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Complexation of fluoroquinolones with magnesium ions

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ABSTRACT

Aim. To evaluate strength of magnesium ion complexes with levofloxacin and moxifloxacin.

Materials and methods. Complexation of levofloxacin, moxifloxacin, and reference ligands (ethylenediaminetetraacetate (EDTA), sodium citrate, and glycine) with magnesium ions in the range from 0.0 to 1.0 mmol / l was studied. The technique developed by the authors (patent RU 2680519 C1) was used to measure the rate of a model formation reaction of a magnesium phosphate coarse dispersion. Complexing activity of ligands was expressed in relation to EDTA activity and compared with the theoretical ion exchange equilibrium constants. The half maximal effective concentration (C_{50}) calculated by the Michaelis − Menten equation was used to evaluate the dependence of the complexing activity on the dose.

Results. A correlation between the activity of EDTA, citrate ions, and glycine and the theoretical equilibrium constants ($R = -0.87$, $p < 0.001$) was found. In the range from 0.0 to 0.4 mmol / l, both levofloxacin and moxifloxacin showed a lesser complexing effect than EDTA ($p < 0.001$), and in the range from 0.6 to 1.0 mmol / l, their complexing effect was comparable ($p > 0.050$). The activity of fluoroquinolones did not differ at any concentration ($p > 0.050$), but moxifloxacin C_{50} (0.13 mmol / l; 95% confidence interval (CI) 0.11–0.15) was significantly lower than that of levofloxacin (0.22 mmol / l; 95% CI 0.19–0.26), ($p < 0.001$). Within the 0.4–1.0 mmol / l concentration range, the activity of levofloxacin was higher than that of citrate ions and glycine ($p < 0.001$). Complexing activity of moxifloxacin was higher than that of citrate ions within the range of 0.2–1.0 mmol / l, and in the range of 0.4–1.0 mmol / l, it was higher than that of glycine ($p < 0.001$).

Conclusion. The proposed method showed that the complexing activity of fluoroquinolones was close to that of EDTA and exceeded the activity of citrate ions and glycine. The complexation of fluoroquinolones may be associated with their ability to induce side effects associated with magnesium deficiency.

Keywords: magnesium deficiency, fluoroquinolones, moxifloxacin, levofloxacin, complex compounds

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Комплексообразование фторхинолонов с ионами магния

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РЕЗЮМЕ

Цель. Провести анализ прочности комплексных соединений фторхинолонов левофлоксацина и моксифлоксацина с ионами магния.

Материалы и методы. В диапазоне концентраций 0,0–1,0 ммоль/л исследовано образование комплексов с ионами магния фторхинолонов левофлоксацина и моксифлоксацина и лигандов сравнения динатрия этилендиаминтетраацетата (ЭДТА), цитрата натрия и глицина. Согласно разработанной авторами методике (патент RU 2680519 C1), измеряли скорость модельной реакции образования грубодисперсной системы фосфатов магния. Комплексообразующую активность лигандов выражали относительно активности динатрия ЭДТА и сопоставляли с теоретическими константами равновесий соответствующих реакций. Зависимость комплексообразующей активности от дозы рассчитывали по уравнению Михаэлиса − Ментен, вычисляли концентрацию, при которой достигался полумаксимальный эффект (C_{50}).

Результаты. Установлена корреляция между активностью динатрия ЭДТА, цитрат-ионов, глицина и теоретическими их константами равновесия ($R = -0{,}87$, $p < 0{,}001$). Левофлоксацин и моксифлоксацин в диапазоне концентраций 0,0–0,4 ммоль/л проявляли меньший комплексообразующий эффект, чем динатрия ЭДТА ($p < 0{,}001$), в диапазоне концентраций 0,6–1,0 ммоль/л их эффект был сопоставим ($p > 0{,}050$). Активность фторхинолонов была одинаковой во всех концентрациях ($p > 0{,}050$), однако C_{50} моксифлоксацина была меньше, чем левофлоксацина: 0,13 ммоль/л (95%-й доверительный интервал (95%-й ДИ) 0,11–0,15) и 0,22 ммоль/л (95%-й ДИ 0,19–0,26) соответственно ($p < 0{,}001$). Активность левофлоксацина в диапазоне концентраций 0,4–1,0 ммоль/л превышала активность цитрат-ионов и глицина ($p < 0{,}001$). Комплексообразующая активность моксифлоксацина в диапазоне концентраций 0,2–1,0 ммоль/л была выше, чем у цитрат-ионов, в диапазоне 0,4–1,0 ммоль/л выше, чем у глицина ($p < 0{,}001$).

Заключение. Предложенный метод показал, что комплексообразующая активность фторхинолонов близка к активности динатрия ЭДТА и превышает активность цитрат-ионов и глицина. Комплексообразование фторхинолонов и ионов магния может сопровождаться серьезными нежелательными реакциями, вызванными дефицитом ионов магния в клетках.

Ключевые слова: моксифлоксацин, левофлоксацин, ионы магния, комплексные соединения

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INTRODUCTION

Fluoroquinolones (FQ) are a class of broad-spectrum antibiotics. The clinical use of FQ is limited due to a number of adverse reactions, such as tendinitis and tendon rupture [1, 2], aortic aneurysm and dissection [3], articular cartilage damage in children [2], and QT prolongation with a risk of ventricular tachycardia [2, 4].

Many assumptions have been made about the development mechanisms of FQ toxic effects [5]. Formation of complexes of FQ with Mg^{2+} ions [5–7] disrupts magnesium-dependent cellular protein function. For example, connective tissue disorder may develop due to dysfunction of magnesium-dependent integrins [8]. In the use of FQ to treat infectious diseases, magnesium metabolism in tissues is disturbed [9, 10]. A

quantitative assessment of the strength of FQ – Mg^{2+} ion complexes is necessary to study the pathogenesis of adverse reactions.

The aim of the study was to analyze the strength of magnesium ion complexes with fluoroquinolones – levofloxacin and moxifloxacin.

MATERIALS AND METHODS

Levofloxacin and moxifloxacin as broad-spectrum antibiotics were chosen as objects of the study. Levofloxacin hemihydrate (Shangyu Jingxin Pharmaceutical Co., Ltd., China) and moxifloxacin hydrochloride (Bayer AG, Germany) were used to prepare solutions. The FQ complexing properties were compared with the effects of disodium ethylenediaminetetraacetate (EDTA), glycine, and citrate ions (such as sodium citrate).

The complexing activity was evaluated by the method developed by the authors (patent for invention RU 2680519 C1 dated 22.02.2019 [11]). The method is based on the model formation reaction of a magnesium phosphate coarse dispersion according to the equation (1):

$$3Mg^{2+} + H_2PO_4^- + HPO_4^{2-} \leftrightarrow Mg_3(PO_4)_2\downarrow + 3H^+. \quad (1)$$

The source of magnesium ions was 1.0 M magnesium sulfate, the source of hydro- and dihydrophosphate ions was a buffer system with pH 8.5–9.2 obtained by mixing 0.1 M K_2HPO_4 and 0.1 M KH_2PO_4 solutions. When a substance forming complex compounds with magnesium ions was introduced into the reaction system, the reaction (2) proceeded simultaneously with the reaction (1):

$$Mg^{2+} + Lig^- \leftrightarrow [MgLig]^+, \quad (2)$$

where Lig is the test substance (ligand).

The reaction of combined heterogeneous and ligand exchange equilibrium is expressed with the equation (3):

$$3[MgLig]^+ + H_2PO_4^- + HPO_4^{2-} \leftrightarrow Mg_3(PO_4)_2\downarrow + 3Lig^- + 3H^+. \quad (3)$$

The introduction of the ligand reduces the number of magnesium ions available for the reaction (1), which is accompanied by a shift of the equilibrium (3) to the left and slowdown in the formation of a coarse dispersion. The process rate was estimated turbidimetrically using the Leki SS2107UV spectrophotometer (MEDIORA OY, Finland) by the decrease in the light transmission of the reaction system in 3 min (ΔT_3).

The experimental technique: light transmission of the system containing 10 ml of an analyte solution at the required concentration and 5 ml of a phosphate buffer with pH 8.5–9.2 (T_0) was measured, then 0.3 ml of 1 M $MgSO_4$ solution was added. After 3 min, light transmission was measured again (T_3). The degree of light transmission reduction (ΔT_3) was calculated by the formula (4):

$$\Delta T_3 = T_0 - T_3. \quad (4)$$

The EDTA solution properties were taken as a complexing activity criterion (1.00). The relative activity of the test substance was calculated by the formula (5):

$$OA = \frac{\Delta T_3(\text{control}) - \Delta T_3(\text{ligand})}{\Delta T_3(\text{control}) - \Delta T_3(\text{standard})}, \quad (5)$$

where ΔT_3 (control) is a decrease in light transmission in the control experiment (distilled water used instead of the solution of the substance), ΔT_3 (ligand) is a decrease in light transmission in the experiment with the test solution, ΔT_3 (standard) is a decrease in light transmission in the experiment with EDTA solution at the same concentration.

The experiment was repeated five times for each substance in order to increase the statistical power of the study. The studied range of ligand concentrations was chosen taking into account the ratio of FQ and magnesium ion concentrations in the blood. The normal content of Mg^{2+} in the serum is 0.66–1.07 mmol / l, the average concentration is 0.87 mmol / l [12]. Levofloxacin concentration after its oral administration at a dose of 500 mg reached a maximum value of 5.19 μg / ml in the plasma of healthy volunteers and during the first 24 h decreased to 0.46 μg / ml, which is 0.014 and 0.001 mmol / l, respectively (the molar mass of levofloxacin is 361 g / mol). The calculated ratio of magnesium ion and levofloxacin concentrations in the plasma was 62.1–870.0 [13]. In a similar pharmacokinetic study, the maximum concentration of moxifloxacin in the plasma was 3.10 mg / l (0.008 mmol / l), the corresponding ratio with the concentration of magnesium ions (108.8) is close to the values for levofloxacin [14].

In this study, the content of magnesium ions in the reaction system was 19.6 mmol / l, the studied ligand concentration decreased by 1.53 times compared with the baseline value after the addition of the phosphate buffer and magnesium sulfate. In this regard, the calculated concentration limits for the baseline solutions of the studied ligands were 0.03 and 0.49 mmol / l.

Meanwhile, the FQ concentration in the tissues is much higher than in the plasma. A study [15] per-

formed on geese showed that levofloxacin exposure, expressed as the area under the "time – concentration" curve (AUC), in the tissues was much more pronounced than in the plasma. The liver-to-plasma AUC ratio was 37.35. In this regard, the concentration range of the baseline ligand solutions was expanded (0.00–1.00 mmol / l).

The experimental method used makes it possible to calculate the activity of the studied ligand in comparison with the EDTA properties, which is considered as a standard with complexing activity of 1.00. To compare the experimental values of glycine and citrate ion activity with the known instability constants of their magnesium complexes, we calculated the equilibrium constants (K_e) for the reactions of the corresponding magnesium complexes with EDTA (reaction (6)).

$$[MgLig]^+ + EDTA^{2-} \leftrightarrow [MgEDTA] + Lig^-. \quad (6)$$

was calculated by the formula (7):

$$K_e = \frac{[[MgEDTA]][Lig^-]}{[[MgLig]^+][EDTA^{2-}]} = \frac{[Mg^{2+}][[MgEDTA]][Lig^-]}{[Mg^{2+}][[MgLig]^+][EDTA^{2-}]} = \frac{K_i([MgLig]^+)}{K_i([MgEDTA])}. \quad (7)$$

The strength of the $[MgLig]^+$ complex is inversely proportional to the of the process (6). The instability constant () of magnesium ion complexes with citrate ions, glycine and EDTA was 6.30×10^{-4}, 3.55×10^{-4}, and 2.04×10^{-9}, respectively [16].

The results were statistically processed using Statistica 13.0 software. (License No. JPZ904I805602ARCN25ACD-6). Normality of data distribution was confirmed using the Shapiro – Wilk test. The complexing activity values were presented as the mean and standard error (M ± S). The correlation of relative citrate and glycine activity in the middle of the studied concentration range (0.60 mmol / l) with theoretical values was evaluated by the Pearson's test. The dose dependence of ligand activity was identified by regression analysis. The mathematical model for dose – response curves was constructed by non-linear estimation using the Michaelis – Menten equation [16] mathematically expressed by the formula (8):

$$OA = \frac{C}{C + C_{50}}, \quad (8)$$

where RA is relative activity of the ligand, C is a concentration of the ligand, and C_{50} is an adjustable parameter corresponding to the concentration at which half maximal complexing effect develops. For C_{50} values, a 95% confidence interval (95% CI) was calculated; the significance of its differences between the ligands was assessed using the two-tailed Student's t-test.

A two-way analysis of variance (ANOVA) was used to construct a model for the dependence of complexing activity on the ligand type and concentration, which were considered as independent predictors. The concentrations of the studied ligands were the same, no correction for covariate was required. Post hoc comparisons were performed using the Scheffé test. The critical level of statistical significance was $p = 0.050$.

RESULTS

The curves for the dependence of relative complexing activity of the ligands on the concentration are shown in the Figure.

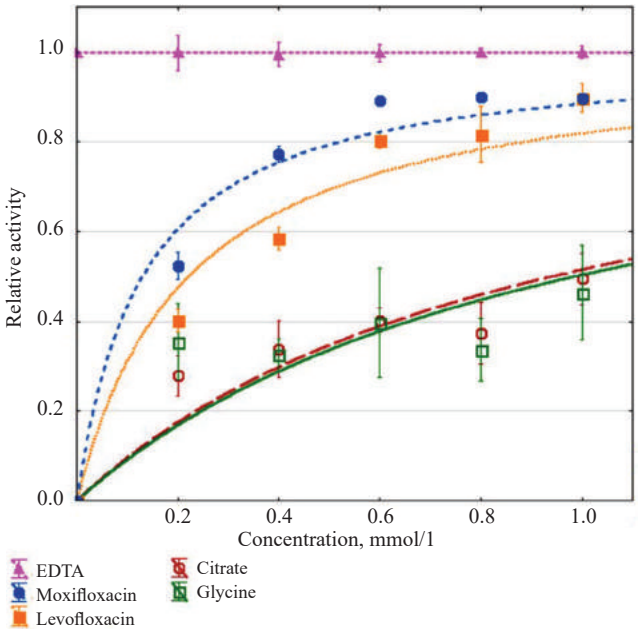

Figure. Relative complexing activity of the studied substances, *M ± S:* Michaelis – Menten fitting curves are depicted [17]

A negative correlation was established between the relative activity of EDTA, citrate, and glycine and the theoretical equilibrium constants of the reaction (6) ($R = -0.87$, $p < 0.001$, Table).

Table

Values for relative activity and calculated equilibrium constants of EDTA, citrate, and, glycine, *M ± S*

Ligand	Theoretical	Relative activity, 0.60 mmol / l
EDTA	–	1.00 ± 0.02
Glycine	1.74×10^5	0.40 ± 0.12
Citrate	3.09×10^5	0.40 ± 0.03

The analysis of variance showed statistically significant differences in the activity of the studied ligands depending on the dose (ANOVA, $p < 0.001$).

The activity of EDTA as a standard did not depend on the concentration (regression analysis, $p = 0.817$) and was graphically expressed by a horizontal line described by the equation: relative activity = 1.00. EDTA had a significantly greater complexing effect than citrate and glycine ($p < 0.001$ at all concentrations, Scheffé test).

Levofloxacin and moxifloxacin in a low concentration range (0.0–0.4 mmol / l) had a significantly lower complexing effect than EDTA ($p < 0.001$ at each concentration, Scheffé test), at high concentrations, their complexing activity was comparable to that of EDTA ($p = 0.058$; 0.134; 0.996 for levofloxacin and $p = 0.990$; 0.997; 0.996 for moxifloxacin at concentrations of 0.6; 0.8, and 1.0 mmol / l, respectively, Scheffé test). The FQ complexing effect did not differ in all the used concentration ranges ($p = 1.000$; 0.938; 0.084; 1.000; 1000; 1.000 at 0.0; 0.2; 0.4; 0.6; 0.8; 1.0 mmol / l, respectively). The graphs for relative activity had the form of typical curves described by the Michaelis – Menten equation. The calculated C_{50} value was 0.22 mmol / l (95% CI: 0.19–0.26) for levofloxacin and 0.13 mmol/L (95% CI: 0.11–0.15) for moxifloxacin, the difference was significant ($p < 0.001$, Student's t-test).

The relative activity of citrate ions and glycine did not differ ($p = 1.000$ at all concentrations, Scheffé test). Levofloxacin showed greater complexing activity than citrate and glycine at concentrations of 0.4 mmol / l and higher ($p < 0.001$, Scheffé test). Moxifloxacin activity was greater than that of glycine, starting at a concentration of 0.4 mmol / l ($p < 0.001$, Scheffé test), and higher than that of citrate at a concentration of 0.2 mmol / l ($p < 0.001$, Scheffé test).

DISCUSSION

The empirical values for relative complexing activity corresponded to the theoretical equilibrium constants in the reaction (6) for EDTA, glycine, and citrate ions, which confirms the possibility of using the proposed experimental technique to evaluate the complexing activity of organic ligands with respect to magnesium ions.

The study carried out using the method developed by the authors showed that the activity of FQ at high concentrations was the same as that of EDTA, which indicates high strength of FQ and Mg^{2+} complex compounds. The activity of levofloxacin and moxifloxacin was greater than that of citrate ions and glycine. The possibility of competition for the magnesium ion between FQ and bioorganic substances, such as carboxylic acids, amino acid residues, nucleotides, and other compounds, was assumed.

The structure of FQ coordination compounds was widely described in the literature. Magnesium ions bind to norfloxacin, levofloxacin, ofloxacin, and gatifloxacin in the mole ratio of 1 : 2 [5, 6]. Ofloxacin (racemate (R, S)) and levofloxacin (S-enantiomer) bind to magnesium ions in the same way [18]. The carboxyl and carbonyl groups of the FQ quinolone ring are involved in the complexation [6, 7, 18]. Nitrogen of the piperazine ring does not interact with magnesium ions [7, 18].

The complexing effect of moxifloxacin developed faster than that of levofloxacin. It is likely that at low concentrations, moxifloxacin significantly disrupts cell metabolism. The study [19] evaluated the cytotoxic effect of levofloxacin, moxifloxacin, and cefuroxime on the corneal epithelium. Lower concentrations of moxifloxacin destroyed half of the corneal epithelial cells if compared with levofloxacin (487 and 578 μg / ml, respectively). Higher cytotoxicity of moxifloxacin was demonstrated in the study [20]. C_{50} determined in this study did not differ significantly, which indicates the relationship between the FQ cytotoxicity and their ability to form complexes with magnesium ions.

The affinity of FQ for magnesium ions determined fluorimetrically correlated with their ability to disrupt the formation of a limb bud in mice *in vitro.* The most pronounced damaging effect was characteristic of sparfloxacin, temafloxacin, and ciprofloxacin, a somewhat lesser effect was characteristic of fleroxacin, lomefloxacin, and ofloxacin, and pefloxacin had almost no damaging effect [21]. Grepafloxacin, which has the highest affinity for divalent calcium and magnesium cations according to the fluorescent analysis, had the greatest teratogenic effect on the formation of a limb bud in mice [22].

In the study on the effect of FQ complexing properties on their antibacterial activity, the affinity constants for magnesium ions were evaluated by nuclear magnetic resonance. The smallest constants were characteristic of sparfloxacin and ofloxacin ($10.1 \pm \pm 0.6 \times 10^{2}\ M^{-1}$), the highest constants were characteristic of ciprofloxacin ($13.0 \pm 0.5 \times 10^{2}\ M^{-1}$), norfloxacin ($13.0 \pm 1.0 \times 10^{2}\ M^{-1}$), and pefloxacin ($21.0 \pm \pm 1.0 \times 10^{2}\ M^{-1}$) [23]. Complexation increased the minimum inhibitory concentration of FQ in relation

to various bacterial strains [18, 23] due to impaired penetration into cells.

The FQ cardiotoxicity is due to impaired potassium current through rapid delayed rectifier IK_r / hERG channels [23], whose activity is modulated by Mg^{2+} [24, 25]. Magnesium ions form ionic bonds with amino acid residues between S2 and S3 linkers of channel α-subunit [24, 25]. It can be assumed that the complexation of FQ with Mg^{2+} reduces the permeability of the hERG channel and inhibits potassium current with impaired membrane repolarization, QT prolongation, and an increased risk of arrhythmia. According to the meta-analysis, moxifloxacin as a FQ with the most pronounced complexing activity with respect to magnesium ions causes arrhythmias more often than other compounds of this series [4].

Therefore, the study conducted using the method developed by the authors showed that high complexing activity of FQ is accompanied by their competition with intracellular substrates for Mg^{2+}, disruption of magnesium-dependent biochemical processes, cell damage, and cell death. The complexing effect of FQ is related to their toxicity. Pronounced complexing properties of FQ with respect to Mg^{2+} may result in severe adverse reactions.

CONCLUSION

The proposed method made it possible to experimentally evaluate the complexing properties of FQ with respect to magnesium ions. The complexing activity of levofloxacin and moxifloxacin was similar to that of EDTA and was greater than that of citrate ions and glycine. FQ may compete with bioorganic ligands for magnesium ions. High complexing activity of FQ may lead to serious adverse reactions caused by intracellular Mg^{2+} deficiency.

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Authors contribution

Bakhtin V.M., Izmozherova N.V., Belokonova N.A. – conception and design, analysis and interpretation of data, critical revision of the manuscript for important intellectual content, final approval of the manuscript for publication. All authors have made an equivalent contribution to drafting of the manuscript.

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Morphological changes in the heart and aorta of rats with diet-induced metabolic syndrome

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ABSTRACT

Aim. To identify early morphological changes in the heart and aorta of rats with experimental metabolic syndrome induced by a high-fat and high-carbohydrate diet (HFHCD).

Materials and methods. The study was carried out on male Wistar rats. The animals were divided into two groups: a control group (n = 10) and an experimental group (n = 10). The rats from the control group were fed with a standard laboratory diet. The rats from the experimental group received HFHCD for 12 weeks. Body weight, blood pressure (BP), and individual parameters of carbohydrate and lipid metabolism were assessed in the rats. A histologic examination of the heart and aorta in the animals was performed.

Results. Feeding rats with HFHCD led to an increase in body weight, elevation of BP, obesity, hyperglycemia, and triglyceridemia. The histologic examination of the heart in the rats of the experimental group showed signs of vascular disease, lipomatosis, and focal myocardial degeneration. Lipid accumulation in the cells of the media, hyperplasia of adipocytes in the adventitia, and depletion and fragmentation of the elastic lamina were revealed in the aortic wall of the rats receiving HFHCD.

Conclusion. The study indicated that HFHCD is an effective way to model metabolic syndrome. Structural disorders in the heart and aorta may be the mainstay for the development of cardiomyopathy and arterial hypertension in diet-induced metabolic syndrome.

Keywords: high-fat, high-carbohydrate diet, metabolic syndrome, myocardium, aorta, obesity

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

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Морфологические изменения в сердце и аорте крыс при диет-индуцированном метаболическом синдроме

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РЕЗЮМЕ

Цель – выявить ранние морфологические изменения в сердце и аорте крыс при экспериментальном метаболическом синдроме, вызванном высокожировой и высокоуглеводной диетой (ВЖВУД).

Материалы и методы. Исследование выполнено на самцах крыс линии Wistar, которые были распределены на контрольную (n = 10) и экспериментальную (n = 10) группы. Крысы контрольной группы получали стандартный корм. Крысы экспериментальной группы в течение 12 нед находились на ВЖВУД. У животных определяли массу тела, артериальное давление (АД) и отдельные параметры углеводного и липидного обмена. Выполняли гистологическое исследование тканей сердца и аорты животных.

Результаты. Установлено, что ВЖВУД вызывает у крыс увеличение массы тела, ожирение, повышение АД, гипергликемию, триглицеридемию. При гистологическом исследовании сердца крыс экспериментальной группы выявлены признаки сосудистого поражения, липоматоза, очаговой дистрофии миокарда. В стенке аорте крыс, получавших высокожировой и высокоуглеводный рацион, выявлено накопление липидов в клетках медии, гиперплазия жировых клеток в адвентиции, истончение и разволокнение эластических мембран.

Заключение. Исследование показало, что ВЖВУД является эффективным способом моделирования метаболического синдрома. Обнаруженные структурные изменения в тканях сердца и аорты могут лежать в основе развития кардиомиопатии и артериальной гипертензии при диет-индуцированном метаболическом синдроме.

Ключевые слова: высокожировая и высокоуглеводная диета, метаболический синдром, миокард, аорта, ожирение

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

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Соответствие принципам этики. Исследование одобрено комиссией по контролю содержания и использования лабораторных животных СибГМУ (протокол № 1 от 07.12.2021).

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INTRODUCTION

Over the past decades, the prevalence of cardiovascular diseases (CVDs) has increased dramatically around the world. According to experts, CVDs would be the cause of more than 23 million deaths around the world by 2030 [1]. Recent epidemiologic studies have shown a relationship between the nature of nutrition and an increase in the prevalence of CVDs [2, 3]. The studies have shown that dietary fats play an important role in the development of cardiovascular disease [4]. Currently, 49% of patients with CVDs are overweight and obese. These components are crucial in metabolic syndrome (MS) along with hyperglycemia, insulin resistance, arterial hypertension, and atherogenic dyslipidemia [5, 6].

Recent studies have proven the possibility and mechanisms of myocardial lipotoxic injury in obesity, which alters both the structure and the functional state of the myocardium [7, 8]. Systemic inflammation and oxidative stress that occur in MS result in myocardial fibrosis and are associated with damage to the endothelial and smooth muscle cells of the vascular wall [9]. In addition, obesity and vascular dysfunction accelerate the development of arterial hypertension, which increases the risk of heart failure [10].

Experimental diet-induced MS models are the most accessible way to study the morphofunctional features of the impact of metabolic disorders on the cardiovascular system [11, 12]. In recent years, a combined high-fat and high-carbohydrate diet has become widespread, since it most closely resembles a diet of a modern person and is considered appropriate for reproducing the pathogenetic factors and phenomenology of MS [13, 14]. A detailed description of the histologic changes that occur in the myocardium and blood vessels at early stages of MS may contribute to the development of potential approaches to CVD treatment in metabolic disorders.

The aim of the study was to identify early morphological changes in the heart and aorta of rats with experimental MS induced by HFHCD.

