.

Computed tomography (CT) (28.05.2021, Fig. 1) detected infiltrative foci of different size in both lungs with a trend toward a merge into areas of consolidation, in the lower medial part of the right lung, a consolidation site measuring 45 x 35 mm with signs of volume reduction and traction bronchiectasis. In the supra-diaphragmatic zone of the left lung at S 8, 9, 10, there were merging foci and areas of consolidation of a similar nature with partially preserved bronchial patency and traction bronchiectasis. At S5, there was a subpleural consolidation site with signs of volume reduction and cylindrical traction bronchiectasis. Cylindrical and cystic bronchiectasis were determined by separate groups in the upper part of both lungs, more often on the right. Conclusion: focal lung dissemination, signs of bilateral polysegmental pneumonia, areas of local pneumosclerosis in the basal parts of both lungs; bronchiectasis of both lungs.

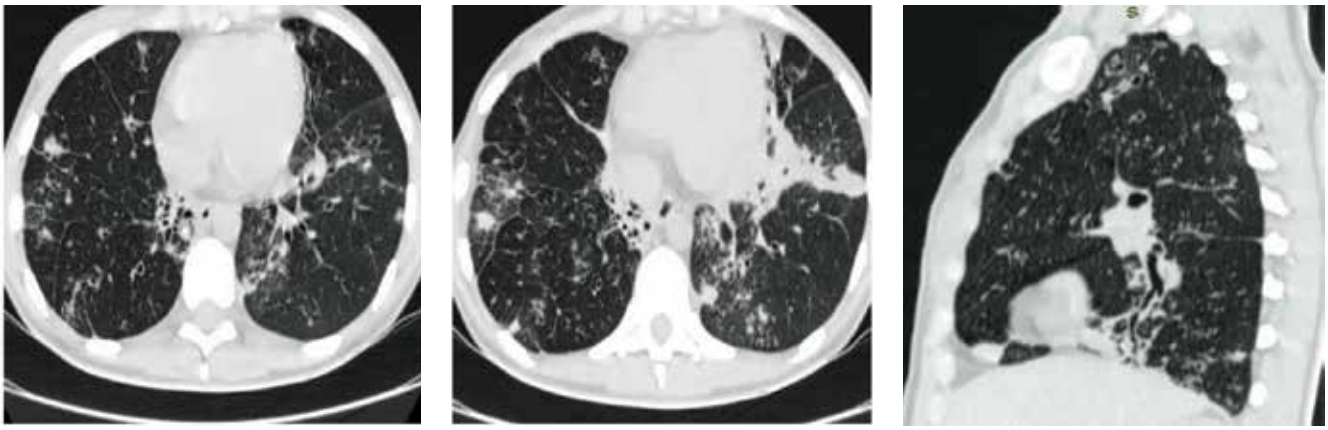

Fig. 1. Chest CT (28.05.2021).Signs of focal dissemination with consolidation around bronchiectasis in moderate pneumofibrosis

The patient was enrolled in the 1st group of follow-up with the diagnosis of disseminated pulmonary tuberculosis, infiltration phase, MTB (+). At the beginning, treatment was prescribed according to the regimen of drug-sensitive tuberculosis with a daily dosage of medicines according to body weight (H 0.6; R 0.6; Z 2.0; E 1.2) with an enhanced regimen for levofloxacin (Lfx) 1.0. After NTMB was identified, clarithromycin 1.0 was added to the treatment regimen.

During treatment, the patient developed adverse reactions to pyrazinamide – joint pain (relieved by the prescription of nonsteroidal anti–inflammatory drugs), and ethambutol decreased visual acuity (it was canceled). Furtheron, the prescribed therapy brought about positive clinical and radiological changes in the form of a decrease (resorption) of focal opacities and areas of consolidation (Fig. 2).

Microbiological analysis data over time: positive microbial culture test for MTB persisted for 8 months until February 2022, positive test for NTMB persisted for 12 months until May 2022. The patient was removed from the register in September 2022. The total course of treatment was 12 months. In the summer of 2023, there was a recurrence of bronchopulmonary infection caused by *Klebsiella pneumoniae,* in the diagnostic titer 10^5. She was examined, including for the recurrence of tuberculosis and mycobacterial infection, no data were obtained. Currently, the patient's condition is satisfactory, she is followed up by a pulmonologist and continues treatment for cystic fibrosis.

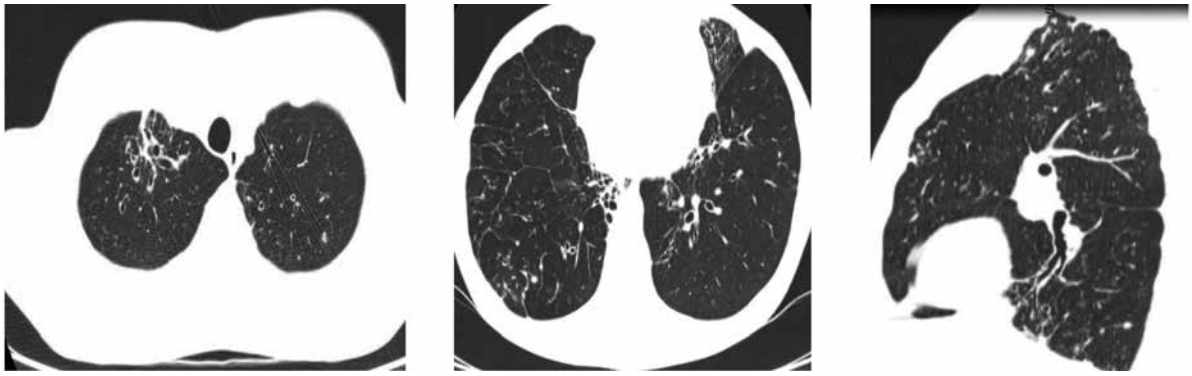

Fig. 2. Chest CT (30.07.2021): in comparison with the CT study of 28.05.2021, there was an improvement in the pulmonary pneumaticity in the lower lobe of the left lung due to a decrease in the number of focal opacities, the area of consolidation, and the severity of the interstitial inflammation

DISCUSSION

Among chronic lung diseases, predisposing factors to the development of mycobacterial infection caused by MABSc include bronchiectasis, cystic fibrosis, chronic obstructive pulmonary disease, primary ciliary dyskinesia, allergic bronchopulmonary aspergillosis, α-1-antitrypsin deficiency, pneumoconiosis, interstitial lung disease, pulmonary alveolar proteinosis, as well as conditions requiring frequent surgical interventions, punctures, injections, prosthetics [6–9, 12, 14, 15]. The symptoms of this pulmonary pathology are nonspecific and very similar to chronic infections of other etiologies, including tuberculosis. Clinical studies have shown that before the diagnosis of mycobacterial infection of the lungs caused by *M. abscessus*, patients in a third of cases have other types of NTMB, in half of cases they had previously experienced pulmonary tuberculosis.

According to CT data, bronchiectasis and single or multiple destruction cavities were detected in more than 50% of cases, and dissemination was detected in every third patient. Almost 70% of the sputum was found to contain acid-resistant bacteria [16]. In addition to the lungs, *M. abscessus* can also cause extrapulmonary infections of the central nervous system, skin, soft tissues, bones, joints, lymph nodes, other parenchymal organs, as well as generalized disseminated processes.

According to available data, mycobacteria of the MABSc complex have numerous virulence factors, ranging from early stages of colonization to intracellular persistence, which actually allow subspecies of *M. abscessus* to be classified as true pathogens, especially in patients with weakened immunity. The hydrophobicity of the mycobacterium cell wall contributes to their strong adhesion to various surfaces with the formation of biofilms, which provide high colonization in natural (bodies of water) and artificial reservoirs of infection, such as various water supply systems at home and in healthcare institutions, for example, on medical devices, surgical instruments / devices, dialysis, ventilators, and other devices [12, 14, 15].