MATERIALS AND METHODS

The studies were carried out in compliance with the principles of humanity set out in the directives of the European Community (86 / 609 / EEC) and the Declaration of Helsinki. The rats were kept at a constant room temperature (23º C) and exposed to 12 h : 12 h light : dark cycles with free access to food and water. Male Wistar rats (20 rats, average weight 270.6 ± 30.1 g, 6 weeks old) were randomly divided into a control group (n = 10) and an experimental group (n = 10). The rats of the control group (CG) were fed with a standard diet (Chara, Assortiment-Agro, Russian Federation, the ratio of proteins, fats, and carbohydrates was 26%, 5%, and 45%, respectively). The rats of the experimental group (EG) received a specially designed HFHCD (the ratio of proteins, fats, and carbohydrates was 16%, 22%, and 54%, respectively) containing a standard food with the addition of animal fat (lard, 17%), fructose (17%), and cholesterol (0,25%). Drinking water was replaced with a 20% fructose solution. The duration of the experiment was 12 weeks, after which the animals were euthanized via CO_2 inhalation.

To confirm MS in the rats fed with HFHCD, body weight and blood pressure (BP) were measured at the end of the experiment (Systola, Neurobotics, Russian Federation). In the euthanized animals, whole blood was taken from the heart; visceral adipose tissue, the heart, and the thoracic aorta were removed. Serum for biochemical studies was obtained by centrifugation of the whole blood (3,000 rpm, 10 min) and stored at −20° C for further analysis. The levels of glucose, triacylglycerols (TAG), and cholesterol (CHOL) in the blood serum were determined by enzymatic colorimetric methods (Chronolab Systems SL., Spain). The concentrations of TAG and CHOL in the aorta (in mg / g of tissue) were measured photometrically using test kits (Chronolab, Spain) after extraction of the lipid fraction from the tissue samples with a chloroform – methanol mixture. Before the analysis, a 20% solution of Thesit (Sigma-Aldrich, USA) dissolved in chloroform was added to the organic phase. Chloroform was removed with a stream of nitrogen, and emulsified lipids were dissolved in distilled water. Working reagents from the TAG and CHOL assay kits were added to the resulting aqueous emulsion.

To study structural changes, the heart and thoracic aorta were fixed in 10% neutral buffered formalin (BioVitrum, Russian Federation) for 24 h, washed from the fixative, and then dehydrated in an isopropanol-based solution IsoPrep (BioVitrum, Russian Federation). The prepared objects were embedded in the Histomix paraffin medium (BioVitrum, Russian Federation), and thin (5–6 μm) sections were made on the HM355 S automatic microtome (Thermo Fisher Scientific, USA). The sections were stained with hematoxylin (BioVitrum, Russian Federation), eosin (BioVitrum, Russian Federation), and orcein (Bio Vitrum, Russian Federation) to identify elastic units of the aorta. To detect lipids, the aortic samples were frozen at –80° C. Then, they were poured into a cryogel, and 20 μm thick sections were made on the HM525 NX cryostat (Thermo Fisher Scientific, USA) at –25° C. The sections were mounted on poly-L-lysine-coated slides and stained with Sudan III according to Herxheimer (BioVitrum, Russian Federation). Micropreparations were examined using the AxioSkop 40 microscope (Carl Zeiss, Germany). Digital images of the sections were obtained using the AxioVision 4.6 software. In histology specimens of the aorta, the thickness of the intima, media, and adventitia (in μm) was measured.

The data were analyzed using the SPSS Statistics 23 software. Normally distributed data were presented

as the mean and standard deviation ($M \pm SD$). Non-normally distributed variables were presented as the median and the interquartile range Me (Q_1; Q_3). The Student's t-test or the Mann – Whitney U-test were used to identify differences between the samples. The differences were considered statistically significant at $p < 0.05$.

RESULTS

Feeding rats with HFHCD for 12 weeks resulted in an increase in body weight, systolic and diastolic BP, specific gravity of abdominal fat, including mesenteric, epididymal, and retroperitoneal adipose tissue in the EG rats. HFHCD led to hyperglycemia, an increase in TAG in the blood serum, as well as an increase in the levels of TAG and CHOL in the aortic wall (Table 1).

Table 1

Physiological and biochemical indicators in rats, $M \pm SD$

Indicator	Group	
	control (n = 10)	experimental (n = 10)
Body weight, g	415.3 ± 35.6	466.1 ± 32.1 (p = 0.002)
Systolic BP, mm Hg	129.1 ± 8.4	138.2 ±8.1 (p = 0.005)
Diastolic BP, mm Hg	92.3 ± 8.2	110.4 ± 12.6 (p = 0.04)
Adipose tissue weight / body weight ratio, g	2.2 ± 0.3	4.8 ± 1.3 (p = 0.001)
Glucose (serum), mmol / l	4.1 ± 0.5	5.6 ± 0.7 (p = 0.002)
TAG (serum), mmol / l	1.2 ± 0.8	3.2 ± 0.9 (p = 0.001)
CHOL (serum), mmol / l	2.2 ± 0.3	2.9 ± 0.4 (p = 0.055)
TAG (aorta), mg / g	4.2 ± 1.2	6.8 ± 1.4 (p = 0.001)
CHOL (aorta), mg / g	1.1 ± 0.4	2.6 ± 0.6 (p = 0.03)

Note: here and in Table 2: p – significance of differences compared with the control group.

The heart wall in the CG rats had normal structure. Ventricular cardiomyocytes had an elongated cylindrical shape, with distinguishable longitudinal and transverse striations; they lacked pathological tortuosity. The interstitium was represented by a few vessels, which were single arteries in the right ventricle (Fig. 1, *A*). The interstitium of the left ventricular myocardium was more pronounced due to plethora of capillaries, dilatation and emptying of venules, and the presence of optically empty slit-like spaces between the cardiomyocytes of the middle and inner layers of the myocardium (Fig. 1, *B*).

Compared with the CG, the micropreparations of the right ventricle obtained from the experimental rats showed local aggregates of adipocytes in the epicardium of the right ventricle (Fig. 1, *C*). In the wall of the left ventricle, foci of fragmented cardiomyocytes and cardiomyocytes with intense cytoplasm staining and loss of longitudinal and transverse striations were identified. Areas of myocardial disarray were found (Fig. 1, *D*). The changes in the vessels described for the CG persisted, but they were more pronounced: increased number of erythrocytes and vascular dilatation were more common; they were present in all small vessels and in larger arteries and veins, in some of the latter, blood separation was noted and the lumen of such veins was filled with plasma (Fig. 1, *D*).

The aortic wall in the CG rats had normal architecture in all three layers (Fig. 2, *A*). In the EG, the structure of the aortic wall was preserved, but aggregates of adipocytes appeared in the adventitia. Besides, in the cytoplasm of many interstitial cells (fibroblasts, smooth muscle cells), multiple small, irregularly shaped, optically empty inclusions were noted, and the interstitial cells took the form of foam cells (Fig. 2, *B*). The arrangement of the foam cells varied according to the depth of the wall: they were almost always localized in the media closer to the adventitia, less often – in the middle and outer parts of the media. The morphometric study revealed an increase in the thickness of the middle and outer layer of the aortic wall (Table 2).

Table 2

Thickness of aortic wall layers, μm, Me (Q_1; Q_3)

Group	Intima	Media	Adventitia
Control, n = 10	5.2 (4.6; 6.9)	63.4 (60.7; 0.4)	10.5 (9.5; 13.1)
Experimental, n = 10	6.1 (5.1; 7.3)	71.6 (67.1; 78.5) (p = 0.01)	14.8 (13.3; 16.5) (p = 0.001)

In the frozen sections of the CG rat aorta, stained with Sudan III according to Herxheimer, Sudan III-stained components were detected exclusively in lipocytes of the adventitia (Fig. 3, *A*) and were not numerous. In the EG, adipocytes in the adventitia of the aorta were characterized by abundant Sudan III-stained inclusions, which were also detected in small amounts in the cells of the outer part of the media (Fig. 3, *B*).

The aortic wall of the CG rats stained with orcein revealed elastic units, such as membranes and fibers, with a normal structure (Fig. 4, *A*). In the EG, signs of elastolysis were observed, such as a decrease in staining, thinning, and defibrated fenestrated elastic laminae. The tortuosity of the elastic components of the media also increased, and their mutual parallel arrangement was disturbed (Fig. 4, *B*).

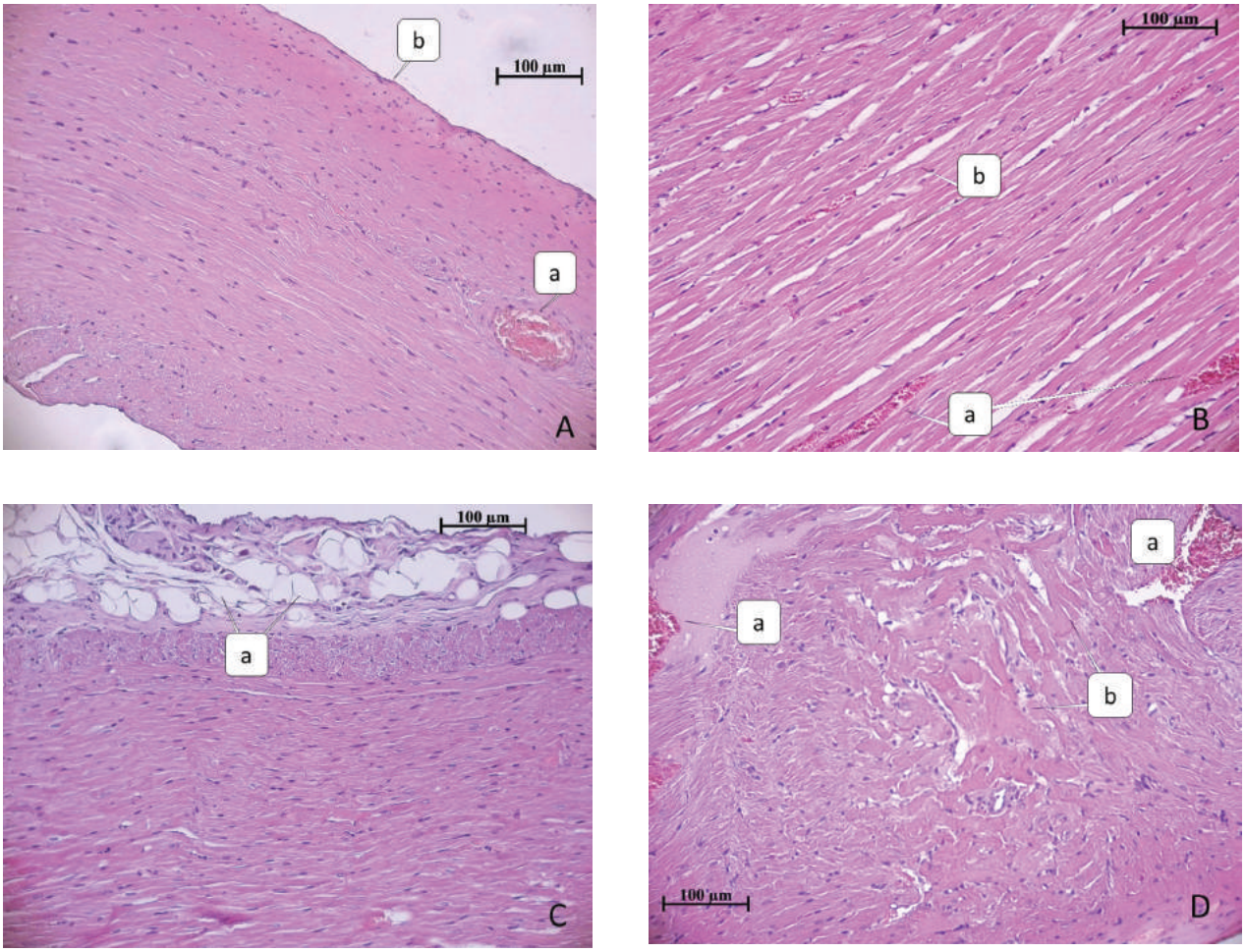

Fig. 1. Effect of HFHCD on the morphological structure of rat myocardium. *A* – fragment of the right ventricular wall of the rat in the CG: plethora of the artery (*a*), absence of epicardial lipomatosis (*b*). *B* – fragment of the left ventricular wall of the rat in the CG: plethora of the capillaries (*a*), interstitial edema (*b*). *C* – fragment of the right ventricular wall of the rat in the EG: epicardial lipomatosis (*a*). *D* – fragment of the left ventricular wall of the rat in the EG: plethora and plasma stasis in the vessels (*a*), myocardial disarray and striations in cardiomyocytes (*b*) ×200. Here and in Fig. 2, staining with hematoxylin and eosin

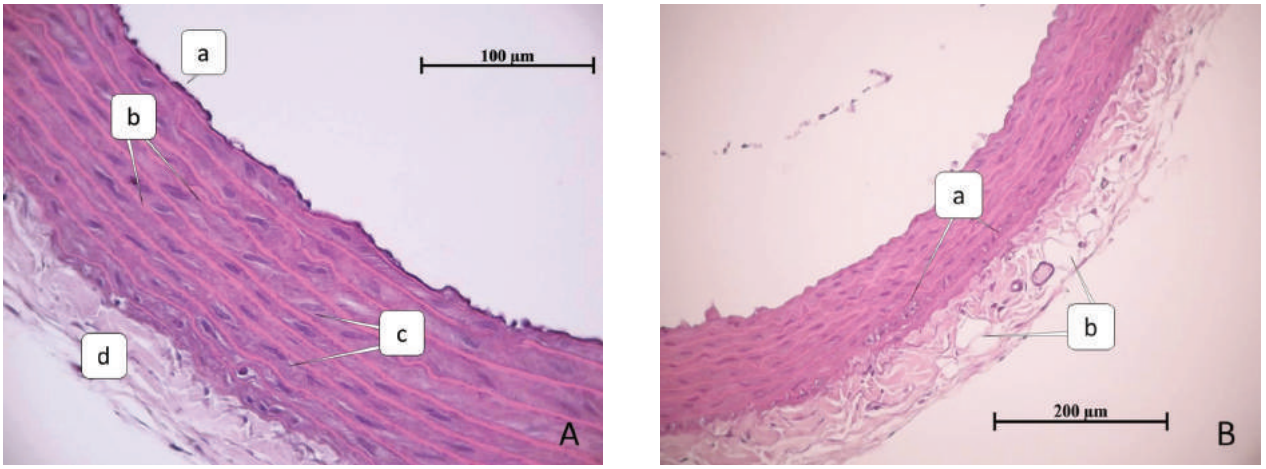

Fig. 2. Effect of HFHCD on the morphological structure of the aortic wall. *A* – transverse section of the CG rat aorta: endothelium (*a*), fenestrated elastic laminae (*b*), interstitial cells (*c*), adventitia (*d*) have a normal structure. ×200. *B* – transverse section of EG rat aorta: xanthomatous cells in the media (*a*), adipocytes in the adventitia (*b*). ×100

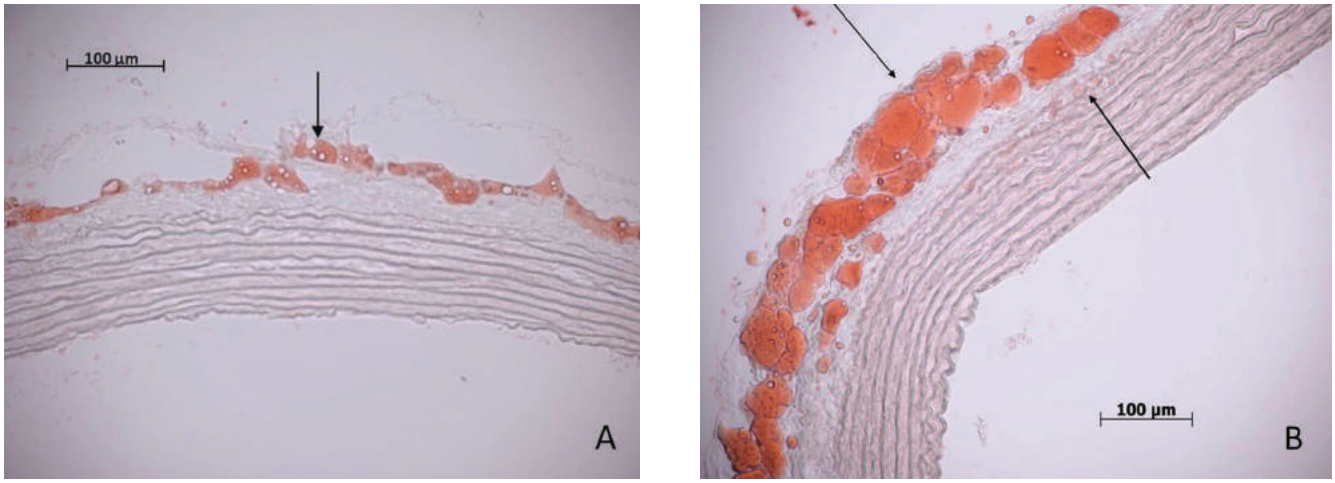

Fig. 3. Accumulation of lipids in the aortic wall of the rats fed with HFHCD. *A* – transverse section of the CG rat aorta. Localization of Sudan III-stained components (lipids) in the adypocytes of the adventitia (black arrow). *B* – transverse section of the EG rat aorta. Accumulation of Sudan III-stained components (lipids) in the adipocytes of the adventitia and cells of the media (black arrow). *A, B* – Sudan III staining according to Herxheimer. ×200

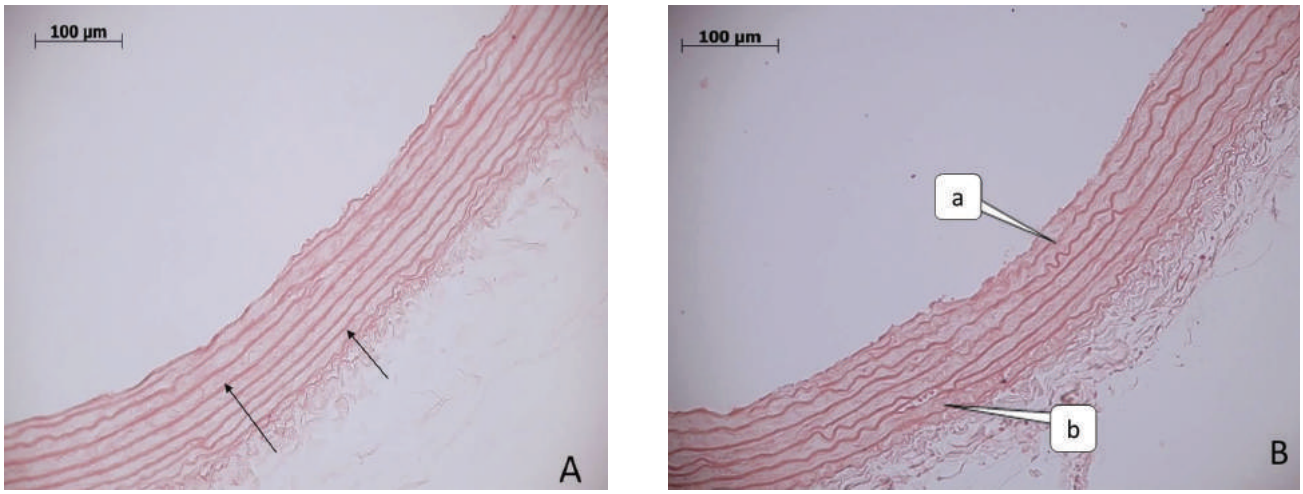

Fig. 4. Changes in the elastic lamina in the aortic wall of the rats fed with HFHCD. *A* – transverse section of the CG rat aorta. Normal structure of fenestrated elastic laminae (black arrow). *B* – transverse section of the EG rat aorta. Increase in tortuosity (*a*), disturbed parallel arrangement of fenestrated elastic laminae (*b*). *A, B* – orcein staining. 200x

DISCUSSION

Animal models represent some of the most effective and available tools for understanding the pathophysiological mechanisms underlying MS. One way to model MS is to use specially designed diets [3, 11]. Diets containing both high-fat and high-carbohydrate components have been reported to be more clinically representative than single-component diets alone [12, 15].

In the present study, MS in the rats was induced with HFHCD. Feeding the animals with HFHCD for 12 weeks resulted in an increase in animals' body weight, obesity, increased BP, development of hyperglycemia, and a rise in the concentration of TAG in the blood serum. The data obtained indicate that feeding the rats with a HFHCD contributes to the formation of MS [4, 13].

Disorders of carbohydrate and lipid metabolism that occur in MS, in combination with chronic inflammation and oxidative stress, lead to the development of pathomorphological changes in the cardiovascular system [16, 17]. It is known that dysregulation of energy homeostasis in the heart in MS is associated with various adaptations and changes in the structure and function of the myocardium, which occur with abnormal lipid accumulation and adipose tissue hyperpla-

sia [18]. Studies on rats receiving high-fat and (or) high-carbohydrate diet also showed that oxidative stress is a potential mechanism for obesity-induced cardiotoxicity [8, 19]. In the cardiac muscle of the animals fed with such a diet, the absence of myofilaments and / or their disorganization, dilatation of the sarcoplasmic reticulum vesicles, a decrease in the diameter of myofibrils, intracellular vacuolization, the presence of lipid inclusions, hypertrophy, and signs of fibrotic changes are morphologically determined [10, 14, 20], the latter being most pronounced with an increased content of saturated fatty acids in food.

At the same time, it has been noted that HFHCD-induced vascular endothelial dysfunction in MS [16] provokes stasis of formed elements and dilatation of the vascular lumen in the myocardium. Our findings, namely focal lesions of cardiomyocytes in the left ventricle in the form of striations and fragmentation, lipomatosis in the epicardium, and microcirculatory disorders (hyperemia) in the hearts of the EG rats are consistent with the known data. However, the revealed changes were not pronounced.

The histologic study of the micropreparations of the EG rat aorta showed the beginning of atherosclerotic vascular wall remodeling, which to a greater extent affected the media and adventitia. A decrease in orcein staining, discontinuity of fenestrated elastic laminae, the presence of foam cells, the emergence of Sudan III-stained inclusions in the interstitial cells of the media, and hyperplasia of adipocytes in the adventitia may be the cause of the increase in the aortic wall thickness in the animals receiving HFHCD, which is also characteristic of experimental MS [9, 21, 22]. Similar morphological changes in the aorta are manifested by changes in its mechanical properties, including a decrease in elasticity and an increase in wall stiffness [23]. This is due to elastolysis and a decrease in production of elastic components with a simultaneous rise in collagen production, resulting in an increase in the collagen / elastin ratio. Thickening of the aortic wall and its stiffness due to an increase in the collagen level, as well as degeneration of smooth muscle cells can be a structural basis that contributes to arterial hypertension. At the same time, accumulation of TAG in the aorta can be considered as an additional prognostic criterion for vascular wall stiffness [24].

CONCLUSION

The study showed that HFHCD is an effective way to simulate MS in rats, causing obesity, increased BP, hyperglycemia, and triglyceridemia. Morphological changes in the heart wall that occur in MS are characterized by damage to the vascular bed, lipomatosis, and focal myocardial degeneration. In the aorta, signs of media remodeling, lipid infiltration of interstitial cells of the media, hyperplasia of adipocytes, and defibrated elastic membranes were revealed. The detected structural disorders may underlie the development of cardiomyopathy and hypertension in diet-induced MS. The resulting experimental model can be used to study the mechanisms of development of metabolic and hemodynamic disorders in MS, as well as to test potential cardioprotective and angioprotective pharmacological agents.

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Authors contribution

Birulina J. G., Ivanov V.V. – conception and design, drafting of the manuscript. Dzyuman A.N., Bykov V.V. – carrying out of the histologic examination, analysis and interpretation of the data. Nosarev A.V., Gusakova S.V. – approval of the manuscript. Buyko V.V., Grigoreva A.V. – simulation of the metabolic syndrome, carrying out of the biochemical studies.

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Radionuclide methods in assessing pulmonary perfusion and ventilation in patients with connective tissue dysplasia

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ABSTRACT

Aim. To perform a scintigraphic assessment of the bronchopulmonary system and pulmonary microcirculation in patients with connective tissue dysplasia.

Materials and methods. The study included 31 male patients of conscription age with connective tissue dysplasia (CTD), diagnosed according to the 2019 clinical guidelines (average age (19.6 ± 2.6 years)), and 25 practically healthy individuals comparable in gender and age, who formed a control group. All patients underwent planar pulmonary ventilation – perfusion scintigraphy with determination of pulmonary alveolar – capillary permeability.

Results. In patients with CTD, the apical to basal perfusion gradient (U/L_Q) was on average 24% lower than in the control group ($p = 0.046$), and alveolar – capillary permeability was higher in both lungs, both at minute 10 and at minute 30.

Conclusion. Static pulmonary ventilation – perfusion scintigraphy allows to identify functional disorders in patients with CTD at the preclinical stage: a decrease in the perfusion gradient on average by 24% compared with the control group and an increase in alveolar – capillary membrane permeability.

Keywords: connective tissue dysplasia, pulmonary ventilation – perfusion scintigraphy

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

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Conformity with the principles of ethics. All patients signed an informed consent to participate in the study. The study was approved by the Ethics Committee at Siberian State Medical University (Protocol No. 8903 of 20.12.2021).

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Радионуклидные методы в оценке легочной перфузии и вентиляции у пациентов с дисплазией соединительной ткани

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РЕЗЮМЕ

Цель. Провести сцинтиграфическую оценку состояния бронхоальвеолярной системы и легочной микроциркуляции у пациентов с дисплазией соединительной ткани.

Материалы и методы. В исследование включен 31 пациент мужского пола призывного возраста с дисплазией соединительной ткани (ДСТ), диагностированной согласно клиническим рекомендациям 2019 г., средний возраст 19,6 ± 2,6 лет, и 25 практически здоровых лиц, сопоставимых по полу и возрасту, составивших группу контроля. Всем пациентам выполняли вентиляционно-перфузионную сцинтиграфию легких в планарном режиме с определением скорости альвеолярно-капиллярной проницаемости.

Результаты. У пациентов с ДСТ апикально-базальный градиент перфузии (U/L_Q) имел более низкие значения по сравнению с группой контроля в среднем на 24% ($p = 0,046$) и более высокие значения альвеолярно-капиллярной проницаемости в обоих легких, как на 10-й, так и на 30-й мин.

Заключение. Вентиляционно-перфузионная сцинтиграфия в статическом режиме позволяет на доклиническом этапе выявить функциональные нарушения у пациентов с ДСТ: снижение градиента перфузии по сравнению с группой контроля в среднем на 24% и повышение проницаемости альвеолярно-капиллярной мембраны.