An important feature of MABSc is the high content of lipids and waxes in the cell wall (glycopeptidolipids, GPL, up to 60% of dry weight), which plays a key role in the intracellular survival of microorganisms. GPL *M. abscessus*, like other virulent mycobacteria, form a cord factor and bind microorganisms together in the form of braids and bundles, bypassing the digestion processes [17]. This is facilitated by an advanced expression network of transport proteins (31) of lipid metabolites for GPL cell wall biosynthesis (MmpL and MmpS), which are known to be virulence factors in slow-growing mycobacteria, such as *M. tuberculosis* and *M.bovis*, severe pathogens for humans and animals [18].

In addition to inhibiting phagocytosis processes, the highly immunogenic GPL of mycobacteria of the MABSc complex shifts the effective initiation of cellular adaptive immunity toward an increased humoral response, which leads to increased apoptosis, extracellular replication microorganisms, and the development of acute inflammation and tissue damage with the formation of abscesses in infected people [19]. It has been proven that MABSc possess a whole set of genes (in particular, *GroEL-ES, hsp, ESX-4, EsxU and EsxT*) responsible for survival of

microorganisms inside macrophages. Their functional expression allows them to avoid intracellular oxygen-dependent destruction processes. such as heat shock or oxidative stress, and switch to a phenotype of slower growth, using various sources as energy, for example, fatty acids [20, 21].

Uniqueness of M. abscessus regarding its pathophysiology is confirmed (in addition to weak permeability of the cell wall) by the presence of efflux pumps capable of removing drugs, as well as enzymes that modify both antibacterial agents and their targets (Table).

Table

Biological determinants of *M. abscessus* drug resistance

Factors of natural / acquired drug resistance (enzymes or mutations)	Antibacterial resistance
Rifampicin-ADP-ribosyltransferase	Resistance to rifampicin
Rifampicin-ADP monooxygenase	Resistance to rifampicin
Rifamycin-glycosyltransferase	Resistance to rifampicin
Aminoglycoside-2-N-acetyltransferase	Resistance to aminoglycosides
Aminoglycoside-2-N-phosphotransferase	Resistance to aminoglycosides
β-lactamase	Resistance to β-lactams, including cephalosporins and carbapenems
Flavin-containing monooxygenase	Tetracycline resistance
The presence of the *erm41 gene* in M. abscessus subsp. *Abscessus and M. abscessus subsp. bolletii* (M. abscessussubsp. massiliense does not have it)	Resistance to macrolides
Induced mutations in the *23S* rRNA gene via the functional erm41 *gene*	Acquired resistance to macrolides (clarithromycin induces erm41 *to* a much greater extent than azithromycin)
Induced mutations in the *16S* rRNA gene	Resistance to aminoglycosides (amikacin, kanamycin, and gentamicin)
Induced mutations in the *eMBB* gene	Resistance to ethambutol
Mutations in the regions determining resistance to quinolones (QRDR, *gyr A, gyr* B)	Resistance to fluoroquinolones

According to available data, since the *M. abscessus* genome was decoded in 2009, this mycobacteriosis pathogen has a wide range of natural drug resistance to antibiotics. Taking into account, among other things, their high mutagenic activity, leading to the formation of secondary resistance, this disease is an "incurable nightmare" for clinicians with low treatment effectiveness (less than 50%) in most patients, even after a combination of 4–5 antibacterial drugs during months of therapy [21, 22]. *M. abscessus* has natural resistance to first-line anti-tuberculosis drugs, such as HRZE. In addition, *M. abscessus* is resistant to the main antimicrobial drugs used in respiratory infections, such as β-lactams, aminoglycosides (gentamicin), cyclins (doxycycline), sulfonamides, and macrolides (erythromycin), and induced or mutational resistance to fluoroquinolones is also manifested [2, 23].

As is known, patients with cystic fibrosis are highly susceptible to bacterial and mycotic infections, most often caused by *Staphylococcus aureus, Pseudomonas aeruginosa*, *Burkholderia cepacia* complex, including NTMB [15, 24]. At the same time, transmission of infection caused by mycobacteria of the MABSc complex can be carried out from person to person [25]. The mechanisms of susceptibility to these infections in patients with cystic fibrosis are explained by the pathogenesis of the disease, where due to a mutation in the *CFTR* gene (cystic fibrosis transmembrane conductance regulator), transport of electrolytes between the cell and the intercellular fluid changes, which leads to the formation of thick viscous sputum, secondary ciliary dysfunction of the bronchial mucosa, and inability to effectively eliminate microorganisms from bronchial secretions.