Ключевые слова: дисплазия соединительной ткани, вентиляционно-перфузионная сцинтиграфия легких

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 этическим комитетом СибГМУ (протокол № 8903 от 20.12.2021).

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INTRODUCTION

Connective tissue dysplasia (CTD) is an inherited condition manifested through defects of the ground substance and fibrous tissue structures. It underlies morphological and functional disorders in various organs and systems and is characterized by a progressive course, which determines the features of associated pathological conditions. Social and clinical significance of these pathological manifestations is determined, on the one hand, by high prevalence of the disease due to accumulation of genetic defects in the population and by a significant impact of anthropogenic and abiotic factors and on the other hand –

by the progressive nature of clinical manifestations, characterized by multi-organ lesions with a risk of severe complications at late stages, up to death of the patient [1, 2].

The main period for a clinical debut of CTD is adolescence, when an increase in the volume of connective tissue corresponds to growth and development of the body [3]. Phenotypic manifestations of CTD are usually divided into groups depending on organs and systems involved in it. The first systematized data on lesions of the respiratory tract in undifferentiated CTD were published in 1994. [3].

The authors identified a group of dysplasia-associated changes of respiratory organs, which were determined by genetic defects in fibrillogenesis. This group included tracheobronchomegaly, tracheobronchomalacia, Williams – Campbell syndrome, pulmonary hypoplasia, bronchiectasis, pulmonary emphysema, and tracheobronchial dyskinesia. The morphofunctional state of the respiratory system in CTD is characterized by both increased elasticity of the bronchial tree and alveolar lung tissue and by extrapulmonary manifestations, such as thoracoabdominal syndrome and lability of the autonomic nervous system and immune system. Later, spontaneous pneumothorax as a consequence of bullous emphysema was included in the group of dysplasia-associated respiratory changes [4, 5].

Some authors believe that the mainstay for impaired binding and supporting function of the connective tissue in CTD is genetic weakness of all lung structures (bronchi, blood vessels, alveoli) that is manifested through several clinical syndromes in the postnatal period under the influence of external factors [5]. Dysplasia is often accompanied by a decreased tone of the bronchial wall with subsequent development of tracheobronchial dyskinesia and bronchiectasis [6]. The most important extrapulmonary factors determining the state of respiratory organs in CTD are chest wall and spinal deformity, weakness of respiratory muscles, features of breathing regulation, and the state of pulmonary hemodynamics [7, 8].

Radiology techniques are known to occupy a prominent place in the diagnosis of bronchopulmonary pathology. However, generally accepted radiology methods allow to detect changes in the lung parenchyma at relatively late stages of the disease. To assess morphofunctional changes at the preclinical stage of the disease, the use of radionuclide methods is promising. They are easy to reproduce and highly sensitive in evaluating both pulmonary ventilation and perfusion [9–11]. At the same time, when studying pathological changes in the lungs, the most complete information can be obtained using combined ventilation–perfusion scintigraphy, which significantly complements the data on the state of the capillary blood flow with lung ventilation parameters, which is essential for the diagnosis of early functional disorders [12].

Therefore, the aim of the study was to perform a scintigraphic assessment of the bronchopulmonary system and pulmonary microcirculation in patients with CTD.

MATERIALS AND METHODS

The study is based on the results of the examination of a group of persons subject to mandatory enlistment in the Armed Forces of the Russian Federation at the Military Medical Commission of Tomsk. The study included 31 male patients aged 16–22 years. The average age was 19.7 ± 2.7 years, the body mass index was 18.2 ± 2.2.

CTD was detected by the anamnestic data to identify previously diagnosed diseases typical of CTD, the clinical examination to identify external signs of systemic connective tissue involvement, including the anthropometric study, and the findings of instrumental methods of examination. Undifferentiated CTD was revealed in all patients. The severity of the disease was assessed using the phenotype table proposed by T.I. Kadurina and V.N.Gorbunova [4]. Exclusion criteria were patients' inability to participate or disagreement with the protocol requirements, bronchopulmonary diseases, acute diseases, and exacerbation of chronic diseases. The control group encompassed 25 healthy non-smoking volunteers, comparable in gender and age with the patients of the experimental group.

All the individuals underwent planar pulmonary ventilation – perfusion scintigraphy with determination of alveolar – capillary permeability according to the previously developed technique [9]. The ventilation – perfusion ratio (V / Q ratio) was determined by dividing the percentage of the accumulated inhaled radioaerosol by each lung separately by the similar parameter of the perfusion radiopharmaceutical (RPC).

Scintigraphic studies were performed using the Forte gamma camera (Philips Medical Systems, Netherlands). Image registration and gamma scan processing were carried out using the JETStream Workspace 3.0 software (PMS, Netherlands).

Statistical data analysis was performed using Microsoft Office and STATISTICA 10.0 software packages (StatSoft Inc, USA). Quantitative data were

presented as the mean (*M*) and the standard deviation (*SD*). The Mann – Whitney test was used to compare independent quantitative variables. The differences were considered statistically significant at $p < 0.05$.

The study was conducted in accordance with the principles set out in the Declaration of Helsinki and approved by the Ethics Committee at Siberian State Medical University and the Ethics Committee at Cardiology Research Institute. All patients signed an informed consent to participate in the study.

RESULTS AND DISCUSSION

The results of the scintigraphic studies are presented in the Table. In the patients with CTD, the mean V / Q value for the right lung was 0.97 ± 0.06 and for the left lung – 1.04 ± 0.07, and these values did not differ from the ones in the control group. The apical to basal ventilation gradient (U/L_V) also did not differ from that in the control group, while the perfusion gradient U/L_Q was 24% lower than in the control group ($p = 0.046$) and averaged 0.52 ± 0.14.

Three physiological processes are involved in effective external respiration: pulmonary ventilation, pulmonary perfusion, and gas diffusion through the alveolar – capillary membrane. In various pathological conditions in the lungs, these processes can be impaired. To assess the disorders, it is necessary to use methodological approaches allowing to determine diagnostically significant parameters, which include the V / Q ratio, alveolar – capillary permeability, etc. [9, 13–15].

In assessing the results of ventilation – perfusion scintigraphy in patients with CTD, the V / Q ratio corresponded to the control values for both lungs (Table). In CTD patients, a decrease only in U / L_Q (0.52 ± 0.14; $p = 0.046$) was observed, while the U / L_V gradient was not significantly different from that in the control group (Table). Concordance of the main functional units of the lungs in patients with CTD was quite satisfactory, and a drop in U / L_Q was probably associated with vasoconstriction, which contributed to a decrease in the blood flow to poorly ventilated lung areas.

Table

Scintigraphic parameters of pulmonary perfusion and ventilation in patients with connective tissue dysplasia and in the control group, *M ± SD*

Parameter		Control group, *n* = 25	Experimental group with CTD, *n* = 31					
			RL	MW test (*p*) 1–2	LL	MW test (*p*) 1–4	RL + LL	MW test (*p*) 1–6
		1	2	3	4	5	6	7
V/Q		0.98 ± 0.03	0.97 ± 0.06	NS	1.04 ± 0.07	NS	–	–
U/L_Q		0.68 ± 0.03	–	–	–	–	0.52 ± 0.14	0.046
U/L_V		0.66 ± 0.04	–	–	–	–	0.63 ± 0.11	NS
ACP, %	10 min	10.6 ± 2.9	18.73 ± 8.51	0.005	15.56 ± 8.58	0.006	–	–
	30 min	21.3 ± 4.3	33.17 ± 9.87	0.001	30.32 ± 9.96	0.004	–	–

Note: NS – nonsignificant; CTD – connective tissue dysplasia; *n* – number of patients in the group; RL – right lung; LL – left lung; MW test – Mann – Whitney test; *p* – significance of differences between the groups; V / Q – ventilation – perfusion ratio; U / L_Q – apical to basal perfusion gradient; U / L_V – apical to basal ventilation gradient; ACP – alveolar – capillary permeability.

It is known that alveolar air and pulmonary capillary blood flow are separated by the alveolar – capillary membrane, which provides gas transport according to the laws of diffusion, and pulmonary diffusing capacity depends on both lung capacity and the corresponding gas exchange surface. To assess alveolar – capillary permeability (ACP) of the lungs, we proposed and used an improved method of pulmonary ventilation – perfusion scintigraphy, which included the use of planar ventilation scintigraphy, assessment of radiopharmaceutical washout from the entire affected / intact lung, as well as examination in the posterior – anterior imaging plane of ACP in RPC washout and its registration at minute 10 and then at minute 30 after RPC inhalation [9, 15].

The study revealed an increase in the ACP rate in patients with CTD both in the right and left lung at minute 10 (18.73 ± 8.51% ($p = 0.005$), 15.56 ± 8.58% ($p = 0.007$), respectively) and at minute 30 of the study (33.17 ± 9.87% ($p = 0.001$), 30.32 ± 9.96% ($p = 0.004$), respectively), compared with the control group (Table).

Therefore, a significant increase in the RPC washout rate in patients with CTD was revealed, compared with the control group, which indicates increased ACP. The observed decrease in the U / L_Q perfusion

gradient in CTD can be explained by the involvement of the vasoconstrictor mechanism in the development of alveolar hypoxia. A statistically significant increase in the ACP rate in both lungs, compared with the control group, is probably a compensatory response of the alveolar – capillary network aimed at increasing gas exchange [12, 14]. At the same time, the increase in ACP can be determined both by an increase in gas exchange surface area, typical of primary emphysema in patients with CTD, and by hyperventilation syndrome, often observed in this group of patients [16].

CONCLUSION

Planar ventilation – perfusion scintigraphy allows to detect functional disorders in patients with CTD at the preclinical stage: a decrease in the U / L_Q perfusion gradient on average by 24%, compared with the control group, and an increase in ACM.

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Authors contribution

Vesnina Zh.V. – collection of the material, analysis of the obtained material, statistical processing of the data, interpretation of the data, drafting of the manuscript. Anashbaev Zh. Zh. – carrying out of radionuclide studies, processing of scintigraphy findings, database filling. Teteneva A.V. – conception of the study, review of the literature, analysis of the disease under investigation. Krivonogov N.G. – development of the radionuclide technique, methodological support, analysis of the findings. Bespalova I.D. – coordination of patient selection, clinical substantiation of the diagnostic method and design of the study, collection of the data (clinical section). Sazonova S.I. – literature search, provision of material facilities for radionuclide studies. Serdyukov N.A. –coordination of patients (obtainment of the informed consent to participate in the study), theoretical and clinical confirmation of the findings. Potapov K.V. – selection of patients, collection of raw data for filling the clinical patient database.

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Executive dysfunction in affective disorders: differences in bipolar affective disorder and depressive episode

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ABSTRACT

Aim. To identify the differences in executive function (inhibitory control, working memory, cognitive flexibility) between patients with bipolar affective disorder and depressive episode.

Materials and methods. A total of 72 patients with affective disorders aged 20–40 years were examined. Of them, 30 patients had bipolar affective disorder, a current episode of mild or moderate depression, and 42 patients had a mild, moderate, and severe depressive episode without symptoms of psychosis. The executive function was evaluated using PsyToolkit, a set of software tools for programming psychological experiments. Computerized Go/No–go tasks (assessment of inhibitory control and psychomotor functions), the Corsi block-tapping test (assessment of visual and spatial working memory capacities), and the Stroop Color and Word Test (assessment of cognitive flexibility) were used.

Results. An intergroup comparison of patients revealed that patients with bipolar disorder significantly more often demonstrated false button press in the Go/No–go task (p = 0.043); however, they exhibited a greater working memory capacity in the Corsi block-tapping test (p = 0.049) compared with patients with a depressive episode.

Conclusion. Important data were obtained regarding the specifics of executive dysfunction depending on the type of affective disorder. The presented data expand and supplement available information about the cognitive characteristics of patients with bipolar affective disorder and depressive episode, which may be useful in clinical practice and serve a focus of future research.

Keywords: affective disorders, bipolar affective disorder, depressive episode, executive function, cognitive deficit

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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 Mental Health Research Institute (Protocol No. 114 of 22.10.2018).

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Нарушения исполнительных функций при аффективных расстройствах: различия при биполярном аффективном расстройстве и депрессивном эпизоде

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РЕЗЮМЕ

Цель – выявить различия в исполнительном функционировании (ингибиторный контроль, рабочая память, когнитивная гибкость) между пациентами с биполярным аффективным расстройством и депрессивным эпизодом.

Материалы и методы. Обследованы 72 пациента в возрасте 20–40 лет с аффективными расстройствами. Из них 30 пациентов с биполярным аффективным расстройством, текущий эпизод легкой или умеренной депрессии, и 42 пациента с депрессивным эпизодом легкой, умеренной и тяжелой степени без психотических симптомов. Оценка исполнительного функционирования осуществлялась с помощью программного пакета для разработки психологических тестов PsyToolkit. Использовались компьютеризированные тесты Go/No–go (оценка ингибиторного контроля и психомоторной реакции), Corsi (определение объема пространственной рабочей памяти) и цветовой тест Струпа (Color Stroop) (оценка уровня когнитивной гибкости).

Результаты. При межгрупповом сравнении пациентов обнаружено, что пациенты с биполярным аффективным расстройством статистически значимо чаще совершали ошибки на сигнал «No–go» (ложное нажатие кнопки) в тесте Go/No–go (p = 0,043), однако у них отмечался больший объем рабочей памяти в тесте Corsi (p = 0,049) по сравнению с пациентами с депрессивным эпизодом.

Заключение. Получены важные данные относительно специфики дефицита исполнительного функционирования в зависимости от типа аффективного расстройства. Представленные данные расширяют и дополняют имеющиеся сведения о когнитивных особенностях пациентов, страдающих биполярным аффективным расстройством и депрессивным эпизодом, что может быть полезным в клинической практике и служить дальнейшим направлением для будущих исследований.

Ключевые слова: аффективные расстройства, биполярное аффективное расстройство, депрессивный эпизод, исполнительные функции, когнитивный дефицит.

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

Источник финансирования. Исследование проведено в рамках выполнения государственного задания № 075-01184-22-00, тема «Биопсихосоциальные механизмы патогенеза и клинического полиморфизма, адаптационный потенциал и предикторы эффективности терапии у больных с психическими и поведенческими расстройствами в регионе Сибири» № 122020200054-8.

Соответствие принципам этики. Все пациенты подписали информированное согласие на участие в исследовании. Исследование одобрено этическим комитетом НИИ психического здоровья (протокол № 114 от 22.10.2018).

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INTRODUCTION

Cognitive impairment is a common symptom of affective disorders (mood disorders). In particular, a number of studies have noted impaired memory, attention, thinking, and other symptoms in patients with affective disorders [1–4]. A number of methods were developed for assessing cognitive functions, but only a few of them are currently used in clinical practice. Given partial coincidence of data on the type and magnitude of cognitive deficits in patients with depressive episode (DE) and bipolar affective disorder (BD), it seems unlikely that standard tests can be used as an additional tool for differential diagnosis. In this regard, studies should focus on searching for specific cognitive neuromarkers of affective disorders. Executive function can become one of such neuromarkers.

Executive functions are a system of higher-level processes that support many everyday activities including planning, selecting, and successfully monitoring behaviors that facilitate the attainment of common goals [5]. Executive functions are basic cognitive processes, which include attention, inhibitory control, working memory, and mental flexibility [5, 6]. According to a number of authors, patients with affective disorders have executive dysfunction of varying severity, if compared with a healthy control group [1, 4]. However, we also failed to find data on the features of executive dysfunction between patients with BD and DE.

Based on various studies [1, 2, 4], we assume that patients with BD will have more pronounced executive dysfunction than patients with DE. Nevertheless, there are a few studies that directly compare patients with BD and DE.

The aim of this study was to identify the differences in executive function (inhibitory control, working memory, and mental flexibility) between patients with BD and DE.

MATERIALS AND METHODS

The study was carried out at the Affective States Department of the Mental Health Research Institute of Tomsk National Research Medical Center of the Russian Academy of Sciences (Mental Health Research Institute of Tomsk NRMC), according to the protocol approved by the local Ethics Committee at the Mental Health Research Institute (Protocol No. 114 of 22.10.2018). All patients signed an informed consent to participate in the study.

As part of the study, 72 patients (average age 28.92±6.97 years) were examined with the established diagnosis of Mood [affective] disorders according to the International Classification of Diseases, Tenth Revision (ICD–10). Of them, 30 patients (14 men, 16 women) had BD, a current episode of mild or moderate depression (F31.3), and 42 patients (18 men, 24 women) had a mild, moderate, and severe DE without symptoms of psychosis (F32.0–2).

Inclusion criteria were the established diagnosis of affective disorder with BD or DE, the age of 20–40 years, and a voluntary consent to participate in the study. Exclusion criteria encompassed the presence of pronounced organic brain disorders, mental retardation, administration of medications that affect brain activity, and refusal to participate in the study.

Information about the patients was obtained by means of a questionnaire which allowed to collect data on mental and somatic health. The diagnosis was made by qualified psychiatrists in accordance with the ICD–10 criteria. Clinical symptoms were assessed using psychometric scales, such as the Hamilton Rating Scale for Depression – 17 (HRSD–17) [7] and the Hamilton Anxiety Rating Scale (HAM-A) [8].

Cognitive deficit was diagnosed using PsyToolkit, a set of software tools for programming psychological experiments [9]. Computerized Go / No–go tasks [10] (assessment of inhibitory control and psychomotor functions), the Corsi block-tapping test [11] (assessment of visual and spatial working memory capacities), and the Stroop Color and Word Test [12] (assessment of cognitive flexibility) were used.

Statistical data processing was performed using Statistica 12 software package (StatSoft Inc., USA). Statistical data were presented as the median and the interquartile range $Me\ [Q_1; Q_3]$. The Mann–Whitney U test was used to assess intergroup differences. The differences were considered statistically significant at $p < 0.05$.

RESULTS

First, we analyzed clinical parameters in the studied groups of patients (Table 1).

Table 1

Clinical evaluation of patients with affective disorders, $Me\ [Q_1; Q_3]$

Parameter	Patients with DE, $n = 42$	Patients with BD, $n = 30$	p
Age, years	28 [23; 34]	27 [21; 36]	0.992
Duration of the disease, years	5 [2; 10]	6 [2; 11]	0.586
HDRS–17	23 [18; 24]	20 [16; 25]	0.503
HARS	16 [10; 22]	15 [12; 23]	0.782

The compared groups of patients did not differ in age (p = 0.992), duration of the disease (p = 0.586), severity of depressive symptoms (p = 0.503), and anxiety level (p = 0.782). Thus, patients with unipolar depression and BD were comparable in the clinical parameters. Additionally, all the study participants had incomplete or complete higher education, which allows us to conduct a full-fledged comparison.

Then we compared the parameters of cognitive function (data from cognitive tests) between patients with unipolar depression and BD (Table 2).

Table 2

Data of cognitive tests in patients with DE and BD, *Me* [Q_1; Q_3]

Test		Patients with DE, n = 42	Patients with BD, n = 30	p
Go/No–go tasks	false button press in the Go tasks	3 [2; 6]	4 [1; 7]	0.890
	false button press in the No–go tasks	0 [0; 1]	2 [1; 3]	0.043*
Corsi block-tapping test (working memory capacity)		5 [5; 6]	6 [5; 7]	0.049*
Stroop Color and Word Test (time), s		63 [54; 74]	65 [52; 73]	0.796

It was found that patients with BD significantly more often demonstrated false button press in the Go/No–go tasks (p = 0.043); however, they exhibited a greater working memory capacity in the Corsi block-tapping test (p = 0.049) compared with patients with DE.

DISCUSSION

In this study, executive function (inhibitory control, working memory, cognitive flexibility) was evaluated in patients with BD and DE. The results of the study clearly showed that in patients with affective disorders changes in executive function of varying severity were noted.

In contrast to patients with unipolar depression, patients with BD made significantly more errors in the No–go tasks, which indicated a pronounced impairment of inhibitory control. A number of studies also found deficient inhibitory control in patients with BD, which is consistent with the results in our study [1, 2]. Deficient inhibitory control is a basic cognitive dysfunction that can underlie psychopathology of mania and lead to more complex behavioral characteristics that are typical of the disease, such as impulsivity, distractibility, and a poor ability to suppress emotional reactions [13]. According to neuroimaging studies, brain regions that are functionally associated with inhibitory processes, such as the ventrolateral prefrontal cortex, dorsolateral prefrontal cortex, and the right inferior frontal gyrus, often underwent structural and / or functional changes in patients with BD [14, 15]. However, no such changes were found in patients with DE with respect to cognitive testing data in our study, which indicates the differences in the psychopathology of the disorders in question.

At the same time, the results of the Corsi block-tapping test revealed a greater decrease in working memory capacity in patients with DE, compared with patients with BD. Working memory is of great importance for performing many cognitive tasks in everyday activities [16]. According to a number of studies, a decline in working memory correlates with overload with distracting information [17, 18]. It is believed that people with stronger working memory filter out distractions better and focus only on important information at a given time. According to D.C. Glahn et al. [18], a decline in working memory is the mainstay for recurrent depressive disorders. No such changes were found in patients with BD, which once again indicates the differences in the psychopathology of the disorders.

On the other hand, we found that the cognitive flexibility parameters in the Stroop Color and Word Test and psychomotor functions in the Go/No–go tasks (the Go signal) were equally lower in patients with BD and DE, compared with the normal values, which indicates similar psychopathogenesis of these disorders. Psychomotor function is related to the cognitive concept of information processing speed. Manifestations of psychomotor retardation may include slow speech and a delay in motor activity. These changes are symptoms characteristic of a depressive state [19].

However, according to the results obtained, in patients with BD, a decline in the psychomotor speed and poor inhibitory control were simultaneously noted. These features may underlie manic (disruption of inhibitory control) and depressive (decreased psychomotor speed) symptoms in patients with BD. Cognitive flexibility is an ability to switch between different tasks depending on changing conditions; it plays an important role in a person's ability to adapt to the environment. According to the literature data, in addition to promoting purposeful behavior, cognitive flexibility is involved in the regulation of emotions, and its impairment is typical of mood disorders [19, 20]. Cognitive flexibility is an important component in the pathogenesis of BD and DE.

CONCLUSION

Following the conducted study, important data were obtained regarding the specifics of executive dysfunction depending on the type of affective disorder. The presented data expand and supplement available information about the cognitive characteristics of patients with BD and DE, which may be useful in clinical practice and serve as a focus of future research.

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Authors contribution

Galkin S.A. – conception and design, collection of the material, analysis and interpretation of the data, drafting of the manuscript. Vasilieva S.N. – collection of the material, clinical and psychopathological examination of patients. Simutkin G.G., Ivanova S.A. – final approval of the manuscript for publication.

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Features of the attitude to vaccination against COVID-19 in Russia

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ABSTRACT

Background. 1.5 years after the registration of the first vaccine against COVID-19 in Russia, national herd immunity reached only 49.7%. It is obvious that the success of vaccination measures depends on the readiness of the population for immunization and their attitude to the vaccine.

The aim of the study was to research the attitude to vaccination against a new coronavirus infection among various socio-demographic population groups in Russia.

Materials and methods. The study was conducted online by distributing via social networks a direct link to an electronic form with questions about the attitude to the COVID-19 pandemic and vaccination. A total of 2,786 people (of whom 66.9% were women) aged 16 to 77 years took part in the online survey.

Results. It was shown that distrust of vaccination was more often expressed by women and younger people. A targeted approach to these population groups can improve the results of awareness-raising and preventive measures in the context of an ongoing pandemic.

Keywords: pandemic, COVID-19, coronavirus infection, attitude to vaccination, awareness-raising and preventive measures

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Особенности отношения к вакцинации против COVID-19 в России

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РЕЗЮМЕ

Введение. Спустя 1,5 года с момента регистрации первой вакцины против COVID-19 в России, коллективный иммунитет населения страны достиг лишь 49,7%. Очевидно, что успешность мероприятий по вакцинации зависит от готовности населения к иммунизации и его отношения к вакцине.

Целью исследования стало изучение отношения к вакцинации против новой коронавирусной инфекции среди различных социально-демографических групп населения России.

Материалы и методы. Исследование проводилось в онлайн-формате посредством распространения в социальных сетях прямой ссылки на электронную форму с вопросами об отношении респондентов к пандемии COVID-19 и вакцинации. В заполнении формы онлайн-опроса приняли участие 2 786 человек (66,9% женщин) в возрасте 16–77 лет.

Результаты. Показано, что недоверие к вакцинации чаще проявляли женщины и лица более молодого возраста. Применение таргетированного подхода к данным группам населения может улучшить результаты информационно-профилактических мероприятий в условиях продолжающейся пандемии.

Ключевые слова: пандемия, COVID-19, коронавирусная инфекция, отношение к вакцинации, информационно-профилактические мероприятия

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

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INTRODUCTION

According to the World Health Organization (WHO), at the end of March 2022, a total of 468,859,830 COVID-19 cases and 5,792,618 related deaths were registered worldwide [1], and these figures continue to grow. As of March 2022, about 17,803,503 COVID-19 cases and 368,025 related deaths were registered in Russia [2]. The number of new coronavirus cases varies significantly between countries. One of the main reasons for this are administrative orders and recommendations of health services to slow the spread of coronavirus disease [3].

Mass vaccination is the most important measure to combat COVID-19, since the use of the vaccine allows to create stable population immunity [4, 5]. However, success of vaccination measures depends on the readiness of the population for immunization and their attitude to the vaccine. Currently, there is some distrust of vaccination and medical technologies in general

among various social groups [6, 7]. The origins of this distrust date back to the 1980s–1990s when a whole movement of anti-vaccinationism emerged, whose members conduct quite aggressive anti-vaccination propaganda. Russia is no exception in this regard. A lot of reports and media coverage have repeatedly mentioned low rates of vaccination among citizens, despite the availability of free vaccines, as well as restrictions regarding the spread of COVID-19 [8, 9]. The first vaccine in Russia was registered on August 11, 2020; 1.5 years later, Russian herd immunity reached only 49.7% (data as of March 25, 2022) [10].

Thus, when after a long period of time society faced an unprecedented new pandemic, characterized by a relatively high risk of death or disability, there was a unique opportunity to analyze what the attitude of the population to medical technologies and, in particular, to vaccination is to protect against a new coronavirus infection. To date, an extensive body of empirical data has been accumulated on psychological, socio-demographic, and behavioral predictors of vaccination decisions [11–13]. For example, common factors associated with refusal and doubts about influenza vaccination include: the idea that the risk is low, uncertainty about the effect or safety of the vaccine, a general negative attitude towards vaccines, denial of the social significance of vaccination and the disease itself, low socioeconomic status, and a lack of knowledge about vaccination [11–13]. It is possible that similar factors are the reason for the low rate of COVID-19 vaccination in Russia. Nevertheless, there are very few studies on factors influencing the attitude to COVID-19 vaccination in the available literature, and their reliability is questionable due to the small sample size.

The aim of the study was to research the attitude to COVID-19 vaccination among various socio–demographic groups of the Russian population.

MATERIALS AND METHODS

To study the attitude of the population to COVID-19 vaccination, in January–March 2021 we conducted a survey in the form of online testing on the platform ivik.org. The respondents were asked to fill out an electronic questionnaire in which they independently answered questions to clarify their attitude to measures aimed at preventing the spread of the novel coronavirus infection.

All respondents signed an informed consent to participate in the study and publish the data in an anonymous and generalized form. The sample was collected by advertisements in social media in accordance with standard network methods for recruiting respondents [14, 15]. A total of 2,786 people aged 16–77 years took part in the study (the average age was 29.57 ± 10.86 years), including 1,864 women (66.9%) and 922 men (33.1%). A total of 42 (1.5%) people had incomplete secondary education, 415 (14.9%) people had secondary general education, 187 (6.7%) people – secondary special education, 1,197 (43%) people – incomplete higher education, and 945 (33.9%) respondents – higher vocational education. The study involved residents of the following federal districts of Russia: Central (40.1%), Northwestern (10.4%), Ural (5.1%), Volga (27.4%), Southern (10.5%), and Siberian (6.5%). Out of 2,786 people, 734 (26.3%) reported having COVID–19, of whom 93% experienced the disease in a mild form.

The results were processed using the Statistica 12 software package. Descriptive analysis methods were used. The data are presented in the form of absolute and relative values of n (%). To identify the significance of the differences in parameters between the groups, the Pearson's $\chi 2$ test was used. The Spearman's rank correlation coefficient (r_s) was used to assess correlations between the studied parameters.

RESULTS

The analysis of the questions reflecting the respondents' attitude to COVID-19 vaccination showed mixed results (Fig. 1). To the question "I will agree to COVID-19 vaccination", 1,604 (57.6%) respondents answered "disagree", 551 (19.8%) people answered "agree", the remaining 631 (22.6%) respondents neither agreed nor disagreed with the statement. At the same time, 1,389 (49.9%) respondents did not agree that vaccination should be mandatory, while only 729 (26.2%) people supported mandatory vaccination. Moreover, 1,076 (38.6%) people did not believe that "the vaccine can help control the spread of COVID-19", however, 829 (29.8%) people agreed with this statement.

Then the survey participants were divided into two groups based on gender. The data obtained as a result of the survey, depending on gender, are presented in Table 1.

According to the survey, women were more likely to be against vaccination than men (61 vs. 50.7%; $\chi 2 = 40.72$; $p < 0.001$); women more often expressed doubts about the effectiveness of vaccination (41.7 vs. 32.4%; $\chi 2 = 37.38$; $p < 0.001$) and also more often disagreed with the statement that vaccination should be mandatory (52.8 vs. 43.9%; $\chi 2 = 30.29$; $p < 0.001$).

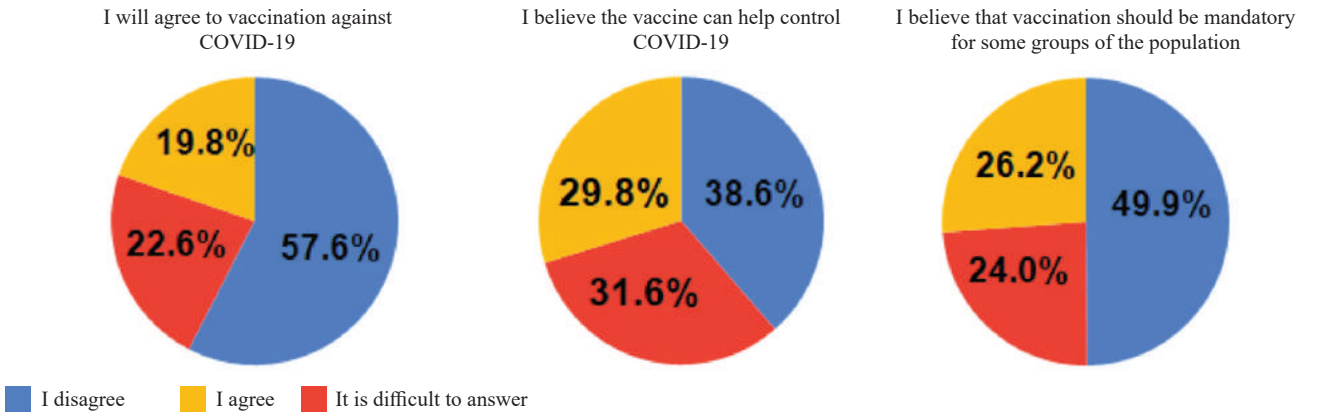

Figure. The attitude of the respondents to COVID-19 vaccination

Table 1

Results of the survey on the attitude to COVID-19 vaccination based on gender, *n* (%)

Do you agree with the following statements?	Response options	Men, *n* = 922	Women, *n* = 1,864	Total, *n* = 2,786
I will agree to COVID-19 vaccination	I disagree	467 (50.7%)	1,137 (61%)	1,604 (57.6%)
	Neither agree nor disagree	213 (23.1%)	418 (22.4%)	631 (22.6%)
	I agree	242 (26.2%)	309 (16.6%)	551 (19.8%)
I believe the vaccine can help control the spread of COVID-19	I disagree	299 (32.4%)	777 (41.7%)	1,076 (38.6%)
	Neither agree nor disagree	282 (30.6%)	599 (32.1%)	881 (31.6%)
	I agree	341 (37%)	488 (26.2%)	829 (29.8%)
I believe that vaccination should be mandatory for some population groups	I disagree	405 (43.9%)	984 (52.8%)	1,389 (49.9%)
	Neither agree nor disagree	218 (23.6%)	450 (24.1%)	668 (23.9%)
	I agree	299 (32.4%)	430 (23.1%)	729 (26.2%)

Then the respondents were divided into 6 groups based on age: group I – young people under the age of 20 (927 individuals), represented by schoolchildren and first- and second-year students; group II – respondents aged 20–29 years (1,219 people), represented by undergraduates, Master's and post-graduate students; group III – people aged 30–39 years (279 people), group IV – people aged 40–49 years (223 people), and group V – people aged 50–59 years (86 people) – people of working age; group VI – respondents aged 60 years and older (52 people), who are all retired. The data obtained as a result of the survey are presented in Table 2 arranged by the age of the respondents.

Table 2

Results of the survey on the attitude to COVID-19 vaccination based on age, *n* (%)

Do you agree with the following statements?	Response options	Age groups					
		I < 20 years	II 20–29 years	III 30–39 years	IV 40–49 years	V 50–59 years	VI 60 and older
I will agree to COVID-19 vaccination	I disagree	551 (59.4%)	755 (61.9%)	138 (49.5%)	113 (50.7%)	26 (30.2%)	21 (40.4%)
	Neither agree nor disagree	221 (23.8%)	255 (20.9%)	75 (26.9%)	48 (21.5%)	22 (25.6%)	10 (19.2%)
	I agree	155 (16.7%)	209 (17.1%)	66 (23.7%)	62 (27.8%)	38 (44.2%)	21 (40.4%)
I believe the vaccine can help control the spread of COVID-19	I disagree	387 (41.7%)	498 (40.9%)	83 (29.7%)	75 (33.6%)	18 (20.9%)	15 (28.8%)
	Neither agree nor disagree	327 (35.3%)	376 (30.8%)	90 (32.3%)	59 (26.5%)	20 (23.3%)	9 (17.3%)
	I agree	213 (23%)	345 (28.3%)	106 (38%)	89 (39.9%)	48 (55.8%)	28 (53.8%)
I believe that vaccination should be mandatory for some population groups	I disagree	455 (49.1%)	629 (51.6%)	149 (53.4%)	107 (48%)	26 (30.2%)	23 (44.2%)
	Neither agree nor disagree	242 (26.1%)	288 (23.6%)	57 (20.4%)	46 (20.6%)	26 (30.2%)	9 (17.3%)
	I agree	230 (24.8%)	302 (24.8%)	73 (26.2%)	70 (31.4%)	34 (39.5%)	20 (38.5%)

According to the data from Table 2, it was found that older people (50 years and older, groups V and VI) more often than others agreed to get vaccinated ($\chi 2 > 34.78$; $p < 0.001$), and in general there was a direct correlation between the age of the respondents and the percentage of those who agreed to get vaccinated ($r_s = 0.244$; $p = 0.018$). A similar trend was observed with regard to the effectiveness of vaccination ($r_s = 0.322$; $p < 0.001$); older individuals (30 years and older, groups III and VI) more often agreed that the vaccine could help control the spread of COVID-19 (more than half of the respondents in each group agreed with this statement; $p < 0.05$). Nevertheless, in almost all the groups, with the exception of group V, the majority were against mandatory vaccination for some population groups, although the percentage of dissenters decreased with age.

Additionally, we analyzed the survey results depending on the level of education of the respondents. We identified five groups: I – individuals with incomplete secondary education (42 people), II – individuals with secondary general education (415 people), III – people with secondary vocational education (187 people), IV – respondents with incomplete higher education (1,197 people), and V – people with higher vocational education (945 people). The data obtained as a result of the survey are presented in Table 3 arranged by the level of education of the respondents.

Table 3

Results of the survey on the attitude to COVID-19 vaccination based on the level of education, *n* (%)

Do you agree with the following statements?	Response options	Groups by the level of education				
		I	II	III	IV	V
I will agree to COVID-19 vaccination	I disagree	29 (69%)	238 (57.3%)	121 (64.7%)	731 (61.1%)	485 (51.3%)
	Neither agree nor disagree	9 (21.5%)	97 (23.4%)	44 (23.5%)	265 (22.1%)	216 (22.9%)
	I agree	4 (9.5%)	80 (19.3%)	22 (11.8%)	201 (16.8%)	244 (25.8%)
I believe the vaccine can help control the spread of COVID-19	I disagree	23 (54.8%)	169 (40.7%)	87 (46.5%)	495 (41.4%)	302 (32%)
	Neither agree nor disagree	9 (21.4%)	133 (32.1%)	60 (32.1%)	414 (34.6%)	265 (28%)
	I agree	10 (23.8%)	113 (27.2%)	40 (21.4%)	288 (24.1%)	378 (40%)
I believe that vaccination should be mandatory for some population groups	I disagree	26 (62%)	192 (46.3%)	94 (50.3%)	601 (50.2%)	476 (50.4%)
	Neither agree nor disagree	8 (19%)	115 (27.7%)	50 (26.7%)	294 (24.6%)	201 (21.3%)
	I agree	8 (19%)	108 (26%)	43 (23%)	302 (25.2%)	268 (28.4%)

According to the data presented, the level of education affected the attitude to the effectiveness of vaccination – the majority of respondents with higher vocational education (group V) agreed that the vaccine could help control the spread of the disease (40 vs. 32%). Besides, this group had the highest percentage of those who agreed to vaccination (25.8%) and introduction of mandatory vaccination for some population groups (28.4%).

DISCUSSION

The COVID-19 pandemic has brought unprecedented challenges for society [14, 16]. Existing health problems among vulnerable population groups may worsen multiple times under the influence of new waves of coronavirus infection [16–18]. Despite this, most of the respondents surveyed had a negative attitude to COVID-19 vaccination (57.6%) or could neither agree or disagree with it (22.6%), which may be due to claims about the quality and effectiveness of the vaccine (38.6%), as well as distrust of vaccination in general. Only a small number of the respondents (19.8%) expressed readiness to use the vaccine, while the spread of opinion correlated with gender and age.

Doubts about vaccination and uncertainty about its necessity and effectiveness are more typical for women than for men. Thus, according to the data obtained, women in comparison with men mostly disagreed to get vaccinated (61 vs. 50.7%), as well as doubted about the effectiveness of the vaccine itself (41.7 vs. 32.4%). For example, the majority of men agreed with the statement that the vaccine could help control the spread of COVID-19 (37 vs. 32.4%), while the majority of women tended to assume the opposite (26.2 vs. 41.7%). This largely corresponds to the data of other studies [19–21].

It was also found that a significant proportion of young people under the age of 49 (55% on average) who were not ready for immunization was opposed to a relatively large proportion of those who agreed to get vaccinated among older people (42.3% on average). This finding is a relatively positive trend, since older people are at risk of developing a serious illness and acute respiratory distress syndrome from the novel coronavirus infection [22]. A similar trend was observed with regard to the effectiveness of vaccination in different age groups. It is also worth noting that the majority of the respondents with higher vo-

cational education agreed that the vaccine could help control the spread of COVID-19. In addition, in this group of the respondents, the highest percentage of people who agreed to get vaccinated was noted.

CONCLUSION

According to the Levada Center [23], even sufficient information and the second wave of the pandemic at the beginning of March 2021 did not make the population confident about getting vaccinated. Our study showed that distrust of vaccination was more often expressed by women and younger people. A targeted approach to these population groups can improve the results of awareness-raising and preventive measures in the context of the ongoing pandemic.

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Lung cancer in patients with COPD and factors associated with reduced survival

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ABSTRACT

Background. A combination of different types of lung cancer and chronic obstructive pulmonary disease (COPD) is very common. COPD, accompanied by ventilation disorders and, often, respiratory failure, is a significant additional risk factor for mortality in these patients. Identification of risk factors for mortality in patients with lung cancer and COPD can potentially be associated with better long-term outcomes.

Materials and methods. Using a Cox regression model based on information about the outcome of the disease and life expectancy after treatment initiation, a survival analysis was performed with an assessment of the contribution of various clinical and anamnestic factors for a group of 118 COPD patients with primary diagnosed lung cancer. These patients received treatment at the Cancer Research Institute in Tomsk in 2013–2019.

Results. The study included 118 patients (87.3% men and 12.7% women). Among them, 77.97% of patients were active or former smokers with smoking index (SI) ≥ 10 pack-years, and 22% of patients had never smoked or had SI < 10 pack-years but had other risk factors for COPD. Peripheral lung cancer was detected in 45.8% of cases. Squamous cell carcinoma was noted in 54.2% of cases, adenocarcinoma – in 34.7%, large cell carcinoma – in 1.7%, small cell carcinoma – in 5.9%, and carcinoid tumors – in 2.5% of cases. Patients were characterized by varying degrees of severity of ventilation disorders in accordance with the GOLD classification: stage 1 was observed in 44% of patients, stage 2 – in 38.1 % of patients, stage 3 – in 16.9 % of patients, and stage 4 – in one patient. Three-year mortality was 28.12%.

Conclusion. According to the results of the Cox regression analysis, factors that significantly reduced the survival rate of patients with lung cancer in combination with COPD were more severe stages in terms of the size of the primary tumor and its localization, the prevalence of metastasis (according to TNM classification), more severe dyspnea (mMRC scale), lower oxygen saturation values, atelectasis, and episodes of pneumonia, including paracancrotic pneumonia, over the previous 12 months. The presence of certain types of metastases, such as metastatic lesions of the pleura, adrenal glands, distant non-regional lymph nodes, and bones should also be noted as negative factors for survival. It is worth noting that surgical treatment of the primary tumor was associated with an increase in the survival rate in patients with lung cancer in combination with COPD.

Keywords: lung cancer, chronic obstructive pulmonary disease, COPD, patient survival

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Рак легкого у больных ХОБЛ и факторы, ассоциированные со снижением их выживаемости

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РЕЗЮМЕ

Актуальность. Сочетание различных форм рака легкого и хронической обструктивной болезни легких (ХОБЛ) встречается очень часто. ХОБЛ, сопровождающаяся вентиляционными нарушениями и нередко дыхательной недостаточностью, является существенным дополнительным фактором риска летальности у этих больных. Выявление факторов риска смертности у пациентов с раком легкого и ХОБЛ потенциально может быть связано с лучшими отдаленными результатами.

Материалы и методы. С помощью регрессии Кокса, основанной на информации об исходе заболевания и продолжительности жизни после начала лечения, проведен анализ выживаемости с оценкой вклада различных клинико-анамнестических факторов для группы из 118 больных ХОБЛ с первично диагностированным раком легкого. Эти больные получали лечение в НИИ онкологии в Томске в период с 2013 по 2019 г.

Результаты. В исследование включены 118 пациентов (87,3% мужчин и 12,7% женщин). Среди них 77,97% пациентов были активными или бывшими курильщиками с индексом курильщика (SPI) ≥ 10 пачек/год, а 22% никогда не курили или с SPI < 10 пачек/лет, но имели другие факторы риска развития ХОБЛ. Периферический рак легкого выявлен в 45,8% случаев. Плоскоклеточный рак отмечен в 54,2% случаев, аденокарцинома – у 34,7% больных, крупноклеточный рак – у 1,7%, нейроэндокринный мелкоклеточный рак – у 5,9%, карциноидные опухоли – у 2,5% пациентов. Больные характеризовались различной степенью выраженности вентиляционных нарушений по классификации GOLD: первая стадия ХОБЛ наблюдалась у 44% больных, вторая – у 38,1%, третья – у 16,9%, четвертая стадия – у одного из пациентов. Трехлетняя выживаемость составила 28,12%.

Заключение. По результатам анализа методом регрессии Кокса в качестве факторов, значительно снижающих выживаемость больных раком легкого в сочетании с ХОБЛ, необходимо выделить более распространенные стадии по размеру первичной опухоли и ее локализации, а также выраженность метастазирования (по классификации TNM), более значительную одышку (по шкале mMRC), более низкое значение сатурации кислорода, наличие ателектаза легкого, эпизодов пневмонии, в том числе параканкрозной, в предшествующие 12 мес. В качестве негативных факторов выживания следует также отметить наличие некоторых видов метастазов, таких как метастатическое поражение плевры, надпочечников, отдаленных нерегиональных лимфатических узлов, костей скелета. Отметим, что хирургическое лечение первичной опухоли было связано с увеличением выживаемости больных раком легкого в сочетании с ХОБЛ.

Ключевые слова: рак легкого, хроническая обструктивная болезнь легких, ХОБЛ, выживаемость больных

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 проведение исследования. Исследование одобрено локальным этическим комитетом НИИ онкологии Томского НИМЦ (протокол № 10 от 26.09.2016).

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INTRODUCTION

Lung cancer (LC) is a malignant neoplasm (MN) characterized by a high mortality rate. Every year in Russia, more than 60,000 people die from it, 80% of them are men. According to statistics, after the initial diagnosis, one-year mortality reaches more than 50%, and the average five-year survival rate, even with adequate treatment, is about 10–16% [1].

Survival in LC depends on many factors, such as the stage of the disease, tumor morphology, patient's age, concomitant chronic pathology, and treatment strategy [2].

Almost 2/3 of men and half of women with newly diagnosed primary LC have signs of airway obstruction [3]. J.P. De-Torres et al. examined outpatients in the United States and Spain (n = 2,125) and developed criteria to predict LC. These criteria include age over 60 years, body mass index < 25 kg / m^2, smoking index (SI) > 60 pack – years, emphysema on CT scan, and signs of chronic obstructive pulmonary disease (COPD) according to spirometry [3]. Thus, airflow limitation and emphysema, which are typical of COPD, should probably be considered as potential risk factors for the development of LC.

LC and COPD are comorbidities with a similar pathogenetic mechanism underlying the development of pathology in the bronchi and lung parenchyma, caused by systematic damaging effects of smoking, radon, asbestos, and pollutants, combined with genetic susceptibility [4–6].

Currently, COPD is considered an independent risk factor for LC [7]. According to epidemiological data, the incidence of LC in smokers with COPD is 4–5 times higher than in smokers without COPD [7, 8]. R. P. Young et al. showed that decreasing forced expiratory volume in 1 second (FEV1) in COPD increased the risk of LC by 4 times compared with smokers with normal lung function, regardless of their age and smoking history [9]. J. Murakami et al. noted that the cancer that occurs with emphysema is more aggressive and has a higher postoperative recurrence [9].

Understanding of the general mechanisms of LC and COPD development suggests that timely diagnosis and long-term effective treatment of chronic inflammation in the respiratory tract associated with bronchial obstruction in patients with COPD in combination with elimination of altering factors can probably prevent and reduce the risk of developing LC [6, 10].

It is COPD that typically leads to pulmonary function decline. It determines the frequency of complications and the risk of death in cancer patients. According to R. Kondo et al. (2011), the five-year survival rate in LC patients with and without COPD was 38% and 54 %, respectively. At the same time, the mortality rate in patients with and without COPD was 63% and 45%, respectively [7].

COPD is a significant perioperative mortality predictor and the most common cause of postoperative pulmonary complications and respiratory failure due to severe respiratory dysfunction. These concerns are the main reason for refusing radical surgery in some patients, since severe COPD and significant concomitant / comorbid pathology in aged patients make it very difficult to conduct mechanical ventilation and manage the early postoperative period, which is associated with an increased risk of mortality in this patient group [10, 11].

The aim of this retrospective study was to research the initial clinical and anamnestic data of patients with COPD and primary LC, assess the disease outcome and life expectancy during treatment, and establish factors associated with survival and mortality in this group of comorbid patients.

MATERIALS AND METHODS

We analyzed demographic, clinical, and anamnestic data from the health records of 118 patients with COPD who underwent examination and treatment (surgery, radiation therapy, chemotherapy or combined) for newly diagnosed MN in the bronchi and lung at the Cancer Research Institute (Tomsk National Research Medical Center, Russian Academy of Sciences) in 2013–2019.

Statistical processing of the obtained data was carried out using Statistica 10.0 and StatCalc 6.0 software packages. Qualitative data were presented as absolute or relative (%) frequencies, quantitative data were shown as $X \pm x$, where X is the arithmetic mean and x is the error of the mean. Results were considered statistically significant at $p < 0.05$. We conducted the survival analysis for these patients and assessed the contribution of various clinical and anamnestic factors using the Cox regression analysis based on information about the disease outcome and life expectancy after treatment initiation (within the assessment of one- and three-year survival).

RESULTS AND DISCUSSION

Currently, there are more men among patients with LC and / or COPD than women, and the incidence increases with age [2, 11]. According to our retrospective study, among 118 patients with COPD and newly diagnosed primary LC, 87.3 % of patients were male and 12.7 % were female. At the initiation of treatment, the age of patients was 63.63 ± 7.25 years, they were diagnosed with LC at the age of 61.92 ± 7.26 years.

It is known that in 90–95% of COPD cases, the cause of the disease is smoking. Smoking also has a carcinogenic effect on lung tissue in 85–95% of LC cases in men and in 65–80% of LC cases in women [6–8]. In the study group, 13 patients (11.1 %) used to smoke and 79 patients (66.9 %) smoked at the beginning of the follow-up period in the retrospective analysis with SI > 10 pack – years. In 26 patients (22 %), SI was < 10 pack – years, however, they had other COPD risk factors: exposure to occupational hazards (dust, chemical agents, acid, and alkali vapors) and industrial pollutants (SO_2, NO_2, furnace smoke, etc.). The average smoking history in the study group was 33.45 ± 13.58 years, and SI was 33.35 ± 14.53 pack – years. The longest smoking history was 55 years, and the maximum SI in the heaviest smokers was 60 pack – years. Peripheral LC was detected in 45.8 % of cases, central LC — in 44.2 % of cases.

We classified patients in accordance with the 7th edition of the International Classification of Malignant Neoplasms, TNM (2011) [12]. According to the spread of the primary tumor (TNM-T), T1, T2, T3, and T4 stages were diagnosed in 19%, 18.7%, 43.2%, and 22% of patients, respectively. Regional lymph node metastases were diagnosed in 67% of patients (N1, N2, and N3 in 22.9%, 34.7%, and 9.3%, respectively). Distant metastases (M1) were diagnosed in 22% of patients in the study.

Thus, metastatic pleurisy was diagnosed in 11.9 % of patients. Unilateral or contralateral pulmonary metastases were detected only in 5.9% of patients. Extrapulmonary metastases were detected in the bones (9.3% of patients), liver (5.9%), brain (3.4%), adrenal glands (6.8%), and non-regional lymph nodes (16%). Aggressive invasive tumor growth that invaded adjacent organs and tissues and great vessels was determined in 48% of patients.

The results obtained indicate that patients with neglected LC (locally advanced and metastatic) prevailed, which can be explained by the long-term low-symptom disease, masking its manifestations with symptoms and exacerbations of COPD in this group of patients, as well as a delay in seeking medical care. According to A.D.Kaprin et al., 68.7% of patients with LC were diagnosed at stages III-IV of the disease, of them, metastasis was determined in 40.8% of cases [1, 10, 13].

According to statistical data, LC most often develops in the right lung (56 %). Moreover, the upper lobe, the lower lobe, and the middle lobe of the lung are affected in 60%, 30%, and 10% of cases, respectively [1, 13]. We obtained similar results in the group of patients with combined COPD and LC. Thus, MN of the right lung was diagnosed in 64.4% of patients, and MN of the left lung – in 33.9% of patients. Only two patients (1.7%) were diagnosed with metachronous LC. Lesions of the upper lobe

were detected most often (in 57.6% of patients). An isolated lesion of the lower lobe was diagnosed in 24.6% of patients. The middle lobe of the right lung was affected in 8.5% of patients. Combined lesions located in two or three lobes were detected in 9.3% of patients.

According to the Russian and foreign literature, adenocarcinoma is the most common type of non-small cell lung cancer, which is diagnosed in about 40% of cases. Squamous cell lung carcinoma (epidermoid cancer) accounts for 25–30% of all LC cases and is detected more often in smokers [6, 7]. This may explain the fact that in the examined patients with combined LC and COPD, the vast majority of whom were heavy smokers, squamous cell LC was the dominant histologic pattern (identified in 54.4% of cases). Adenocarcinoma was detected in 35.5% of patients. Large cell LC (1.7%), small cell LC (5.9%), and carcinoid tumors (2.5%) were significantly less common.

It should be noted that patients with combined LC and COPD often have comorbidities that prevent from radical treatment (due to concomitant coronary artery disease, essential hypertension, cerebral atherosclerosis, peripheral arterial disease, gastric ulcer, and osteoporosis) [13, 14]. In addition to LC and COPD, concomitant pathology as a combination of several diseases was often noted in most of the patients examined. Arterial hypertension (66.9%), coronary artery disease (48.3%), gastric and / or duodenal ulcer (active disease or history in 28% of patients) were the most common. Obesity of varying severity was detected in 16.9% of patients, diabetes mellitus was revealed in 6.8% of cases. Concomitant chronic lung pathology other than COPD, but including asthma and bronchiectasis, was noted in 7.6% of patients. Some patients had history of myocardial infarction and stroke: 13.6% and 10.2%, respectively.