In addition, macrophage dysfunction is also observed in patients with cystic fibrosis, leading to impaired phagocytosis and excessive production of inflammatory mediators. More and more scientific evidence is being accumulated of a certain predisposition of these patients to infection caused by *M. abscessus* [9, 12, 14, 15, 26]. A typical treatment regimen includes intensive therapy using ≥ 2 parenteral antibiotics (options include amikacin, imipenem, tigecycline) for 6–8 weeks, followed by maintenance therapy with clofazimine and inhaled amikacin with or without azithromycin (taking into account its immunomodulatory effect) [27].

Unlike mycobacterial infection, pulmonary tuberculosis is potentially possible, but occurs in single cases of patients with cystic fibrosis. Data obtained

from Russian and French cystic fibrosis centers report 11 cases (8 in Russia, 3 in France) of coexisting pulmonary tuberculosis and cystic fibrosis. It is worth noting that in none of the described cases, the diagnosis was based on clinical and radiological symptoms, but only on the bacteriological examination of sputum, and in almost half of the cases, Russian patients had positive microscopy and multidrug resistance of the pathogen [28].

Simultaneous detection of MBTC and NTMB in mycobacterium cultures is not common, only a few publications are devoted to this issue. One of the latest foreign studies in South Korea determined that out of 6,201 culture samples, 2,456 (59.0%) were identified as MBTC, 2,456 (39.6%) as NTMB, and only 86 (1.4%) as mixed. At the same time, in the last samples, *M. intraculturale* (29.0%) and *M. abscessus* (29.0%) were determined in equal proportions together with *M. tuberculosis* [29]. Data obtained in Russia are similar. In 5,531 patients secreting mycobacteria, *M. tuberculosis* was detected in 3,829 (69.2%) mycobacterium culture samples, while NTMB was detected in 1,638 (29.6%). Mixed populations were found in 64 cases (1.2%), of which four (6.2%) had *M. tuberculosis* + *M. abscessus*[30]. Detailed information on patients secreting mixed mycobacterial cultures has not been published.

CONCLUSION

Thus, a clinical case demonstrates a favorable clinical course of mycobacterial infection and pulmonary tuberculosis caused by coinfection of *M. abscessus* and *M. tuberculosis* in the patient with pulmonary manifestations of cystic fibrosis one year after delivery. In the available publications, there was only one case of such coinfection in an immunocompetent patient without cystic fibrosis, whose favorable outcome would be determined only after pneumonectomy [31].

In our case, it should be noted that the algorithm for diagnosing tuberculosis in patients with chronic infectious processes in the lungs is justified. The development of molecular identification of mycobacteria species in the regions makes it possible to verify mycobacterial infection in the lungs, which was challenging in the recent past, and all cases with positive microscopy were classified as tuberculosis. Despite the fact that drug-sensitive NTMB has not been identified, effective therapy allows to conclude that *M. abscessus subsp. abscessus* were sensitive to macrolides (clarithromycin). It is worth noting that bacteria continue to excrete for a long period of time: MTB – 8 months of treatment, NTMB – 12 months.

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Authors' contribution

Filinyuk O.V. – analysis of a clinical problem, work with scientific literature. Teteneva A.V. – conception and design. Kruk E.A. – analysis of a clinical case. Loginova Yu.A., Kostoyakova E.P. – selection of clinical material, design. Bespalova I.D., Tetenev K.F., Karzilov A.I., Mishustina E.L. – consulting, registration, translation.