Based on the medical records, we noted variability in lung ventilation disorders caused by COPD in the patients. Mild and moderate bronchial obstruction were the most common, which could be due to the fact that patients without severe decompensated underlying diseases and concomitant pathology were primarily selected for treatment. Mild airflow limitation (FEV1 salbutamol challenge test [post-FEV1] > 80%) was detected in 44% of the patients, moderate (post-FEV1 50–80%) – in 38.1% of the patients, severe (post-FEV1 30–50%) – in 16.9% of the patients. Only one patient was diagnosed with very severe COPD (post-FEV1 < 30%). Baseline peripheral oxygen saturation in the blood in the patients was 96.86 ± 1.25%.

Based on the CT data, emphysema, chronic bronchitis, and a mixed phenotype of COPD were determined in 55.9%, 13.6%, and 30.5% of the cases, respectively. Within a year preceding the study, 72.9% of the patients had at least one episode of COPD exacerbation, and 53.4% of the patients had ≥ 1 episode of pneumonia, including paracancrotic pneumonia (in 29.7% of the patients). Antibiotic therapy (≥ 1 time in 12 months)for respiratory infections was required in 65.3% of the patients.

The absence of regular maintenance therapy for COPD remains a significant challenge in the efforts against the disease progression. It is especially typical of patients with mild symptoms [6, 11]. According to our study results, only 29.7 % of the patients received inhaled therapy for COPD routinely or episodically. Only 6.8% of the patients received long-acting bronchodilators which are the mainstay of modern COPD treatment (in combination with inhaled corticosteroids or without them).

Treatment strategy in non-small cell lung cancer (NSCLC) is ambiguous and depends on the stage of the disease, the histologic pattern of the tumor, differentiation of the tumor, regional and distant metastases, as well as somatic symptom pathology and functional reserves of vital organs and systems [16]. The most effective technique is surgery performed for resectable LC. Pneumonectomy or lobectomy with removal of all mediastinal lymph nodes on the side of the affected lung is considered standard surgical therapy. In addition to the lung resection, combined surgeries include resection of a neighboring organ invaded by the tumor (pericardium, chest wall, diaphragm, vagus or phrenic nerve, less often the superior vena cava, atrium, esophagus, and pulmonary artery). In patients with decreased pulmonary function (stage III–IV COPD) and severe cardiac pathology, a smaller extent of lung resection is acceptable, such as atypical resection and segmentectomy. However, it subsequently leads to a threefold increase in regional recurrence of LC [15].

Currently, patients with stage 0–IIIA NSCLC are considered operable, but patients with

stage IB, II, and IIIA and stage N1, N2 lymph node metastases are prescribed adjuvant chemotherapy to suppress the activity of the tumor cell and its effects on subclinical micrometastases in the lymph nodes and distant organs. At stage IV, a patient is prescribed palliative chemotherapy, immunotherapy, or their combination with radiation therapy [16]. In 2010, the Thoracic Cancer Department of Cancer Research Institute (Tomsk NRMC) developed a method for combination treatment of patients with stage III NSCLC, including two courses of neoadjuvant chemotherapy, radical surgery, and subsequent prescription of postoperative personalized adjuvant chemotherapy according to a scheme based on analysis of monoresistance gene expression. There was a decrease in the local recurrence rate and distant metastasis rate and an improvement in recurrence-free survival by 29.1% compared with the control group, which demonstrates the effectiveness of this approach in disease management [17].

According to the analysis performed, 76 (64.4%) patients underwent surgery: 42 (55.26%) patients underwent lobectomy, 9 (11.84%) patients – bilobectomy, 21 (27.63%) patients – pneumonectomy, 2 (2.63%) patients – atypical resection; one patient underwent diagnostic thoracotomy, and one patient – transbronchial endoscopic tumor resection. In 72 (94.74%) cases, radical surgery was performed in combination with ipsilateral mediastinal lymph node dissection. Reconstructive organ-preserving plastic surgery was performed in 23 (30.26%) patients. In accordance with modern guidelines [16], 95 (80.5%) patients received chemotherapy, 28 (23.7%) patients – external beam radiation therapy.

According to the literature, in most leading thoracic surgery clinics, where LC surgery is performed, the incidence of postoperative complications remains 15–25% [13, 18]. Complications can be surgical (bronchial anastomotic leakage, bronchial fistula, obstructive atelectasis, pleural empyema, bleeding) and non-surgical (pneumonia, acute cardiac failure, cardiac arrhythmia, myocardial infarction, pulmonary embolism, ischemic stroke). Pneumonia is more often observed after lung resection than after pneumonectomy (in 11.7% and 3% of the patients, respectively). It occurs due to impaired bronchial drainage, formation of atelectasis due to incomplete expansion of the remaining lobe or segment of the lung, circulatory disorders, and underlying chronic inflammation of the bronchi, especially in COPD. The most common cardiovascular complication is cardiac arrhythmia, namely atrial fibrillation and ventricular extrasystole [13, 18].

In our study, 44 (57.9%) of 76 operated patients developed surgical complications or a combination of them in the postoperative period. In particular, these complications included pneumonia (17.1%), pleurisy (17.1%), obstructive atelectasis (3.9%), cardiac arrhythmias (38.16%), acute myocardial infarction (2.63%), COPD exacerbation (34.21%), anastomotic leakage (10.53%), bronchopleural fistula (9.2%), pleural empyema (13.16%), and pulmonary hemorrhage (2.27%).

According to statistics, after the initial diagnosis, one-year mortality rate reaches more than 50%, and the average five-year survival rate, even with adequate treatment, is about 10–16% [1]. As part of our study, we assessed one- and three-year mortality of the patients. During the first year of the follow-up after the initiation of the treatment, 29 (24.58%) out of 118 patients died. Three-year mortality in the group of 96 patients was 71.88%; 69 patients died. The follow-up lasted less than 36 months for 22 patients from the study group. According to the literature analyzing 348 operated patients, the best survival in LC patients was observed in the absence of regional lymph node metastases, radical surgery, and squamous cell lung cancer [18].

The results of the Cox regression analysis showed that factors that significantly reduced the survival of patients with combined LC and COPD included more severe stages of the disease in terms of the size of the primary tumor and its localization, invasion into adjacent organs and tissues, severity of regional and distant metastasis (according to the TNM classification), and ventilation disorders, manifested through more severe dyspnea (according to the mMRC scale), reduced baseline values of peripheral blood oxygen saturation, pulmonary atelectasis, and episodes of pneumonia (including paracancrotic types) in the previous 12 months. The presence of metastases in the pleura (pleurisy), adrenal glands, and distant non-regional lymph nodes should also be considered as negative factors for survival. It should be noted that surgical removal of the primary tumor was associated with an increase in the survival rate of patients with combined LC and COPD (Table).

Table

Survival-associated factors in patients with combined LC and COPD

Factor	Beta*	OR	l_CI95	u_CI95	R^2	*p*
Tumor Spread (TNM-T)	0.4047	1.4988	1.1445	1.9628	0.078	0.0033
Distant metastases (TNM-M)	0.866	2.3775	1.4185	3.9847	0.081	0.001
mMRC	0.596	1.8149	1.279	2.5753	0.09	0.0008
Pronounced invasion into adjacent organs and tissues according to SCT (T3–T4 according to TNM classification)	0.429	1.5363	1.1132	2.1202	0.058	0.0089
Peripheral oxygen saturation	–0.2867	0.7507	0.6417	0.8783	0.088	0.00034
Pleural metastases	1.4122	4.1049	2.1791	7.7324	0.13	0.00012
Metastases in the adrenal glands	1.7178	5.5721	2.5707	12.0774	0.11	0.00013
Non-regional lymph node metastases	0.9620	2.6169	1.5176	4.5127	0.087	0.00054
Pneumonia in previous 12 months	0.8141	2.2571	1.3811	3.6888	0.094	0.0012
Pulmonary atelectasis	0.8648	2.3744	1.4746	3.8233	0.11	0.00037
Paracancrotic pneumonia in previous 12 months	0.8396	2.3156	1.4211	3.7732	0.089	0.00075
Surgical therapy for the primary tumor	–1.3606	0.2565	0.1594	0.4127	0.25	<0.00001

Note: beta is a coefficient indicating how much the units of standard deviation in the dependent variable changed to alter the unit of standard deviation in the independent variable of interest with all other controlled variables; OR – odds ratio; l(u)_CI95 is lower (upper) confidence interval; R^2 – the accuracy degree of the process description by the model; *p* – statistical significance of the differences; SCT – spiral computed tomography.

CONCLUSION

The combination of COPD and LC is still an important medical problem. On the one hand, COPD can be considered as an independent risk factor for LC. On the other hand, severe bronchial obstruction and emphysema often hinder radical LC treatment.

The study established that combined COPD and LC was more common among men (approximately 6.8 times more common than among women) with a long history and high intensity of smoking, which correlated with the predominance of squamous LC (54.4%) in the analyzed population.

The study highlighted the problem of late diagnosis of LC (at stage III–IV with the disseminated tumor and severe invasive tumor growth in adjacent organs and tissues), as well as the lack of adequate maintenance COPD therapy in most patients, despite of severe respiratory symptoms, significant obstructive ventilation disorders, and emphysema.

Factors associated with lower survival of patients with combined LC and COPD, besides advanced tumor stages, invasive tumor growth in adjacent organs and tissues, and severity of regional and distant metastasis (according to the TNM classification), included severity of ventilation disorders and dyspnea (according to the mMRC scale), reduced baseline peripheral oxygen saturation, pulmonary atelectasis, and episodes of pneumonia (including paracancrotic pneumonia) in the previous 12 months. Higher mortality was also associated with metastases in the pleura, adrenal glands, and distant non-regional lymph nodes.

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Authors contribution

Dobner S.Yu., Fedosenko S.V. – conception and design; analysis and interpretation of the data; varying out of the research; justification of the manuscript and critical revision of the manuscript for important intellectual content; drafting of the manuscript. Tuzikov S.A. – analysis and interpretation of the data; final approval of the manuscript for publication. Yarovoy N.D., Petrov V. A. – analysis and interpretation of the data, editing of the manuscript. Rodionov E.O., Samykina I.A. – analysis and interpretation of the data, editing, critical revision of the manuscript for important intellectual content. Starovoitova E.A. – analysis and interpretation of the data.

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Variants of creating heterotopic and orthotopic PDX models of human colorectal cancer

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ABSTRACT

Aim. To create heterotopic and orthotopic patient-derived xenograft (PDX) models of colorectal cancer (CRC) by transplantation of patient's tumor samples into immunodeficient BALB / c Nude mice.

Materials and methods. The study was performed on 15 female BALB / c Nude mice aged 6–8 weeks weighing 21–25 g. All animals underwent transplantation of the tumor material obtained from CRC patients into the following sites: heterotopic transplantation (under the skin of the thigh and into the omentum), orthotopic transplantation (into the descending and ascending colon and into the cecum). Weight and general condition of the animals and the size of the tumor nodule had been monitored for 80 days. The success of each model was assessed by the degree of engraftment, the dynamics of tumor growth, and the reproducibility of histopathologic characteristics. At the end of the experiment, the animals were euthanized by cervical dislocation.

Results. 100% survival of the animals and similar tumor growth dynamics in the xenograft models were observed throughout the experiment. The analysis of histologic specimens obtained from the xenografts and patient's tumor showed their correspondence to moderately differentiated intestinal adenocarcinoma. The main advantages and disadvantages of different variants of PDX models were described.

Conclusion. Heterotopic and orthotopic PDX models reproduce the morpho-histologic characteristics of human tumors and demonstrate stable growth dynamics. Therefore, they are a suitable tool for the development, testing, and validation of potential anticancer drugs.

Keywords: PDX model, xenograft, colorectal cancer, adenocarcinoma, orthotopic transplantation, heterotopic transplantation, BALB / c Nude

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

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Conformity with the principles of ethics. The patients signed an informed consent to transfer of the biological material. The study was approved by the Bioethics Committee at the National Medical Research Center of Oncology (Protocol No. 6/84 of 30.06.2020).

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Варианты создания гетеротопических и ортотопических PDX-моделей колоректального рака человека

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РЕЗЮМЕ

Цель. Создание гетеротопических и ортотопических моделей ксенографтов колоректального рака (КРР), полученных от пациентов (Patient-derived xenograft, PDX-модель), путем трансплантации образцов опухоли пациента иммунодефицитным мышам линии Balb/c nude.

Материалы и методы. Проведено исследование на 15 самках мышей линии Balb/c nude, возраст 6–8 нед, масса тела 21–25 г. Всем животным проведена трансплантация опухолевого материала, взятого от пациентов с КРР, в следующие сайты: гетеротопические (под кожу бедра, в сальник); ортотопические (в нисходящий и восходящий отделы толстой кишки, в слепую кишку). В течение 80 сут у животных контролировали следующие параметры: массу тела, общее состояние, объем опухолевого узла. Успешность каждой из моделей оценивали по степени приживления, динамике опухолевого роста и воспроизводимости гистопатологических характеристик. По завершению эксперимента животным выполнена эвтаназия методом цервикальной дислокации.

Результаты. На протяжении всего эксперимента наблюдалась 100%-я выживаемость животных и схожая динамика роста ксенографтов. Анализ гистологических препаратов ксенографтов и опухоли пациентов показал их соответствие умеренно дифференцированной аденокарциноме кишки. Описаны основные преимущества и недостатки создания различных вариантов PDX-моделей.

Заключение. Гетеротопические и ортотопические PDX-модели воспроизводят морфогистологические признаки человеческой опухоли и обладают устойчивой динамикой роста, следовательно, являются подходящим инструментом для разработки, тестирования и валидации потенциальных лекарственных препаратов против рака.

Ключевые слова: PDX-модель, ксенографт, колоректальный рак, аденокарцинома, ортотопическая трансплантация, гетеротопическая трансплантация, Balb/c nude

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. Пациенты подписали информированное согласие на передачу биологического материала. Исследование одобрено комиссией по биоэтике ФГБУ «НМИЦ онкологии» Минздрава России (протокол № 6/84 от 30.06.2020).

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INTRODUCTION

Colorectal cancer (CRR) is the fourth most common cancer worldwide with high mortality rates at advanced stages [1, 2]. 85% of colon tumors occur sporadically, and 15% are associated with hereditary predisposition [3, 4].

The choice of the correct strategy for colon cancer treatment largely depends on the anatomical location of the tumor [5]. Right-sided colon cancer (proximal

tumor location) and left-sided colon cancer (distal tumor location) exhibit different molecular characteristics and histology and, accordingly, show different therapeutic responses. Therefore, effective medical therapy should be developed taking into account all the differences between these tumors [6].

Histologic and morphological characteristics of right-sided and left-sided colon tumors are determined by their different embryonic origin. The cecum, appendix, ascending colon, hepatic flexure, and proximal two-thirds of the transverse colon develop from the midgut, while the distal third of the transverse colon, splenic flexure, descending colon, sigmoid colon, and rectum develop from the hindgut. Right-sided tumors are represented by sessile serrated adenomas or mucinous adenocarcinomas with polyp morphology, while left-sided tumors include tubulovillous and conventional adenocarcinomas [7].

Genomic heterogeneity of these tumors is due to the fact that right-sided CRC displays high microsatellite instability (MSI-high), while left-sided CRC is associated with high chromosomal instability (CIN-high) [8]. Adjuvant chemotherapy (5-fluorouracil (5-FU)) and targeted anti-epidermal growth factor receptor (EGFR) therapy are the most effective strategies for patients with left-sided CRC. Patients with right-sided CRC respond much better to immunotherapy due to high antigen load in these tumors [9].

It is advisable to study the effectiveness of anticancer drugs using preclinical models that fully reflect all clinically significant characteristics of the original human tumor. Cancer cell lines and their xenografts are used for screening tests of drugs *in vitro* and *in vivo*. However, growing neoplastic cells in an artificial environment leads to a change in genetic, transcriptomic, and histologic parameters of the tumor due to its adaptation to the altered conditions [10]. Disadvantages of cancer cell lines are reduced tumor heterogeneity, their belonging mainly to highly aggressive malignant tumors, and low predictive value in the clinical practice [10, 11].

To more accurately reproduce the biological characteristics of a donor human tumor, patient-derived xenograft (PDX) models were created. PDX models are created by transplanting malignant tumors from patients into immunodeficient mice [12]. The fragments of the patient's surgical material retain intercellular interactions, so these models contain not only malignant cells, but also components of the tumor microenvironment [13–16]. Many recent studies have described the successful creation of PDX models for various cancers, such as colorectal cancer [17], breast cancer [18], kidney cancer [19], stomach cancer [20], and non-small cell lung cancer [21].

In PDX models, tumor fragments are transplanted into a recipient mouse either heterotopically or orthotopically. Heterotopic xenografts are created by implanting a tumor fragment into a site not associated with the site of the original tumor, usually subcutaneously [14, 22]. In orthotopic transplantation, the patient's tumor tissue is transplanted into the corresponding organ. Subcutaneous transplantation of PDX models rarely causes metastasis in mice and does not reproduce the organ-specific tumor microenvironment [14]. Since orthotopic transplantation reproduces a natural environment in which the tumor developed in humans, it is considered the highest priority for testing highly selective targeted drugs [22]. At the same time, sites for orthotopic transplantation of the surgical material differ depending on the experiment objectives. The use of this model system takes into account not only an organ specific for a tumor, but also left-sided and right-sided location in case of CRC.

The aim of this study was to create five variants of PDX models of CRC by transplantation of patient's tumor samples into immunodeficient BALB / c Nude mice, as well as to analyze the advantages and disadvantages of each model.

MATERIALS AND METHODS

Human tumor material was transplanted into 15 female BALB /c Nude mice aged 6–8 weeks, weighing 21–25 g. The mice were maintained in an SPF vivarium at 22–24 °C and relative humidity of 60%, with a day / night light regime, in mechanically ventilated cages with sterilized food, water, and bedding. All surgical manipulations in the experiment were performed in compliance with the rules for the use of laboratory animals. The mice were observed for 80 days, then the animals were euthanized.

Tumor samples were obtained from CRC patients receiving treatment at the National Medical Research Center of Oncology from October, 2020 to January, 2021. All patients gave their written informed consent to transfer of the biological material. The donor tumor material for orthotopic transplantation into the ascending colon and cecum was obtained from mucinous colorectal adenocarcinoma, while a tumor fragment for transplantation into the descending colon was obtained from the patient with conventional intestinal adenocarcinoma. For heterotopic transplantation, the

material was also obtained from conventional intestinal adenocarcinoma.

The volume of subcutaneous tumor nodules was measured according to the Shrek's formula for an ellipsoid:

$$V = a \times b \times c \times \pi / 6$$

where V is the tumor volume (mm3), and a, b, and c are the maximum ellipsoid diameters in three planes (mm).

The mice were injected with Xyla at the concentration of 20 mg / ml as premedication and Zoletil at the concentration of 22.57 mg / ml as the general anesthetic. Immediately after excision, the human tumor tissue was placed in 5% gentamicin dissolved in Hanks' balanced salt solution. Immediately before the transplantation, tumor pieces of approximately 3 mm x 3 mm x 3 mm were excised with scissors removing all necrotic tissues. The time from the tumor material resection to the transplantation into the large intestine of a mouse did not exceed 40 minutes. Immediately before the surgery, the mouse skin in the dissection area was treated with a povidone – iodine 10% solution. The skin and peritoneum were closed using a 4-0 Prolene suture; the fragments of the patients' tumors were sutured to the intestinal wall and omentum using 5-0 Prolene ligation.

Heterotopic transplantation of the tumor samples under the skin of the thigh. Tumor fragments were transplanted subcutaneously, dorsally, into the posterior thigh, since this site facilitates tumor monitoring. An incision was made in the thigh using scissors. A fragment of the donor tumor was placed in a pocket made using blunt dissection. The surgery was completed by suturing the skin at the incision site.

Heterotopic transplantation of the tumor samples into the omentum. Transrectal access to the omentum was provided by layer-by-layer dissection of the skin and peritoneum along the rectus abdominis muscle at a distance of 2.5 cm from the xiphoid process. A section of the large intestine suspended by the omentum was placed into the surgical wound. A tumor fragment was sutured to the omentum. The intestine was placed back in the abdominal cavity, and the peritoneum and skin were sutured in layers using the glover's suture.

Orthotopic transplantation of the tumor samples into the cecum. Layer-by-layer dissection of the skin and peritoneum was performed along the midline of the body. The incision was started 20 mm from the end of the xiphoid process and ended 5 mm from the urethra. The cecum was exposed by passing it in the surgical wound. A purse-string serous – muscular suture was applied without tightening the ligature on the end of the cecum at a distance of 5 mm from the edge. Then, the serosa and the muscular layer of the cecum were dissected, the end of the cecum was invaginated creating a pocket, and a fragment of the human tumor was placed into the pocket. The ligature was sequentially tightened, the cecum was placed in the abdominal cavity, and the peritoneum and skin of the mouse were sutured in layers.

Orthotopic transplantation of the tumor samples into the ascending colon. Similarly to the method described earlier, the cecum and the ascending colon were passed into the surgical wound, and the serosa and the muscular layer of the intestinal wall were dissected longitudinally. Next, a tumor fragment was sutured above the dissection site. The intestine was placed back in the abdominal cavity, and the peritoneum and skin were sutured in layers using the glover's suture.

Orthotopic transplantation of the tumor samples into the descending colon. A 10-mm long incision was made in the skin and peritoneum with scissors at a distance of 7 mm from the base of the tail, retreating 3 mm from the spine to the right. Then, the rectum was exposed using anatomical forceps. Longitudinal dissection of the intestinal wall was performed at a distance of 5 mm from the site where the descending colon connects to the rectum. The human colon tumor tissue was ligated to the mouse colon. The peritoneum and skin of the mouse were sutured in layers.

Weight and general condition of the animals and the volume of the tumor nodules were monitored for 80 days after the surgery. The degree of engraftment and tumor growth in orthotopic and heterotopic xenograft models were assessed by control laparotomy on day 40, 60, and 80 after the transplantation. Successful xenotransplantation was evidenced by tumor growth to 1–1.5 cm3. The size of the tumor nodules was measured using a caliper.

After necropsy, fragments of the tumor material were fixed in 10% formalin for 24 hours, and at the processing stage, they were dehydrated and impregnated with paraffin. Then, the paraffin blocks and sections were prepared, mounted on glass slides, and stained with hematoxylin and eosin. Light microscopy was used for a histologic examination of the donor human tumor and heterotopic and orthotopic xenograft models. The Aperio Scan Scope XT slide scanner was used to obtain images of entire areas at 200x magnification.

A statistical analysis of the data was performed using the STATISTICA 10 software package. To assess

the significance of differences, the Mann – Whitney test was applied.

RESULTS AND DISCUSSION

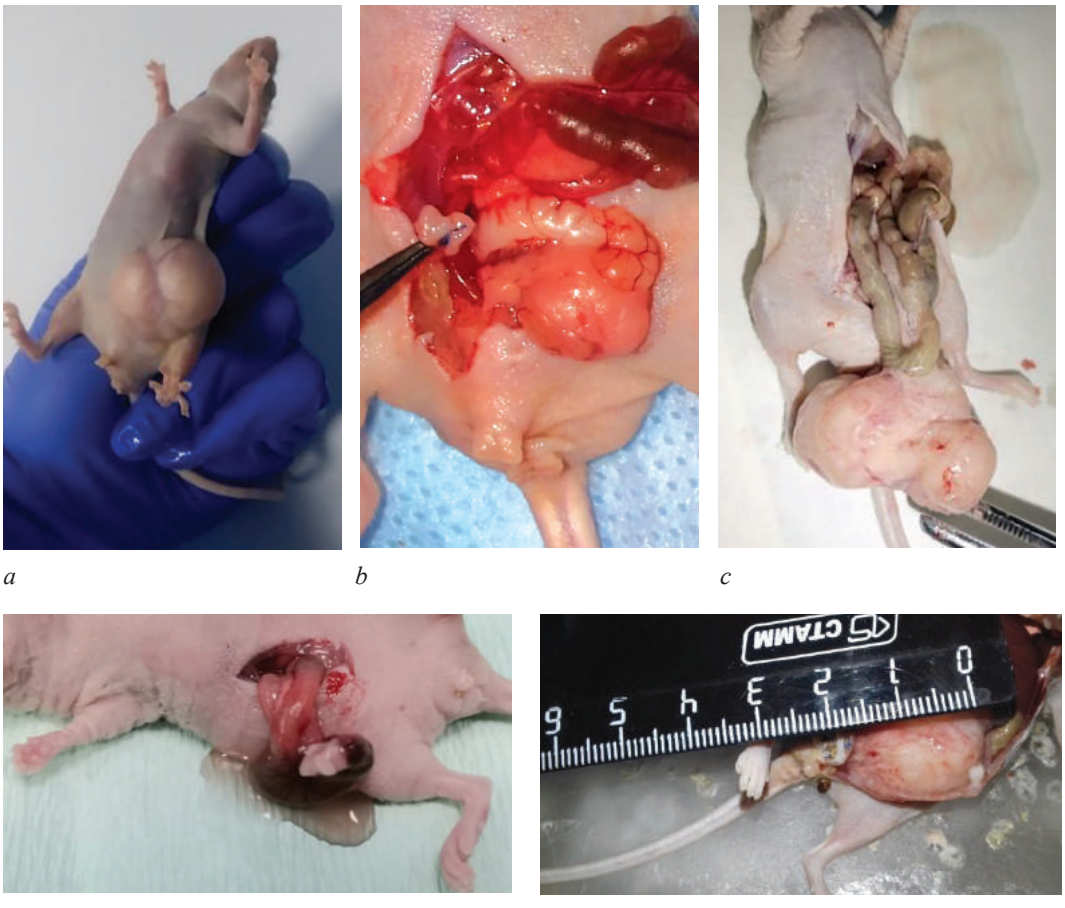
During the experiment, we transplanted the tumor material into immunodeficient mice to the following sites: under the skin and into the omentum (heterotopic transplantation), to the descending and ascending colons and to the cecum (orthotopic transplantation) (Fig.1). Depending on the site of transplantation, the animals were divided into 5 groups (3 females in each group). Throughout the experiment, the survival of the animals was 100%, and their condition was satisfactory. Growth of the engrafted PDX tumors was observed throughout the experiment.

12 of 15 heterotopic and orthotopic transplantation procedures resulted in creating a PDX model, i.e. the overall engraftment rate was 80%. Figure 2 demonstrates the growth dynamics of the tumor nodules in the heterotopic and orthotopic PDX models of human CRC. The statistical analysis using the Mann – Whitney test showed that the volumes of the tumor nodules in all 5 experimental groups of animals on day 40, 60, and 80 of the experiment differed significantly from the baseline volume of the transplanted donor tumor fragment. Therefore, a statistically significant increase in the heterotopic and orthotopic xenograft models was observed throughout the experiment.

A histologic analysis showed similar characteristics between different PDX models of CRC and original donor tumors. Tumor differentiation, necrosis, and stroma were assessed for each xenograft model and in 3–5 different samples of the original patient tumor. Microimages of the histologic preparations of the developed PDX models are shown in Fig.3.

Control laparotomy of the orthotopic xenograft model (transplantation into the ascending colon) on day 80 demonstrated liver metastasis (Fig. 4).

The table summarizes the main advantages and disadvantages observed when creating and using different variants of PDX models in preclinical studies.



a *b* *c*

d *e*

Fig. 1. Xenotransplantation of the patient's tumor material into immunodeficient mice using various sites: heterotopic transplantation (day 80 after the surgery): *a* – under the skin of the thigh, *e* – into the omentum; orthotopic transplantation (day 20 after the surgery): *b* – into the descending colon; *d* – into the cecum; *c* – orthotopic transplantation (day 80 after the surgery) into the ascending colon

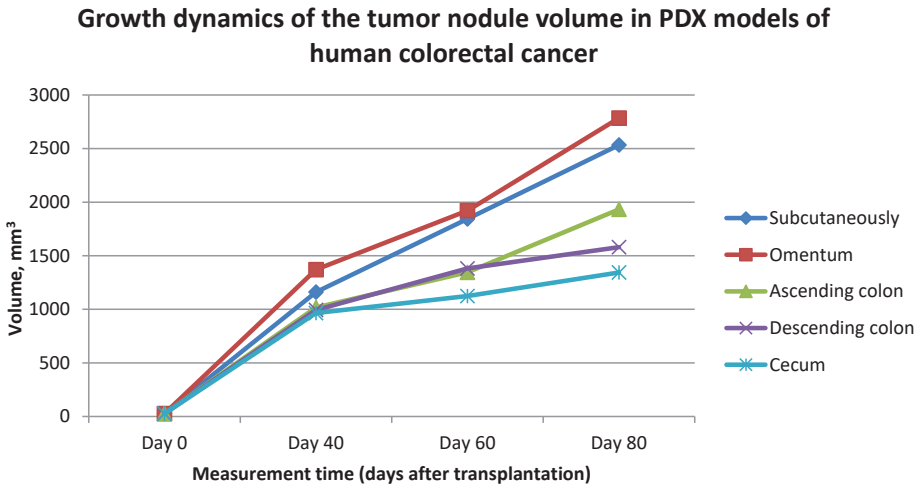


Fig. 2. Growth dynamics of tumor nodule volume in PDX models of human colorectal cancer, mm^3

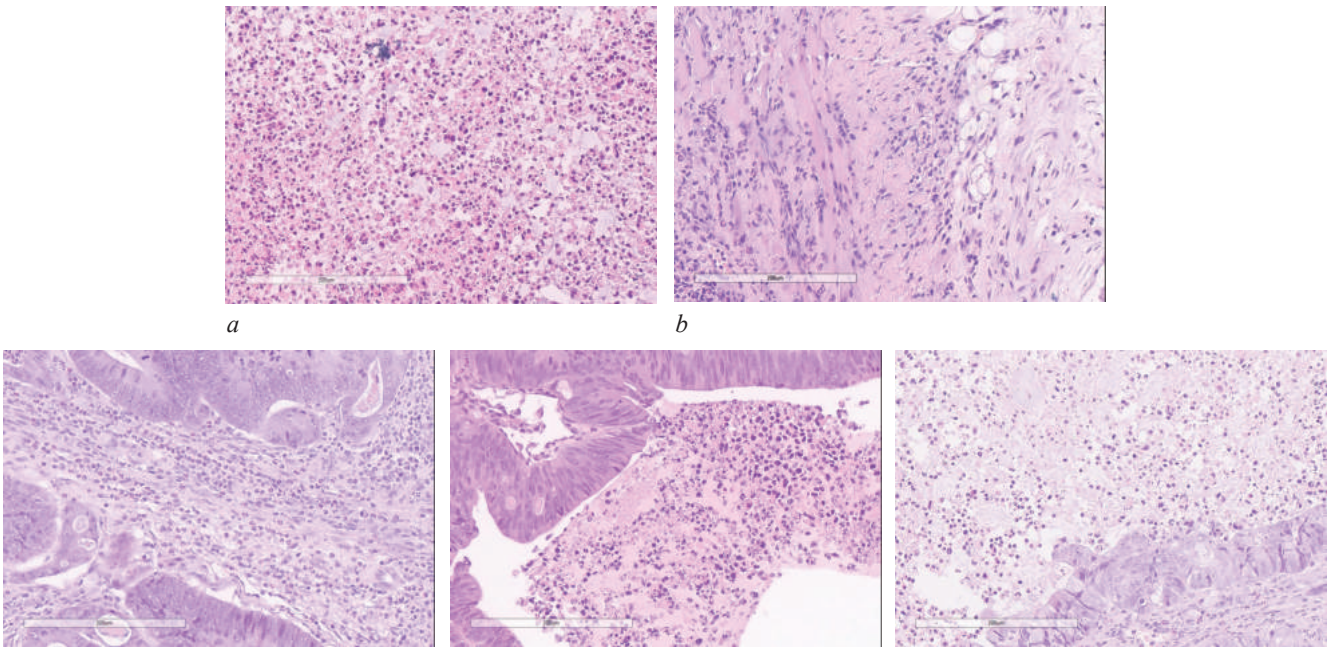


Fig. 3. Histologic preparations of the tumor material. Heterotopic transplantation of tumor xenograft model: *a* – under the skin, *b* – into the omentum, *c* – into the descending colon, *d* – into the ascending colon, *e* – into the caecum. Stained with hematoxylin and eosin, x200

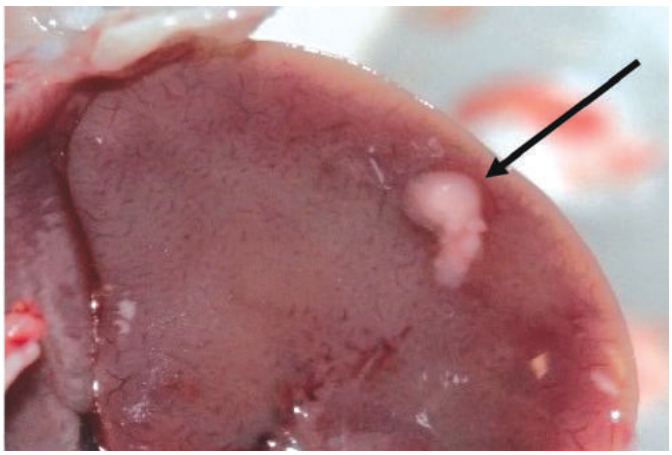


Fig. 4. Liver metastasis (marked with an arrow) during the control laparotomy of the orthotopic xenograft model on day 80 after the transplantation

Table

Advantages and disadvantages of creating different variants of PDX models of colorectal cancer

Type of PDX model transplantation	Characteristics of the transplantation site	Model advantages	Model disadvantages
Heterotopic	Created by transplanting a fragment of a human colon tumor under the skin of the thigh of an immunodeficient mouse	Simple and fast surgery. Easy to visually assess the dynamics of tumor growth. A wide variety of reproducible human tumors. Less traumatizing for the animal.	Replacement of tissue-specific tumor stroma. Microenvironment is not specific for the tumor. No metastasis.
	Created by transplanting a fragment of a human colon tumor into the omentum of an immunodeficient mouse	Good blood supply ensuring good trophism and engraftment	Microenvironment is not specific for the tumor. Complicated surgery. Complicated assessment of the tumor growth dynamics.
Orthotopic	Created by transplanting a fragment of a human colon tumor (mucinous adenocarcinoma) into the ascending colon and cecum of an immunodeficient mouse; created by transplanting a fragment of a human colon tumor (typical adenocarcinoma) into the descending colon of an immunodeficient mouse	Specific intraorgan microenvironment. Suitable for studying metastasis.	Complicated surgery. Complicated assessment of the tumor growth dynamics.

Heterotopic transplantation requires less time and efforts. The surgery is less traumatizing for the experimental animal. In our experiment, this transplantation type demonstrated 100% survival rate. Subcutaneous transplantation allows for easy monitoring of the tumor growth dynamics by measuring the tumor size with a caliper. The most significant disadvantage is a non-specific organ microenvironment resulting from tumor transplantation into a site not typical of the original patient's tumor.

Orthotopic transplantation into the intestinal wall requires significant technical skills and is quite traumatizing for the animal; therefore, this model has a limited capacity and reproducibility. It is worth noting that, when creating a xenograft model for the descending colon, it was still impossible to palpate the tumor and visually assess the results on days 40–80 of the experiment. During the control laparotomy, the access to the xenograft was complicated due to the descending colon location near the lumbosacral region of the spine.

The main advantage of orthotopic transplantation is a specific transplantation site inside the organ, which makes it possible to reproduce the features of the tumor microenvironment, thereby preserving the main molecular genetic signaling pathways.

The use of orthotopic and intraperitoneal xenograft models in studies on the effectiveness of drugs also requires certain technical facilities, since an assessment of tumor growth dynamics requires such imaging methods as magnetic resonance imaging (MRI) and computed tomography (CT).

Despite the required technical facilities, orthotopic PDX models can also be used as spontaneous metastasis models to evaluate the metastatic potential of a tumor and the effectiveness of anti-metastatic drugs, which is an important aspect in fundamental studies on biology of oncogenesis. It is important to take into account that rapid growth of an inoculated tumor orthotopically transplanted into the intestine of an immunodeficient mouse can lead to intestinal obstruction and death of the animal and, consequently, to forced termination of the experiment.

The experiment showed that the choice of a transplantation method in creating PDX models depends on many factors, such as the aim and objectives of the experiment, the quality and biological characteristics of the donor tumor material and model organism, technical facilities of the laboratory, staff training, funding, time frame, etc.

CONCLUSION

The study allows to conclude that, regardless of the site of donor tumor material transplantation into an immunodeficient mouse, xenograft models show a high engraftment rate and largely reproduce the morphological and histologic characteristics of the original human tumor. This determines the possible use of PDX models in searching for biomarkers and assessing the effectiveness of new anticancer drugs.

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Authors contribution

Kiblitskaya A.A., Egorov G.Y., Zaikina E.V., Lukbanova E.A., Volkova A.V. – analysis and interpretation of the data. Maksimov A.Y. – conception and design, final approval of the manuscript for publication. Goncharova A.S. – conception and design. Nepomnyashchaya E.M., Zlatnik E.Y. – substantiation of the manuscript and critical revision of the manuscript for important intellectual content.

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Calprotectin in the blood plasma as a new biomarker for assessing the activity of rheumatoid arthritis

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ABSTRACT

Aim. To study the potential use and information value of calprotectin in the blood plasma as a new biomarker for determining the activity of rheumatoid arthritis (RA).

Materials and methods. The study included 113 people. The treatment group consisted of 79 patients diagnosed with RA; the average age was 58 (± 11.66) years, the median duration of the disease was 10 [6; 15] years. The control group encompassed 34 healthy volunteers; the average age was 40 (± 11.14) years. RA activity was determined according to the Disease Activity Score (DAS) 28 and the Clinical Disease Activity Index (CDAI). The concentration of calprotectin in the blood plasma was determined by the solid-phase enzyme-linked immunosorbent assay. The obtained results were compared with laboratory and clinical parameters, as well as with composite indices (DAS28, CDAI) of RA activity. For mathematical data processing, Spearman's rank correlation coefficient, linear discriminant analysis, and ROC analysis were used.

Results. In the group of patients with RA, the level of calprotectin in the blood was higher than in the control group. A statistically significant relationship was revealed between the level of calprotectin in the blood and all standard parameters of RA activity. The ROC analysis showed that the sensitivity, specificity, and diagnostic accuracy in assessing articular syndrome, as well as moderate and high RA activity according to the composite indices DAS28 and CDAI were higher for calprotectin than for erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP). The linear discriminant analysis showed that a combination of ESR and calprotectin levels was the most informative; following it, the probability of correct classification of RA activity, according to the DAS28 index, was 71%. For the CDAI index, only one marker, calprotectin, resulted in a statistically significant classification with a probability of 70.5 %.

Conclusion. Calprotectin in the blood plasma is a promising laboratory biomarker for assessing synovitis activity in RA demonstrating higher accuracy, sensitivity, and specificity than traditional acute-phase reactants.

Keywords: rheumatoid arthritis, activity, calprotectin

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 Almazov National Medical Research Center of the Ministry of Health of the Russian Federation (Protocol No. 06-19 of 13.05.2019).

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Кальпротектин в плазме крови как новый биомаркер в оценке активности ревматоидного артрита

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РЕЗЮМЕ

Цель. Изучить возможность применения и информативность кальпротектина плазмы крови в качестве нового биомаркера для оценки активности ревматоидного артрита (РА).

Материалы и методы. В исследование включены 113 человек, 79 пациентов с диагнозом РА (основная группа); средний возраст 58 (± 11,66) лет, медиана длительности заболевания 10 [6; 15] лет. Группу контроля составили 34 здоровых добровольца; средний возраст 40 (± 11,14) лет. У пациентов с РА активность заболевания определялась по индексу DAS28 (Disease Activity Score), а также по клиническому индексу CDAI (Clinical Disease Activity Index). Концентрация кальпротектина в плазме крови определялась методом твердофазного иммуноферментного анализа. Полученные данные сопоставлялись с лабораторными и клиническими параметрами, а также композитными индексами (DAS28, CDAI) активности РА. Для математической обработки данных использовались ранговая корреляция по Спирмену, дискриминантный и ROC-анализы.

Результаты. В группе больных РА содержание кальпротектина в крови было более высоким по сравнению с контрольной группой. Выявлена значимая связь уровня кальпротектина крови со всеми параметрами активности РА. При проведении ROC-анализа диагностическая точность, чувствительность и специфичность уровня кальпротектина в плазме крови были выше для оценки суставного синдрома, а также композитных индексов CDAI и DAS28 в сравнении с содержанием скорости оседания эритроцитов (СОЭ) и С-реактивного белка (СРБ). По данным дискриминантного анализа, наиболее информативным оказалось сочетание уровней СОЭ и кальпротектина, для которых вероятность правильной классификации активности РА, согласно индексу DAS28, составила 71%. Для индекса CDAI статистически значимую классификацию давал только кальпротектин с вероятностью 70,5%.

Заключение. Кальпротектин плазмы крови – перспективный лабораторный биомаркер в оценке активности синовита при РА, демонстрирующий более высокую информативность, чем традиционные острофазовые показатели.

Ключевые слова: ревматоидный артрит, активность, кальпротектин

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

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INTRODUCTION

Rheumatoid arthritis (RA) is the most common human systemic autoimmune disease, where inflammation mainly targets peripheral joints with development of erosions and destructive changes in them. Currently, determining RA activity is an important clinical task and a time-consuming and lengthy process [1]. In order to determine RA activity, a number of clinical and laboratory evaluation parameters are used, such as the tender joint count (TJC) and swollen joint count (SJC), acute-phase reactants (erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP)), as well as subjective assessments by the patient (patient global assessment (PGA), the visual analogue scale (VAS)) and the physician (physician global assessment (PhGA)) [2, 3].

Since these parameters separately do not fully reflect RA activity, composite indices are currently widely used for assessing the disease activity, such as Disease Activity Score (DAS) 28 and Clinical Disease Activity Index (CDAI). However, the conducted studies show that indices do not always allow researchers to reliably determine disease activity. According to the studied composite indices, in some patients with RA in remission, X-ray detected disease progression. The information value of traditional acute-phase reactants is insufficient, and composite indices are difficult to use and subjective to a certain extent, which makes it relevant to search for and introduce into clinical practice new biomarkers that accurately reflect immunological and inflammatory processes in the joints.

Calprotectin is a heterodimeric complex protein consisting of two subunits (S100A8 / S100A9) with the molecular mass of 36 kDa and Ca2+ / Zn2+ binding sites. Currently, this marker is used in gastroenterology to assess inflammatory infiltrates in the intestine in patients with inflammatory diseases [6]. This biomarker is an alarmin with proinflammatory properties [7]. Earlier studies on the new biomarker suggest that it can be quite effective in assessing RA activity [8–15].

The aim of this study was to investigate the potential use and information value of calprotectin in the blood plasma as a new biomarker for determining the activity of RA.

MATERIALS AND METHODS

We examined 113 people. The treatment group included 79 patients diagnosed with RA, and the control group encompassed 34 healthy volunteers. The inclusion criterion was compliance of patients with RA of any disease activity, regardless of the chosen treatment, with the criteria of the American College of Rheumatology / the European League Against Rheumatism (ACR / EULAR) published in 2010 [16]. The exclusion criteria were patient's refusal to participate in the study; the presence of active infection; cancer during the period of the study; other autoimmune diseases, except for secondary Sjogren's syndrome; decompensated chronic non-communicable diseases; pregnancy and lactation.

Upon admission, all patients were tested for the articular syndrome using TJC, SJC, VAS, PGA, and PhGA. RA activity was assessed using composite indices, such as DAS28-ESR and CDAI. Laboratory tests included an analysis of acute-phase reactants for ESR and CRP, rheumatoid factor (RF), and cyclic citrullinated peptide antibodies (anti-CCP). The level of plasma calprotectin was measured with the enzyme-linked immunosorbent assay (ELISA). A reagent kit for the analysis was developed by the staff of Sechenov Institute of Evolutionary Physiology and Biochemistry of the Russian Academy of Sciences. The kit included biotin-conjugated protein antibodies derived from rats. ELISA was conducted in accordance with standard protocols on the multi-mode microplate reader CLARIOstar Plus (BMG LABTECH, Germany).

Gastrointestinal pathology is detected in 13–62% of patients with RA and is one of the main extra-articular manifestations of this disease. Associated gastrointestinal lesion in patients with RA can significantly affect the concentration of both fecal and plasma calprotectin. Therefore, we thoroughly collected complaints and anamnestic data regarding the gastrointestinal pathology in all patients participating in this study and assessed the presence of symptoms for intestinal and extraintestinal manifestations of possible inflammatory bowel diseases. All patients with RA who participated in this study underwent esophagogastroduodenoscopy (EGD) during hospitalization as part of an inpatient examination. Colonoscopy (CS) was not a mandatory examination for the participation in this study in the absence of indications for it.

The results obtained were analyzed using Prism 8.0 and Statistica 12.0 software packages. The normality test was used to determine if a data set was well-modeled by a normal distribution. In the absence of normal distribution, the Mann – Whitney *U*–test and the Spearman's rank correlation coefficient were used. The differences between the groups were considered significant at $p < 0.05$. ROC analysis was performed; we calculated the area under the curve (AUC) and marker sensitivity, specificity, and diagnostic accura-

cy. A discriminant analysis was used to determine the differences between the new biomarker and traditional acute-phase reactants.

RESULTS

The average age of the patients with RA was 58 ± 11.66 years, this group included 11 men and 68 women; the control group included 15 men and 19 women whose average age was 40 ± 11.14 years. The demographic characteristics of the groups did not differ significantly. In the group of patients with RA, the median duration of the disease was 10 years [6; 15]. In this group, a total of 68 people (86%) were tested positive for anti-CCP, and 66 patients (83.5%) were tested positive for RF. The patients in the treatment group were distributed according to the degree of RA activity based on the studied indices (DAS28, CDAI). The clinical characteristics of patients with RA and laboratory data of the examined groups are presented in Tables 1 and 2.

Table 1

Clinical, laboratory, and demographic data of patients in the treatment group

Parameter	Value
Age, years, $M \pm SD$	58 (± 11.66)
Disease duration, years, $Me[Q_1; Q_3]$	10 [6; 15]
Male / female ratio, *n*	11 / 68
TJC, $Me\ [Q_1; Q_3]$	10 [6; 18]
SJC, $Me\ [Q_1; Q_3]$	4 [2; 6]
VAS, score, $Me\ [Q_1; Q_3]$	5 [4; 6.5]
DAS28 index, score, $Me\ [Q_1; Q_3]$	5.1 [4.38; 6.11]
DAS28–ESR activity, *n* (%):	
remission	5 (6.3)
low	4 (5)
moderate	29 (36.8)
high	41 (51.9)
CDAI activity, $Me\ [Q_1; Q_3]$	23.5 [16; 30]
remission	4 (5.06)
low	4 (5.06)
moderate	30 (37.97)
high	41 (51.89)
Steinbrocker staging of the disease, n:	
I	2
II	33
III	25
IV	19

Table 2

The comparison of acute-phase reactants in patients of the studied groups, $Me\ [Q_1; Q_3]$

Parameter	Control group	RA patients	*p*
ESR, mm / h	9 [5.5; 12.5]	29 [18; 51]	< 0.05
CRP, mg / l	2 [1.2; 2.1]	7.9 [2.5; 17.5]	< 0.005

The calprotectin level was significantly higher in patients with RA compared with the control group (Fig.1). According to the Mann – Whitney test, the differences in biomarker concentrations in both groups were highly statistically significant ($p < 0.0001$). It should be mentioned that calprotectin levels of four patients did not correspond to the calprotectin level range in the treatment group. A detailed analysis of the results showed that these RA patients were in remission. Remission was detected by a lower plasma calprotectin concentration.

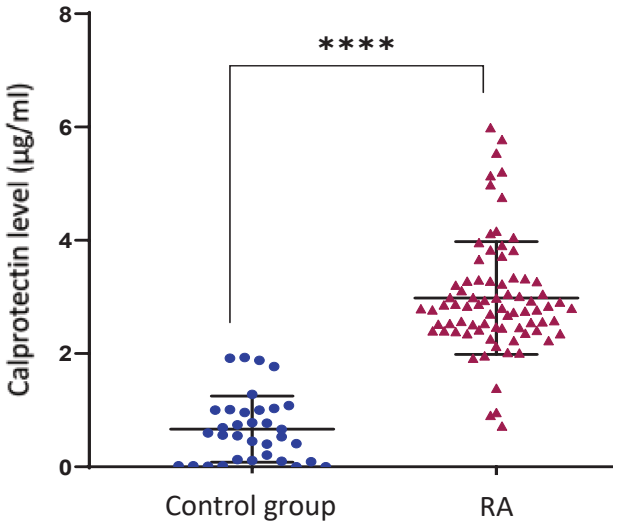


Fig. 1. Comparison of blood calprotectin levels in RA patients and the control group. $Me\ [Q_1; Q_3]$, $p < 0.0001$ (****)

With the Spearman's rank correlation coefficient, a statistically significant correlation was revealed between plasma calprotectin concentration and all components of RA activity. However, the correlation between the new biomarker and the articular component as well as composite indices was more statistically significant, while the correlation between traditional acute-phase reactants and the mentioned parameters was less statistically significant. The rank correlation coefficients are presented in Table 3.

Since DAS28 includes three main domains, including one of the acute-phase reactants (ESR / CRP), in order to exclude the markers that could affect test results, we used CDAI index containing only two domains in subsequent statistical tests.

To conduct ROC analysis, RA patients were divided into 2 groups depending on the articular syndrome. The first group consisted of patients who had TJC ≤ 8 and SJC ≤ 1. The second group included patients with TJC > 8 and SJC > 1. ROC curve plots are shown in

Figures 2 and 3. According to the assessment of TJC, calprotectin had the greatest sensitivity, specificity, and diagnostic accuracy, while ESR and CRP were characterized by the lowest values (Fig. 2*a*). The calprotectin level with higher diagnostic accuracy, sensitivity, and specificity reflected the association with SJC if compared with ESR and CRP (Fig. 2*b*). The data on the test are given in Table 4.

Table 3

Spearman's rank correlation coefficients for calprotectin and acute-phase reactants with clinical data, VAS, as well as DAS28 and CDAI indices

Parameter	Calprotectin	ESR	CRP	TJC	SJC	VAS	CDAI	DAS28
Calprotectin	–	0.316*	0.198	0.441*	0.227*	0.310*	0.419*	0.494*
ESR	0.316*	–	0.651*	0.143	0.124	0.282*	0.236*	0.597*
CRP	0.198	0.651*	–	0.078	0.113	0.191	0.158	0.443*
TJC	0.441*	0.143	0.072	–	0.559*	0.414*	0.838*	0.702*
SJC	0.227*	0.125	0.113	0.559*	–	0.324*	0.715*	0.639*
VAS	0.310*	0.282*	0.191	0.414*	0.324*	–	0.582*	0.597*
CDAI	0.419*	0.236*	0.158	0.838*	0.715*	0.582*	–	0.875*
DAS28	0.494*	0.597*	0.443*	0.702*	0.637*	0.597*	0.875*	–

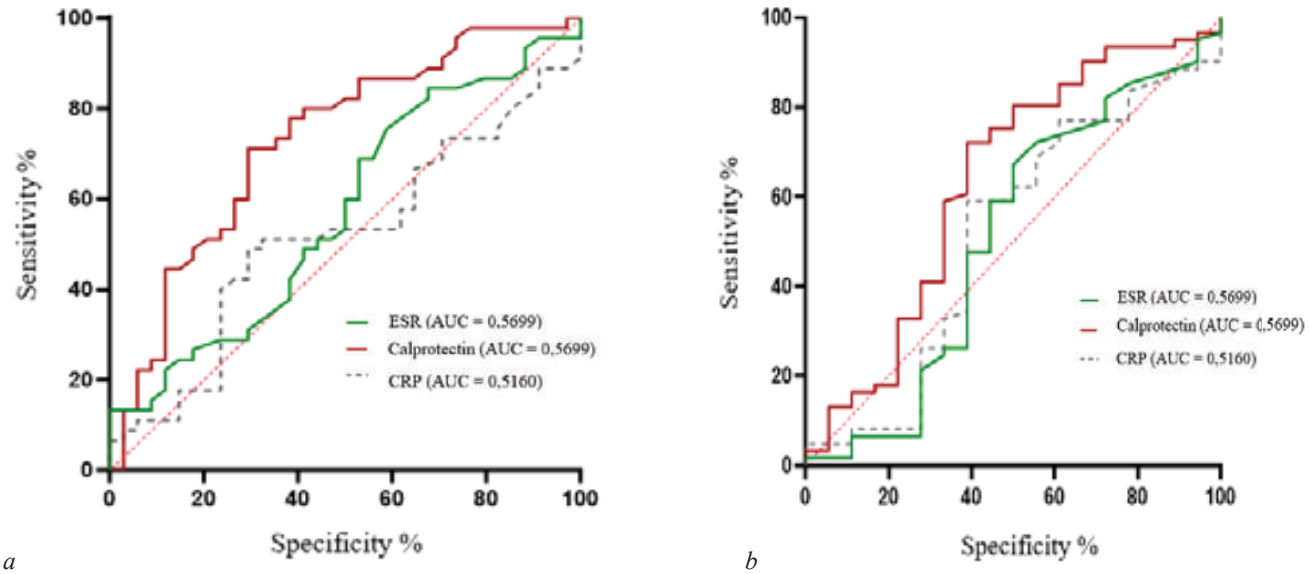
* values with $p < 0.05$.