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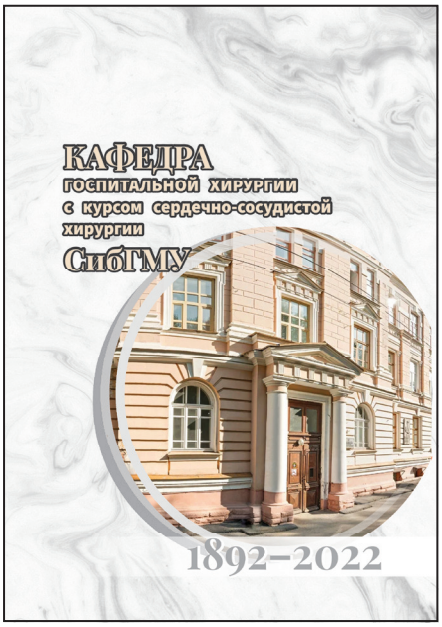
Издательский дом Сибирского государственного медицинского университета представляет серию книг

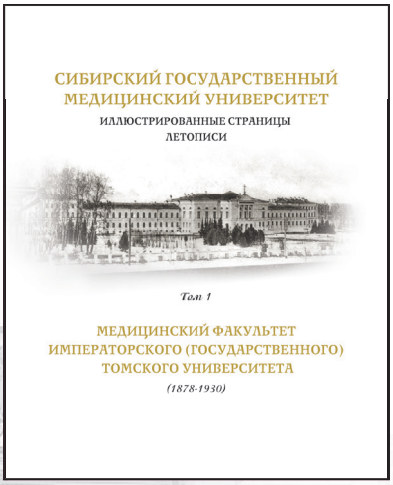
«Наследие томской медицины»



Книга посвящена 130-летнему юбилею кафедры госпитальной хирургии СибГМУ. Приведены биографические данные 79 сотрудников клиники и кафедры госпитальной хирургии в период с 1892 по 2022 г. Им предшествует подробная статья, характеризующая основные научно-практические достижения коллектива на каждом историческом отрезке. В издании упомянуты не только выдающиеся хирурги, звезды мировой величины, но и рядовые профессора, доценты, ассистенты, врачи-ординаторы, многие из которых связали с кафедрой и клиникой всю свою трудовую биографию. При изложении материала наряду с традиционными источниками информации использованы автобиографические документы, данные из семейных архивов, производственные характеристики нередко с сохранением авторского стиля.

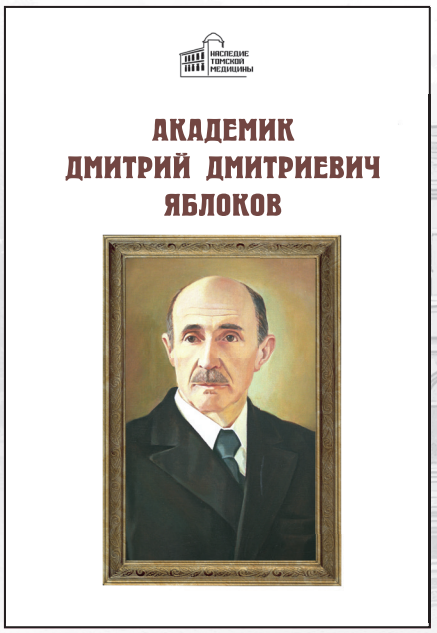
Это позволяет полнее ощутить атмосферу в обществе и рабочем коллективе в разные годы существования клиники. Текстовая информация сопровождена богатым иллюстративным материалом, многие фотографии опубликованы впервые.

Издание предназначено для хирургов, студентов старших курсов врачебных факультетов, специалистов по истории медицины.



Трёхтомная иллюстрированная летопись одного из старейших и наиболее авторитетных медицинских вузов России – Сибирского (Томского) государственного медицинского университета является по сути первой серьёзной попыткой осветить более чем 140-летнюю историю этого прославленного университета.Особенностью издания является его богатейший иллюстративный материал, включающий более четырёх тысяч фотографий (в том числе ранее практически неизвестных), и никогда не публиковавшиеся до этого крайне любопытные и интересные факты о жизни университета, его студентов и профессоров, воспоминания и рассказы выпускников и преподавателей вуза.

Для самого широкого круга читателей, интересующихся историей российских университетов, отечественного высшего медицинского образования и науки, развитием клинических и научно-медицинских школ, здравоохранения, историей Томска, Сибири, России…



В книге представлены биография и обзор научной, педагогической и общественной деятельности выдающегося ученого, терапевта, клинициста, академика АМН СССР, Героя Социалистического труда, лауреата Сталинской премии Дмитрия Дмитриевича Яблокова (1896-1993).

Для врачей, студентов, всех интересующихся историей медицины.

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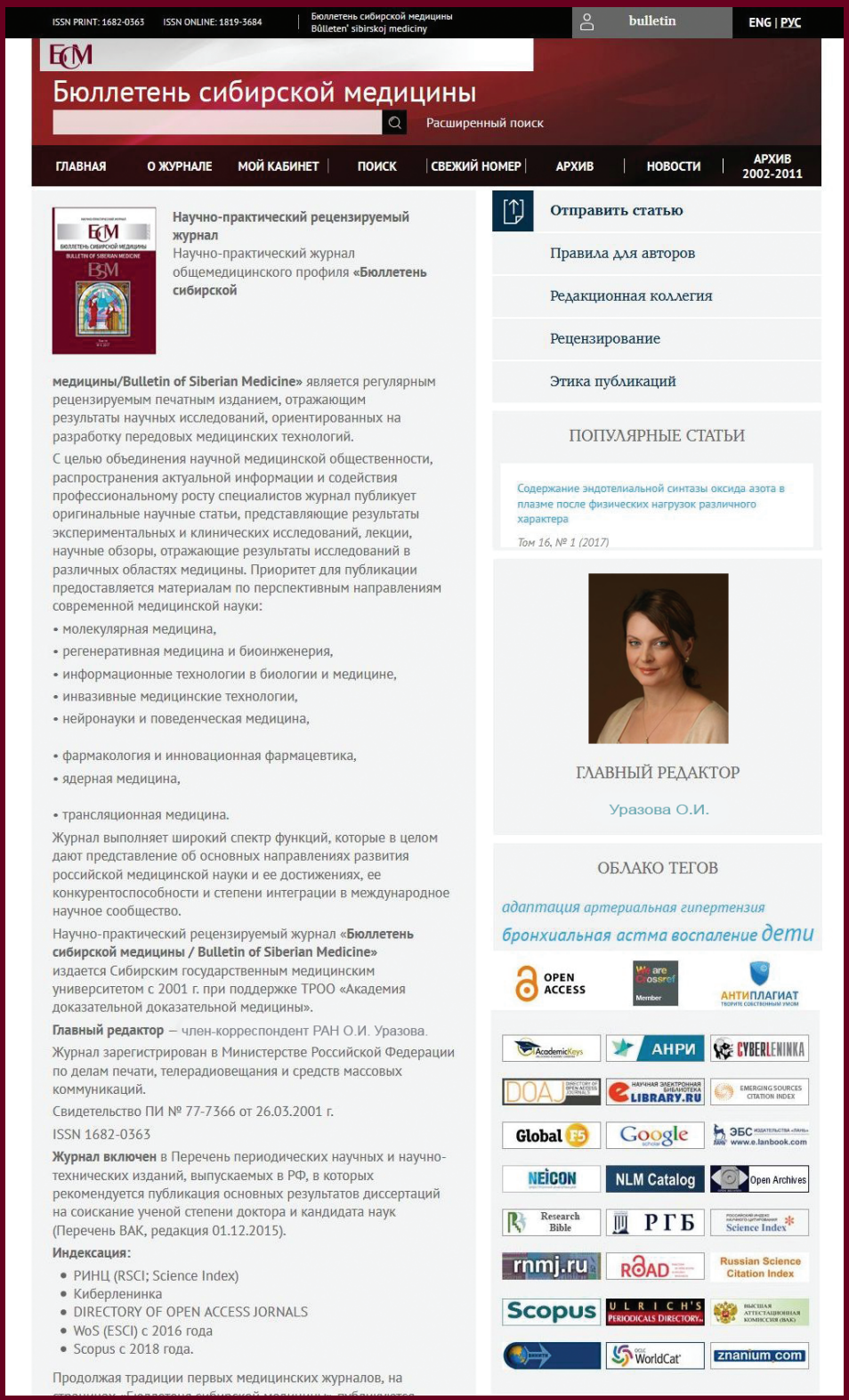

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