Table 4

ROC analysis parameters for calprotectin compared with acute-phase reactants in the assessment of articular syndrome

Parameter	Calprotectin		ESR		CRP	
	TJC	SJC	TJC	SJC	TJC	SJC
Threshold	2.78	2.55	19.5	21	9.8	6
Sensitivity	71.11	72.13	75.56	67.21	48.89	59.02
Specificity	70.59	61.11	41.18	50.00	70.59	61.11
Diagnostic accuracy	70.85	66.62	58.37	58, 6	59.74	60.06
Area under the curve	0.73	0.63	0.57	0.51	0.52	0.51
LB	0.61	0.47	0.44	0.34	0.39	0.35
UB	0.84	0.79	0.70	0.68	0.65	0.68
p	0.0005	0.0868	0.2894	0.9162	0.8083	0.8562

Note: TJC is the tender joint count; SJC is the swollen joint count. LB is the lower bound and UB is the upper bound of the 95% confidence interval.



a *b*

Fig. 2. ROC curves for the correlation between laboratory markers and RA activity: *a* – with TJC; *b* – with SJC

b

When determining the correlation between the studied markers and VAS, we found no differences between the parameters for calprotectin (AUC = 0.63, $p = 0.16$), ESR (AUC = 0.64, $p = 0.14$) and CRP (AUC = 0.56, $p = 0.57$), but the values obtained were not statistically significant ($p > 0.05$).

ROC analysis was conducted to assess the correlation between all markers and the degree of RA activity determined by CDAI. Taking into account the predominance of moderate and high RA activity, we included patients in remission and those with low and moderate RA activity in one group (CDAI < 22) and compared it with patients with high RA activity (CDAI > 22). The results of the analysis showed that calprotectin levels had higher diagnostic accuracy, sensitivity, and specificity (ACC = 73.04) compared with ESR (ACC = 65.82) and CRP (ACC = 61.32) (Fig. 3).

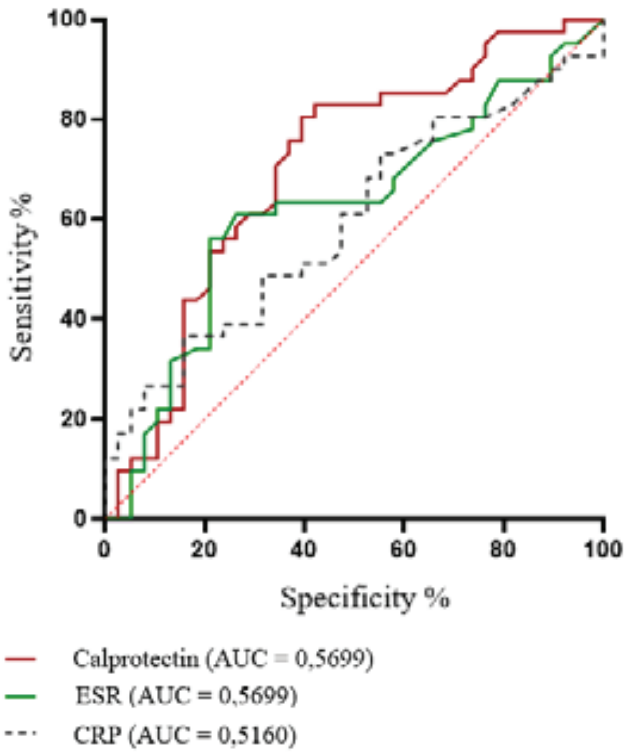


Fig. 3. ROC curves for all markers depending on the disease activity (CDAI >22)

The ROC analysis showed that the sensitivity, specificity, and diagnostic accuracy in assessing articular syndrome, as well as moderate and high RA activity according to the CDAI were higher for calprotectin than for traditional acute-phase reactants.

When the discriminant analysis was conducted, patients in remission and those with low RA activity according to DAS28 and CDAI were grouped together. According to the test results, only the combined use of ESR and calprotectin values was informative; following it, the probability of correct classification of RA activity, according to the DAS28 index, was 71%. According to the CDAI index, only calprotectin resulted in a statistically significant classification with a probability of 70.5 %.

Thus, according to a multivariate statistical analysis of data on the level of new RA activity biomarker, the diagnostic value of calprotectin not only remained competitive, but also showed better results.

According to the results of EGD, 38% of patients with RA showed signs of chronic gastritis without any signs of active inflammation. Gastric ulcer was visualized in 21% of patients, and duodenal ulcer was visualized in 8% of patients. According to EGD, in 1% of patients of the treatment group, gastric epithelial polyps were detected without atypical histologic features. According to EGD, the remaining 32% of patients had intact stomach and initial sections of the duodenum. During the period of hospitalization in the rheumatology department, none of the patients had indications for colonoscopy.

DISCUSSION

We identified statistically significant differences in the calprotectin level in patients with RA and healthy volunteers, since calprotectin is a proinflammatory cytokine involved in the development of immunological and inflammatory processes in RA. Calprotectin plays an important role in inflammation, as it interacts with the receptors of innate immunity, therefore, it was identified as a marker associated with RA [8, 9].

In our study, the calprotectin level correlated with the degree of RA activity, but especially close relationships were observed with the articular component of composite indices, as opposed to ESR and CRP. Our findings are confirmed by literature, where the study of local and systemic calprotectin production showed a statistically significant correlation of the marker level with the intensity of local inflammation, as well as with the clinical parameters of RA activity [10–15]. A meta-analysis conducted by S.-C. Bae et al. also showed that serum and synovial fluid calprotectin level correlated with RA activity [11]. Studies by H.B. Hammer et al. and J. Hurnakova et al. indicated that, based on ultrasound, serum calprotectin level correlated with synovitis, which did not correlate with the levels of ESR and CRP in patients with RA [12, 13]. Indeed, unlike CRP, calprotectin is produced locally and is not produced by hepatocytes in response to

stimulation by inflammatory cytokines, which makes it more informative and reliable [7–9, 15].

According to the conducted clinical and laboratory comparisons of data in ROC analysis, the level of the new biomarker showed the best results in assessing the articular syndrome and moderate and high disease activity, according to CDAI. According to the results of a large SONAR study, it was noted that the concentration of serum calprotectin was associated with SJC, as well as with CDAI. The biomarker level was statistically different in patients with different degrees of disease activity, according to DAS28, which weakly correlated with CRP. The multivariate analysis showed that calprotectin was independently associated with ultrasound parameters of synovitis, unlike CRP.

According to ROC analysis and DAS28, calprotectin turned out to be a more accurate marker in assessing RA activity in contrast to CRP, with AUC of 0.8 and 0.71 [14]. Thus, the authors of the study suggest that serum calprotectin levels accurately reflect the degree of local inflammation. These observations were confirmed by a prospective study, the results of which revealed the association between serum calprotectin level and RA activity according to DAS28 index in patients with normal CRP levels.

CONCLUSION

Plasma calprotectin level is a promising laboratory biomarker for assessing synovitis activity in RA demonstrating higher accuracy, sensitivity, and specificity than traditional acute-phase reactants.

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Authors contribution

Korolkova A.A. – database formation, analysis and interpretation of the results obtained, drafting of the manuscript. Khizha V.V. – laboratory research, database formation. Kozlova D.I. – organization of laboratory research, database formation, analysis and interpretation of the data. Maslyansky A.L. – conception and design, substantiation of the manuscript, critical revision of the manuscript for important intellectual content, final approval of the manuscript for publication. Vavilova T.V. – substantiation of the manuscript, critical revision of the manuscript for important intellectual content, final approval of the manuscript for publication.

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Features of the functioning of innate immunity in children with chronic idiopathic urticaria

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ABSTRACT

Aim. To study the features of the functioning of innate immunity in children with chronic idiopathic urticaria.

Materials and methods. The study included 28 children of both sexes aged 6–16 years with chronic idiopathic urticaria (CIU). The median age of the patients was 8 years (p = 0.045). Clinical research methods included an analysis of complaints and anamnestic data, as well as an objective examination of the child (dynamics of urticaria, severity of itching, the presence of angioedema). Immunological techniques included determination of the number of monocytes expressing $CD14^+CD282^+$, $CD14^+CD284^+$, $CD14^+CD289^+$, the number of peripheral blood lymphocytes expressing $CD3^+CD16^+$, the levels of immunoglobulin (Ig) E, lactoferrin, interferon (IFN) γ, interleukin (IL)-4, and IL-6, and a nitroblue tetrazolium test.

Results. In the course of the study, an increase in the expression of Toll-like receptors TLR2 and TLR4 by monocytes, a decrease in the expression of TLR9 by monocytes, a significant rise in lactoferrin levels, a slight decrease in the number of natural killer (NK) cells, a decrease in microbicidal activity and adaptive reserves, a rise in IgE levels, a decrease in IL-4 levels, and an increase in IFNγ and IL-6 were revealed in children with CIU.

Conclusion. The immunological changes revealed during the study indicate multidirectional expression of Toll-like receptors, disturbances in the work of the cellular components of innate immunity, and a launch of a proinflammatory cytokine cascade in children with CIU, which can serve as a mainstay for the development of new schemes for personalized therapy of CIU in children.

Keywords: chronic idiopathic urticaria, children, innate immunity

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

Source of financing. The authors state that they received no funding for the study.

Conformity with the principles of ethics. All study participants or their parents signed an informed consent. The study was approved by the local Ethics Committee at RostSMU (Protocol No. 10/21 of 27.05.2021).

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Особенности функционирования врожденного иммунитета у детей с хронической крапивницей

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РЕЗЮМЕ

Цель. Изучить особенности функционирования врожденного иммунитета у детей с хронической крапивницей (ХК).

Материалы и методы. В исследование включены 28 детей обоих полов в возрасте от 6 до 16 лет с хронической идиопатической (спонтанной) крапивницей. Медиана возраста – 8 лет (p = 0,045). Клинические методы исследования: анализ жалоб, анамнестических данных, объективный осмотр ребенка (динамика уртикариев, выраженности кожного зуда, наличие ангиоотеков). Иммунологические методы исследования: определение количества моноцитов, экспрессирующих $CD14^+CD282^+$, $CD14^+CD284^+$, $CD14^+CD289^+$, количества лимфоцитов периферической крови, экспрессирующих $CD3^+CD16^+$, содержания иммуноглобулина Е, уровня лактоферрина, интерферона (IFN) γ, интерлейкина (IL) 4 и 6, проведение теста с нитросиним тетразолием.

Результаты. В ходе исследования у детей с ХК выявлено повышение экспрессии моноцитами Toll-рецепторов TLR2 и TLR4, подавление экспрессии моноцитами TLR9, значительное увеличение уровня лактоферрина, незначительное сокращение количества NK-клеток, снижение микробицидной активности и адаптационных резервов, повышение уровня IgE, IFNγ и IL-6, уменьшение уровня IL-4.

Заключение. Выявленные в ходе исследования иммунологические изменения свидетельствуют о разнонаправленной экспрессии Toll-рецепторов, нарушениях в работе клеточного звена врожденного иммунитета, запуске цитокинового каскада воспаления у детей с хронической идиопатической крапивницей, что может послужить основой для разработки новых схем персонифицированной терапии ХК у детей.

Ключевые слова: хроническая крапивница, дети, врожденный иммунитет

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 участники исследования или их родители подписали добровольное информированное согласие. Исследование одобрено локальным независимым этическим комитетом РостГМУ (протокол № 10/21 от 27.05.2021).

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INTRODUCTION

Chronic urticaria (CU) is a current problem in pediatrics due to increasing incidence, frequently developing resistance to treatment methods, and negative impact on children's and adolescents' quality of life (impaired sleep, poor school performance) [1, 2]. The prevalence of CU in the general population varies between 0.1 and 1.5% [3, 4]; according to different researchers, it may increase up to 3% among children and adolescents [5–8].

It is known that CU is characterized by spontaneously developing blisters and / or angioedema persisting for more than 6 weeks [9]. Clinical manifestations of CU are similar in all patients, while the pathogenesis of the disease is varied. However, the leading pathogenetic factor in half of the cases cannot be determined, and such CU is considered to be idiopathic or spontaneous [10, 11].

Since the role of innate immunity in the pathogenesis of urticaria is poorly understood, it seems promising to study the features of its functioning in children with chronic urticaria [12]. At the same time, treatment efficacy is predominantly determined by the possibility of conducting pathogenetically grounded, targeted therapy, which becomes truly effective only when the mechanisms of CU are revealed [13, 14].

The aim of the study was to investigate the features of the functioning of innate immunity in children with chronic idiopathic urticaria (CIU).

MATERIALS AND METHODS

Twenty-eight children of both sexes aged 6–16 years with CIU were examined. The median age was 8 years (p = 0.045). Inclusion criteria were clinical manifestations of urticaria (skin rash, itching, angioedema) lasting more than 6 weeks. Exclusion

criteria were signs of an acute infectious disease, signs of chronic disease exacerbation, a positive autologous serum skin test, elevated serum levels of thyroid peroxidase antibodies, and treatment with systemic glucocorticoids within one month before the examination. The control group included 30 almost healthy boys and girls aged 6–16 years (median age was 9 years) without chronic diseases. The patients were examined on the first day of admission before the initiation of inpatient therapy.

The studies were carried out according to the principles of the Declaration of Helsinki developed by the World Medical Association (WMA) "Ethical principles for medical research involving human subjects" amended by the 52nd WMA General Assembly (2000) and "Principles of Clinical Practice in the Russian Federation", approved by the Order of the Ministry of Health of Russia No.266 of 19.06.2003.

Clinical research methods included an analysis of complaints and anamnestic data, as well as an objective examination of the child (dynamics of rash, severity of itching, the presence of angioedema). Immunological techniques included determining the number of monocytes expressing $CD14^+CD282^+$, $CD14^+CD284^+$, $CD14^+CD289^+$ by flow cytometry with monoclonal antibodies (Caltag Laboratories, USA), determining the number of peripheral blood lymphocytes expressing $CD3^+CD16^+$ using the FC500 laser flow cytometer (Beckman Coulter Inc., USA), measuring the levels of immunoglobulin (Ig) E by the Manchi technique (Microgen, Russia), determining the levels of lactoferrin, interferon (IFN) γ, interleukin (IL)-4, and IL-6 (Cytokine, Russia) by enzyme immunoassay, and performing a nitroblue tetrazolium test (DIA-M, Russia). All laboratory tests were performed once. An informed consent was obtained from parents of children under 15 years and from adolescents 15 years of age and older to conduct clinical research and draw blood from veins.

For quantitative variables, the data were presented as the median and the interquartile range *Me* (Q_1; Q_3), maximum and minimum values *Min; Max*. The Mann – Whitney test was used to compare the medians in the groups. The differences were considered statistically significant at $p < 0.05$. All calculations were performed using R Foundation for Statistical Computing 3.2 software (Austria).

It was found that all children in the experimental group had typical presentation of chronic urticaria exacerbation (rash and itching), in 12 children (42.9%), urticaria was accompanied by angioedema.

RESULTS

The results of studying innate immunity parameters in children with CIU and in the control group are presented in the Table.

Table

Comparison of quantitative parameters of innate immunity in children with CIU and in the control group

Parameter	CIU, *n* = 28		Control group, *n* = 30		*p* (Mann –Whitney test)
	Me (Q_1; Q_3)	*Min; Max*	*Me* (Q_1; Q_3)	*Min; Max*	
$CD14^+CD282^+$, %	38 (26; 45.5)	18; 88	28.5 (27; 32)	22; 34	0.003
$CD14^+CD284^+$, %	45.5 (39; 52.5)	24; 60	7 (5; 8)	5; 9	< 0.001
$CD14^+CD289^+$, %	1.7 (1.5; 1.9)	1.2; 2.6	30.3 (26.8; 33.2)	22.3; 36.5	< 0.001
Lactoferrin, ng / ml	4,378 (2,189; 5,004)	1,087; 6,581	985 (750; 1,084)	582; 1,158	< 0.001
$CD3^+CD16^+$, %	8 (5; 11)	4; 7	10 (7; 13)	5; 16	0.062
IgE, g / l	173 (104; 197)	10.9; 477	45 (38; 75)	34; 88	< 0.001
Spontaneous nitroblue tetrazolium test (NBT), c.u.	96 (84; 112)	67; 167	102 (98; 105)	89; 112	0.077
Stimulated NBT-test, c.u.	143 (114; 162)	87; 200	180 (174; 192)	172; 203	< 0.001
Stimulation coefficient, units	1.3 (1.2; 1.7)	1.2; 2.4	1.8 (1.75; 1.9)	1.7; 1.96	< 0.001
IFN γ, pg / ml	16 (11.1; 18)	8.9; 23.3	5.8 (5.29; 7.5)	4.25; 8.7	< 0.001
IL-4, pg / ml	2.08 (1.9; 2.48)	1.1; 2.8	5.3 (4.2; 11.3)	3.8; 13.7	< 0.001
IL-6, pg / ml	9.89 (2.83; 48.7)	1.71; 67.5	4.98 (4.12; 5.35)	3.5; 5.72	0.154

In children with CIU, we revealed a statistically significant increase in TLR4 expression by monocytes by more than 6 times compared with the control group and extremely low values of TLR9 expression by monocytes – a decrease by almost 18 times. At the same time, the level of TLR2 expression by monocytes was increased by 1.3 times compared with the control group. In the group of children with CIU, a 4.5-fold rise in the level of lactoferrin was recorded compared with the control group. The number of natural killer (NK) cells in the blood of children with CIU was reduced by 1.3 times compared with the control

group. The median of total serum IgE in children with CIU was 3.8 times higher than in the control group. Children with CIU showed a decrease in the values of spontaneous NBT test associated with reduced adaptive reserves of phagocytes combined with decreased values of stimulated NBT test, which was manifested through a significantly low stimulation coefficient. The level of IFNγ was elevated by 2.8 times compared with the control values. At the same time, the content of IL-4 was reduced by more than 2 times compared with the control group. The level of IL-6 exceeded the control values by 2 times, although it was not statistically significant.

DISCUSSION

Studies describing the role of Toll-like receptors (TLRs) in the pathogenesis of CIU published by foreign researchers are few [15]. In our study, we noted an increase in TLR2 and TLR4 expression by monocytes in children with CIU, which is in line with the data published by Russian authors. So, E.V. Sorokina et al. (2020) found that the TLR2 and TLR4 levels were 6 and 4 times higher, respectively, in patients with CIU lasting at least 6 months than in the group of healthy individuals, while the duration of the disease did not affect TLR9 expression [16]. Our study showed a decrease in expression of TLR9 by monocytes in children with CIU compared with the control group. Similar results were obtained in the comparative study published by foreign authors who revealed functional failure of TLR9 expressed by dendritic cells in patients with CU compared with the control group [17].

The properties of serum lactoferrin as one of the transferrin family proteins involved in transport and metabolism of iron are fully described in the literature [18]. Due to this unique ability, lactoferrin exerts its antioxidant and membrane stabilizing effects [19]. We did not find available literature on the contribution of lactoferrin to the pathogenesis of CIU. In our study, a significant increase in the lactoferrin level in children with CIU may be explained by its anti-inflammatory effect – by iron binding in the focus of inflammation, which eventually reduces cell damage by oxygen free radicals and decreases the intensity of inflammation.

Currently, NK cells, in addition to their direct cytolytic effects, are known to exert a regulatory effect on immune responses through secretion of cytokines, chemokines, and growth factors [20]. We did not find any information on the contribution of NK cells to the mechanism of CIU development in the available literature. Pathological presentation of urticaria is characterized by a perivascular infiltrate consisting of lymphocytes, monocytes, mast cells, eosinophils, and neutrophils [21]. A slight decrease in the number of NK cells in the blood of children with CIU in our study may be due to their migration to the focus of inflammation.

We did not find information about the role of phagocytosis in the genesis of CIU in the literature. There are a few works devoted to the study of phagocytosis in allergic diseases. Thus, the study by E.G. Moiseeva et al. (2005) showed a decrease in the intensity of phagocytosis in allergic inflammation, which is regarded as a stage of its depletion [22]. In our study, we revealed low microbicidal activity and a decrease in adaptive reserves in children with CIU, which also characterizes diminishment of phagocytosis.

Determination of IgE level in various variants of urticaria development has always been of great interest for researchers. Thus, A. Kessel et al. (2010) revealed an increase in the level of IgE in patients with CU compared with the control group [23]. In a comparative study, K.L.Chang et al. (2011) found that in children with CU, an increase in IgE levels was not as intense as in patients with acute urticaria [24]. Besides, increased IgE values were found in patients who had CIU with concomitant atopic diseases [16]. The results of our study on determining the level of IgE in children with CIU comply with the results of the works discussed above.

Changes in the cytokine profile play an important role in the pathogenesis of somatic symptom disorders, including the pathogenesis of CIU. IL-4 is often called an anti-inflammatory cytokine, as it is able to suppress the immunostimulatory effect of Th1 and reduce TNF and IFNγ synthesis. However, according to A.S. Simbirtsev (2021), IL-4 is a typical proinflammatory and proallergic cytokine with a wide spectrum of biological activity, which is involved in the functioning of innate and adaptive immunity [25]. The decrease in the serum IL-4 level in children with CIU revealed in our study demonstrates possible reduction or suppression of synthesis of this cytokine.

The role of IL-6 in severe CU and its direct relationship with acute-phase proteins and the fibrinolytic system are shown in the study by A. Kasperska – Zajac (2011) [26]. Other foreign researchers also consider that IL-6 plays one of the key roles in immune and inflammatory responses and can be a biomarker of CU activity [27–29]. The role of IFNγ in the patho-

genesis of CU was also confirmed by foreign scientists. Thus, a positive relationship was found of basophil activation and an increase in TLR4 expression on mast cells with an increase in the serum IFNγ level in CIU [30]. The results of our study also revealed overproduction of proinflammatory cytokines IFNγ and IL-6 in children with CIU, which, in our opinion, can be considered not only as a marker of the inflammatory response intensity, but also as a predictor of CIU in children.

CONCLUSION

The immunological changes revealed in the study indicate multidirectional expression of TLRs, disturbances in the work of cellular components of innate immunity, and a launch of a proinflammatory cytokine cascade in children with CIU. This, in turn, causes activation of regulatory components of inflammation and adaptive defense mechanisms of the body, which results in a chronic course of idiopathic urticaria. An integrated approach to the study of innate immunity will provide a more complete understanding of the immunopathogenesis of CU and may serve as a mainstay for the development of new targeted treatment strategies for this disease in children.

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Authors contribution

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The effect of age and a high-fat, high-carbohydrate diet on the development of arterial hypertension and kidney disease in the experiment

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ABSTRACT

Aim. To identify the structural foundations of the pathogenesis of arterial hypertension and kidney disease associated with a high-fat, high-carbohydrate diet and age.

Materials and methods. The study was carried out on male Wistar rats aged 60 and 450 days. The animals were divided into 4 groups: group 1 (n = 14) – intact rats (60 days old) fed with a standard diet for 90 days; group 2 (n = 14) – rats (aged 60 days) receiving a high-fat, high-carbohydrate diet for 90 days; group 3 (n = 14) – intact rats (aged 450 days) receiving a standard diet for 90 days; group 4 (n = 14) – rats (aged 450 days) fed with a high-fat, high-carbohydrate diet for 90 days. Clinical and instrumental research methods, enzyme-linked immunosorbent assay, and immunohistochemistry and histology techniques were used in the study.

Results. Feeding 60-day-old animals with a high-fat, high-carbohydrate diet resulted in an increase in body weight and abdominal fat, a rise in systolic blood pressure, and moderately pronounced histologic changes in the kidneys. In intact 450-day-old rats, age-related changes prevailed: changes in the myocardial mass, an increase in TGF-β1, morphological changes in the renal tubules and glomeruli. In 450-day-old rats receiving a high-fat, high-carbohydrate diet, the most pronounced increase in both systolic and diastolic blood pressure, a significant rise in serum fibronectin, and destructive changes in the renal tissue were noted.

Conclusion. Functional and biochemical signs of arterial hypertension and morphological changes in the kidneys were the most pronounced in 450-day-old rats fed with a high-fat, high-carbohydrate diet.

Keywords: arterial hypertension, age-related changes in the kidneys, high-fat, high-carbohydrate diet

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Влияние возраста, высокоуглеводной и высокожировой диеты на развитие артериальной гипертензии и поражения почек в эксперименте

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РЕЗЮМЕ

Цель: выявить структурные основы патогенеза артериальной гипертензии и поражения почек, связанных с высокоуглеводной высокожировой диетой (ВУСЖД) и возрастом у крыс линии Wistar.

Материал и методы. Исследование проводили на самцах крыс линии Wistar в возрасте 60 и 450 сут. Животных распределяли на четыре группы: 1-я (n = 14) – интактные крысы (возраст 60 сут), содержащиеся на стандартном рационе в течение 90 сут; 2-я (n = 14) – крысы (возраст 60 сут), содержащиеся на ВУВЖД в течение 90 сут; 3-я (n = 14) – интактные крысы (возраст 450 сут), содержащиеся на стандартном рационе в течение 90 сут; 4-я (n = 14) – крысы (возраст 450 сут), содержащиеся на ВУВЖД в течение 90 сут. Использовались клинико-инструментальный, иммуноферментный, иммуногистохимический и гистологический методы исследования.

Результаты. Высокоуглеводная высокожировая диета приводила у 60-дневных животных к увеличению массы тела и абдоминального жира, нарастанию систолического артериального давления, появлению умеренно выраженных гистологических изменений почек. У интактных 450-дневных крыс преобладали изменения, связанные с возрастом: увеличение массы миокарда, увеличение TGF-β1 в сыворотке крови, морфологические изменения почечных канальцев и клубочков. У 450-дневных крыс, содержащихся на ВУВЖД, отмечалось наиболее выраженное нарастание как систолического, так и диастолического артериального давления, значительное увеличение концентрации фибронектина в сыворотке крови, выраженные деструктивные изменения в почечной паренхиме.

Заключение. Функциональные и биохимические признаки артериальной гипертензии и морфологические изменения в почках были наиболее выражены у 450-дневных крыс, содержавшихся на ВУВЖД.

Ключевые слова: артериальная гипертензия, возрастные изменения почек, высокоуглеводная высокожировая диета

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

Risk factors for arterial hypertension include age, obesity, and associated pathologies, such as diabetes mellitus, diabetic nephropathy, and atherogenic dyslipidemia. Overweightness and obesity are currently equated with a global epidemic and are the main factors underlying a number of metabolic disorders in organs and tissues, which contribute to progression of insulin resistance and development of metabolic syndrome, increasing the risk of diabetes, cardiovascular diseases, hyperlipidemia, non-alcoholic fatty liver disease, and kidney disease [1, 2].

The prevalence of metabolic syndrome and its components has alarmingly increased over the past decade, becoming a public health problem. According to numerous studies, the incidence of metabolic syndrome ranges from 7.5 to 42.2% in different countries [3, 4], which is associated with increased prevalence of bad habits (smoking, overeating, etc.) and a sedentary lifestyle.

Powerful risk factors for the development of kidney disease in metabolic syndrome include not only diabetic nephropathy and arterial hypertension, but also obesity, which, due to the direct effect of oxidative stress on the renal parenchyma, is believed to lead to chronic kidney disease and end-stage renal failure [5]. The latter is a serious health problem, which may result in such cardiovascular complications as arterial hypertension, heart failure, myocardial infarction, and sudden cardiac death [6]. However, the mechanisms of kidney disease in obesity are still unclear.

Therefore, the aim of the study was to identify the structural foundations of the pathogenesis of arterial hypertension and kidney disease associated with a high-fat, high-carbohydrate diet and age in the experiment.

MATERIALS AND METHODS

The study was carried out on male Wistar rats aged 60 and 450 days weighing 350–400 g and 400–600 g, respectively. All procedures were carried out in accordance with the Directive 2010/63 / EU of the European Parliament and the FASEB statement on the principles for the use of animals in research and education. The animals were divided into 4 groups: group 1 (n = 14) – intact rats (aged 60 days) fed with a standard diet for 90 days; group 2 (n = 14) – rats (aged 60 days) receiving a high-fat, high-carbohydrate diet (HFHCD) for 90 days; group 3 (n = 14) – intact rats (aged 450 days) receiving a standard diet for 90 days; group 4 (n = 14) – rats (aged 450 days) fed with HFHCD for 90 days.

HFHCD contained 16% proteins, 21% fats, and 46% carbohydrates, including 17% fructose and 0.125% cholesterol [7]. Water was replaced with a 20% fructose solution. The rats of groups 1 and 3 (intact animals) were fed with standard rodent food (24% proteins, 6% fats, 44% carbohydrates) and pure water *ad libitum*. After the end of HFHCD, the animals received a standard diet and regular drinking water for one week in order to exclude osmotic load due to the consumption of fructose.

Body weight, as well as systolic and diastolic blood pressure (BP) were assessed weekly by tail cuff plethysmography using the MP35 data acquisition system (Biopac Systems Inc., USA). The animals were removed from the experiment by decapitation following anesthesia with chloralose (100 mg / kg intraperitoneally). Before the decapitation, blood samples were taken from the common carotid artery. Immediately after the sacrifice, the heart, both kidneys, and visceral fat were removed and weighed. The study was approved by the Ethics Committee at the Cardiology Research Institute of Tomsk NRMC (Protocol No. 201 of 30.07.2020).

The blood samples were centrifuged (15 min 3,000 rpm), the serum samples were stored in a freezer at –70 °C. Fibronectin and TGFβ-1 in the serum were determined by enzyme-linked immunosorbent assay (ELISA) using the rat fibronectin (ab108850) and rat TGFβ (ab119558) ELISA kits (Abcam, USA), respectively. Sample measurements were performed using the Infinite 200 PRO microplate reader (Tecan GmbH, Salzburg, Austria).

For a histologic examination, kidney samples were taken, which were fixed in 10% neutral buffered formalin and embedded in paraffin according to the standard technique. The sections were stained with hematoxylin and eosin, Van Gieson's stain, and periodic acid – Schiff (PAS) stain with nuclear counterstaining with hematoxylin. Immunohistochemistry (IHC) was performed using monoclonal antibodies Ki-67 (Abcam, USA). The histologic sections were visualized and photographed using the Axiostar plus light microscope (Carl Zeiss, Germany) at 400x and 1000x magnification. The percentage of Ki-67$^+$ positive cells was counted in each glomerulus. A morphometric analysis was performed using the ImageJ software for image analysis and processing (National Institutes of Health, USA); the area of the renal glomeruli and the width of the Bowman's space were calculated.

A statistical analysis was performed using the Statistica 13.0 software package (StatSoft Inc., USA).

The analysis of the obtained data was carried out by the methods of descriptive statistics with the calculation of the median and the interquartile range *Me* (Q_1–Q_3) for non-normally distributed variables. For normally distributed variables, the data were presented as the mean and the error of the mean ($M \pm m$). The differences between the groups were determined using the Kruskal – Wallis test. The differences were considered statistically significant at $p < 0.05$; $0.05 \geq p \leq 0.06$ was taken as a trend.

RESULTS

Body weight increased in the experimental animals of groups 2–4 compared with group 1 (Table 1).

Table 1

Weight of the animals, weight of organs and visceral fat of rats of different age fed with HFHCD, g, $M \pm m$

Weight	Group 1	Group 2	Group 3	Group 4
Body	430.3 ± 5.3	481.2 ± 12.4[1]	517.3 ± 13.0[1]	520.0 ± 35.0[1]
Heart	1.39 ± 0.1	1.31 ± 0.053	1.54 ± 0.04[1, 2]	1.44 ± 0.1[3]
Kidneys	2.8 ± 0.1	2.8 ± 0.1	3.51 ± 0.2[1]	3.05 ± 0.2[3]
Visceral fat	8.32 ± 1.1	16.46 ± 1.6[1]	9.91 ± 0.9	17.47 ± 3.8[3]

[1, 2, 3] statistical significance of the differences compared with group 1,2,3,4 (here and in Tables 2, 3).

Note: here and in Tables 2–4: group 1 – intact rats aged 60 days; group 2 – rats aged 60 days receiving HFHCD; group 3 – intact rats aged 450 days; group 4 – rats aged 450 days receiving HFHCD.

At the same time, there was a pronounced increase in the weight of abdominal fat in the groups of animals fed with HFHCD compared with similar parameters in intact animals of different age. When the heart was weighed, its maximum weight was observed in 450-day-old intact rats, which slightly differed from the values in the group of 450-day-old rats fed with HFHCD. The kidney weight was significantly elevated in the animals of group 3 compared with group 1.

Systolic BP in the rats increased in group 2 and showed a trend toward an increase in group 4 after the prescription of HFHCD, while diastolic BP increased only in group 4 (Table 2).

The study of the blood serum showed a two-fold increase in the concentration of fibronectin in the rats of group 4 (Table 3) compared with the animals in groups 1–3. An increase in the serum concentration of TGFβ-1 was observed in the animals of groups 3 and 4 (Table 3).

The histologic examination of the kidneys revealed foci of pronounced perivascular and periductal lymphocyte and monocyte infiltration of the stroma in group 3; in the lumen of some distal convoluted tubules and collecting ducts, PAS-positive casts were observed, while epithelial cells in the nephron tubules flattened toward the lumen or exfoliated in the lumen. In single epithelial cells of the proximal tubules, lipofuscin granules and nuclear damage were noted.

Table 2

Blood pressure in rats of different age fed with HFHCD, mm Hg, *Me* (Q_1–Q_3)

BP	Group 1	Group 2	Group 3	Group 4
Systolic	129 (124–136)	141 (137–143)[1]	133 (129–136)[2]	140 (135–144)[1, 3]
Diastolic	87 (83–89)	85 (77–89)	86 (80–88)	97 (95–101)[1, 2, 3]

Table 3

Concentration of fibronectin and TGFβ-1 in the blood serum of rats of different age fed with HFHCD, $M \pm m$

Parameter	Group 1	Group 2	Group 3	Group 4
Fibronectin, mg / dl	21.23 ± 1.55	27.58 ± 1.78	29.89 ± 2.38	43.00 ± 3.12[1, 2, 3]
TGFβ-1, ng / ml	14.0 ± 3.0	19.1 ± 2.6	35.3 ± 5.2[1]	31.9 ± 4.1

Morphological changes in the rat kidneys in groups 2 and 4 included pronounced plethora in the vessels of both renal glomeruli and stroma with signs of stasis. In group 2, moderate perivascular fibrosis was noted, while in group 4, both perivascular and focal peritubular fibrosis in the stroma with surrounding lymphoid infiltration and thickening of the outer layer of the Bowman's capsule were identified. Damage to the brush border of the epithelial cells in the proximal tubules was observed in both groups fed with HFHCD, which was the most extended in group 4 (Fig. 1). The distal convoluted tubules in group 4 contained PAS-positive casts, which was accompanied by flattening or death of the epithelial cells (Fig. 2).

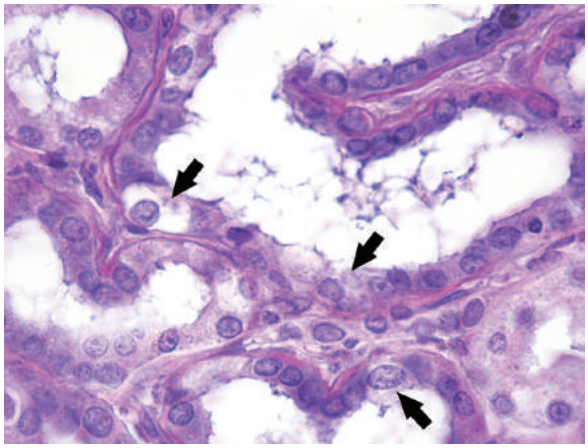

Fig. 1. Damage to the brush border of the epithelial cells in the proximal tubules (indicated by arrows) in the kidneys of 450-day-old rats after the prescription of HFHCD. Staining with PAS and hematoxylin, x400

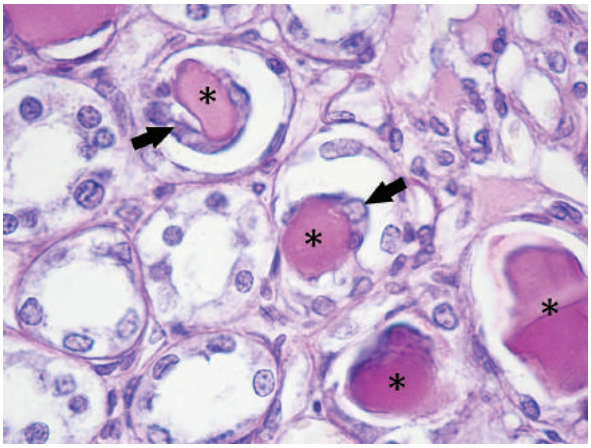

Fig. 2. PAS-positive casts (indicated by asterisks) and exfoliated epithelial cells (indicated by arrows) in the lumen of the distal convoluted tubules of 450-day-old rats after the prescription of HFHCD. Staining with PAS and hematoxylin, x400

A quantitative assessment of changes in the renal glomeruli showed an increase in their size both with age and with the prescription of HFHCD (Table 4). However, the area of the glomeruli in the animals fed with HFHCD was significantly smaller than in the animals receiving a standard diet.

The width of the Bowman's space, located between the inner and outer layers of the Bowman's capsule, also increased with age in all groups, while the urinary space in groups 2 and 4 was significantly wider than that in groups 1 and 3, respectively (Table 4).

Table 4

Area of the renal glomeruli and width of the Bowman's space in rat kidneys, µm 2/ µm, *Me* (Q_1–Q_3)

Parameter	Group 1	Group 2	Group 3	Group 4
The area of the renal glomeruli,×10^3	$23.54^{2,3}$ (19.94–27.21)	$20.41^{1,4}$ (16.98–22.52)	27.04^{1} (23.32–32.03)	24.71^{3} (21.67–29.32)
The width of the Bowman's space	$9.36^{2,3}$ (7.59–11.77)	$12.54^{1,4}$ (10.45–15.10)	$12.21^{1,4}$ (9.88–15.37)	$15.42^{2,3}$ (12.98–17.16)

^{1,2,3,4} significance of the differences compared with group 1, 2, 3, 4.

Ki-67 IHC staining was detected in cells of the renal glomeruli (in mesangial cell processes and flattened endothelial cells) (Fig. 3, 4). At the same time, a rise in the number of Ki-67-positive cells was revealed, which coincided with an increase in the age of the animals fed with HFHCD (Fig. 5).

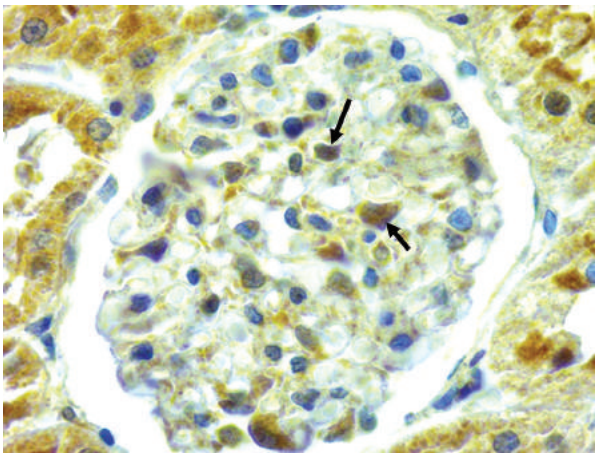

Fig. 3. Ki-67$^+$ cells (indicated by arrows) in the glomeruli of 60-day-old intact rats. IHC staining with Ki67 antibodies and hematoxylin, x1000

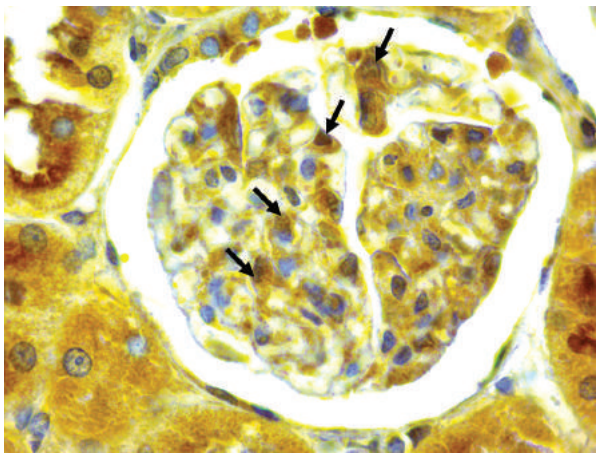

Fig. 4. Ki-67$^+$ cells (indicated by arrows) in the glomeruli of 450-day-old intact rats after the prescription of HFHCD. IHC staining with Ki67 antibodies and hematoxylin, x1000

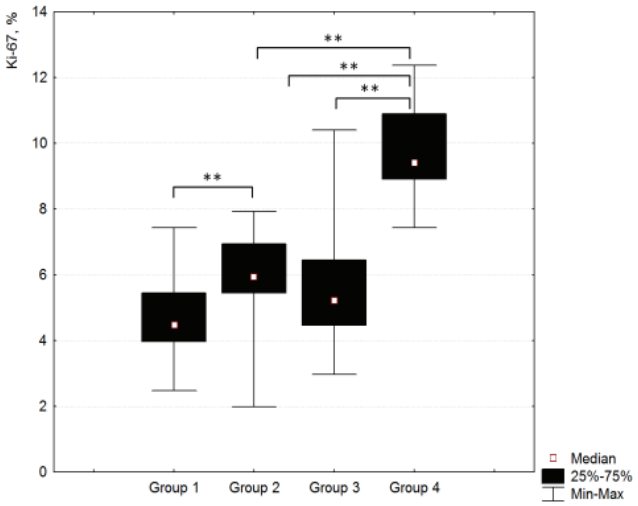

Fig. 5. The percentage of Ki-67+ cells in the renal glomeruli: ** the differences between the groups

DISCUSSION

The HFHCD used in this study corresponds to the so-called cafeteria diet, which, along with obesity, causes a decrease in glucose and insulin tolerance, dyslipidemia, an increase in lipid peroxidation, a decrease in antioxidant activity of the liver, kidneys, and brain, as well as interstitial nephritis [8]. The increase in body, heart, and kidney weight in 450-day-old rats fed with HFHCD and a standard diet could be a consequence of not only a diet, but also an age-related increase in organ mass, while a significant increase in the weight of visceral fat in the rats receiving HFHCD, obviously, was the result of only HFHCD.

Changes in the systolic and diastolic BP values in the animals fed with HFHCD might be caused by an increase in the load on the myocardium associated with obesity, while a rise in diastolic BP may be associated with impaired renal function, which might lead to a decrease in the kidney mass in the animals fed with HFHCD. The histologic signs of kidney disease that we established in aged animals were aggravated by the prescription of HFHCD: a decrease in the area of the renal glomeruli combined with an increase in and thickening of the Bowman's capsule might indicate glomerular hyperfiltration.

A similar negative effect on the kidney structure was also demonstrated when studying the effect of long-term intake of fructose with glucose or sucrose [9]. The main pathophysiological mechanism underlying the negative effect of fructose is associated with a by-product of its metabolism – uric acid, which has a direct effect on endothelial cells and vascular smooth muscle cells. In this case, the bioavailability of endothelial nitric oxide is inhibited, and the renin – angiotensin system is activated, which results in glomerular hypertension and tubulointerstitial injury [10]. Subsequently, renal vasoconstriction and systemic hypertension develop. The latter develops due to an increase in the reabsorption of salts and water and may also be accompanied by inflammation and tubulointerstitial injury [11].

Expression of the proliferation marker Ki-67 was observed in mesangial and, probably, endothelial cells of renal glomerular capillaries, since proliferation is not typical of podocytes [12]. The increase in the number of Ki-67$^+$ cells in the group of aged animals that were fed with HFHCD can also be associated with both the age-related accumulation of advanced glycation end products and the damaging effect of uric acid, which causes glomerular hyperfiltration and hyperplasia of mesangial cells after 2 weeks of feeding animals with fructose, as shown in the experiments [13, 14]. The process was accompanied by the accumulation of extracellular matrix proteins, thickening of the glomerular basement membrane, and the development of glomerular hypertension.

Proliferation of mesangial cells and an increase in synthesis of the extracellular matrix are stimulated by α-SMA and TGF-β1, so both factors were identified as the key mediators of glomerular and tubulointerstitial pathology in chronic kidney disease [15]. An increase in the concentration of TGF-β1 was observed in nephropathy caused by induced diabetes mellitus in rats [16]. However, in our study, the increase in the serum concentration of TGF-β1 occurred in all 450-day-old rats, regardless of HFHCD. Therefore, this parameter is more likely associated with age-related changes. Mesangial cells themselves can produce extracellular matrix proteins, including fibronectin, which leads to renal fibrosis in pathological conditions [17]. In our study, in the aged animals fed with HFHCD, the increase in the Ki-67 expression in mesangial cells was combined with the increased serum levels of TGF-β1 and fibronectin, which also supports the hypothesis on the development of glomerular hypertension.

The results obtained are comparable with the studies that established a relationship between an increase in the concentration of fibronectin and diabetic nephropathy [18], increased concentration of fibronectin in the blood plasma and type 1 diabetes mellitus [19], hypertension, nephropathy [20], as well as obesity and triglyceridemia in patients with type 2 diabetes mellitus [21].

CONCLUSION

The revealed histologic changes in the kidneys may indicate an aggravating effect of HFHCD on aging and, subsequently, cause development of renal hypertension associated with disruption of the normal structure and functioning of the kidneys. The results obtained suggest that serum fibronectin level can be used as a marker of kidney disease in diabetes mellitus in elderly patients, however, this assumption needs clinical confirmation.

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Authors contribution

Mustafina L.R. – carrying out of the morphological and immunohistochemical studies, morphometry, statistical processing and interpretation of the morphological data. Logvinov S.V. – conception, drafting of the morphology section of the article, substantiation of the manuscript, critical revision of the manuscript for important intellectual content, and final approval of the manuscript for publication. Naryzhnaya N.V. – conception and design, carrying out of the research, drafting of the article, substantiation of the manuscript, critical revision of the manuscript for important intellectual content. Kurbatov B.K. – design of the study, carrying out of the research, statistical analysis and interpretation of the data. Maslov L.N. – conception, drafting of the article, substantiation of the manuscript, critical revision of the manuscript for important intellectual content, and final approval of the manuscript for publication.

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The effect of depressive disorder on the clinical presentation of coronary artery disease and five-year survival of patients after myocardial infarction

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ABSTRACT

Aim. To assess the effect of depressive disorder (DD) on the clinical presentation of coronary artery disease (CAD) and five-year survival rate of patients with chronic CAD.

Materials and methods. The study included 79 patients with functional class II–III exertional angina who experienced myocardial infarction more than 6 months before. The patients were divided into two groups: group 1 (n = 45) consisted of patients with CAD and depression and group 2 (n = 34) encompassed patients with CAD without depression. The clinical presentation of CAD was assessed by the results of filling out the angina pectoris self-control diary and exertion tests. The presence and severity of DD were determined using psychometric scales, such as Hospital Anxiety and Depression Scale (HADS) and Beck Depression Inventory (BDI), and verified by the psychiatrist. Information about five-year survival was obtained via telephone interviews with the patients and their relatives.

Results. Patients with CAD and DD were characterized by more frequent episodes of angina pectoris during a week (10 [8; 14] vs 6 [4; 7], p = 0.000004), an increased demand for nitroglycerin (4 [0; 10] tablets vs 0 [0; 4] tablets, p = 0.001), and lower exercise tolerance (50 [25; 75] W vs 75 [50; 75] W (p = 0.06), 350 [250; 400] meters vs 435 [350; 500] meters (p = 0.01) than CAD patients without DD. The five-year survival rate was significantly lower in group 1 than in group 2 (69 [62; 72] vs 71 [68; 72] months (p = 0.04)), 35 (77.8%) vs 32 (94.1%) patients survived. In group 1, a greater number of deaths from cardiovascular accidents (10 (22.2%) vs 2 (5.9%)) was noted (log-rank test, p = 0.03).

Conclusion. In patients with CAD, associated depression results in aggravation of the clinical course of CAD and poor disease prognosis, which requires timely diagnosis and treatment of DD.

Keywords: coronary artery disease, depressive disorders, myocardial infarction, survival, mortality

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 Cardiology Research Institute, Tomsk NRMC (Protocol No. 177 of 30.10.2018).

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Влияние депрессивного расстройства на клиническую картину коронарной болезни и пятилетнюю выживаемость больных после перенесенного инфаркта миокарда

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РЕЗЮМЕ

Цель. Оценить влияние депрессивных расстройств (ДР) на клиническую картину коронарной болезни и пятилетнюю выживаемость больных с хронической ишемической болезнью сердца (ИБС).

Материалы и методы. В исследование включены 79 больных со стенокардией напряжения II–III функциональных классов, перенесшие инфаркт миокарда давностью более 6 мес. Сформированы две группы: 1-я – 45 больных ИБС с депрессией и 2-я – 34 больных ИБС без депрессии. Клиническая картина ИБС оценивалась по результатам заполнения дневника самоконтроля стенокардии, по пробам с физической нагрузкой. Наличие и выраженность ДР определялись с помощью психометрических шкал (Госпитальная шкала тревоги и депрессии (HADS) и Шкала депрессии Бека (BDI)) и верифицировался психиатром. Информация о пятилетней выживаемости была получена методом телефонного интервью с пациентами и их родственниками.

Результаты. Больных ИБС с ДР в сравнении с пациентами без ДР чаще беспокоили ангинозные приступы в течение недели (10 [8; 14] vs 6 [4; 7], p = 0,000004), отмечалась большая потребность в приеме нитроглицерина (4 [0; 10] vs 0 [0; 4] таблеток, p = 0,001), более низкая толерантность к физической нагрузке (50 [25; 75] Вт vs 75 [50; 75] Вт (p = 0,06), 350 [250; 400] м vs 435 [350; 500] м (p = 0,01). В первой группе пятилетняя выживаемость была значительно ниже, чем во второй (69 [62; 72] vs 71 [68; 72] мес (p = 0,04)), выжило (35 (77,8%) vs 32 (94,1%), отмечалось большее число летальных исходов от сердечно-сосудистых катастроф (10 (22,2%) vs 2 (5,9%)) (лог-ранг тест p = 0,03).

Заключение. У больных ИБС присоединение депрессии приводит к ухудшению клинического течения коронарной болезни и прогноза, что требует своевременной диагностики и коррекции ДР.

Ключевые слова: ишемическая болезнь сердца, депрессивные расстройства, инфаркт миокарда, выживаемость, летальность

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 этическим комитетом НИИ кардиологии Томского НИМЦ (протокол № 177 от 30.10.2018).

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INTRODUCTION

Coronary artery disease (CAD) and depressive disorders (DD) have always been of great interest for science. According to the World Health Organization (WHO) forecasts, by 2030, CAD and depression will have become the leading causes of disability and invalidism in high-income countries around the world [1, 2]. Depression itself contributes to the occurrence of CAD; at the same time, it is more often observed

in patients with confirmed cardiovascular diseases [3] and is a strong predictor of a poor disease prognosis [4]. The risk of death in patients with cardiovascular pathology directly depends on the severity of the affective disorder [5–7]. In CAD with comorbid DD, more severe angina pectoris is noted and social functioning of patients is significantly reduced [5]. DD is verified in 40% of patients after acute myocardial infarction, which increases the risk of death by 3–6 times [6].

Studies have shown that less than 30% of patients have psychological complaints, leading to the development of depressive disorders in the future, due to which underdiagnosis of DD and untimely prescription of appropriate therapy for it are noted [9, 10]. Besides, patients with CAD with associated affective disorders show worse compliance with doctor's recommendations on a healthy lifestyle [11] and regular baseline therapy for CAD, which leads to frequent visits to the clinic and hospitalizations [12]. The relevance of studying the comorbidity of CAD and DD is beyond doubt, therefore, the aim of this study was to assess the effect of DD on the clinical presentation of CAD and five-year survival rate of patients with chronic CAD.

MATERIALS AND METHODS

The study included 79 patients with angina pectoris who experienced myocardial infarction more than 6 months before. The patients were divided into two groups: group 1 (n = 45) consisted of patients with exertional angina and depression and group 2 (n = 34) encompassed patients with exertional angina without depression. The diagnosis of DD was made based on the analysis of scales for detecting the presence of DD (Hospital Anxiety and Depression Scale (HADS)) and its severity (Beck Depression Inventory (BDI)). Depressive symptoms were considered elevated with the score of more than 8 on HADS and / or more than 19 on BDI. The final diagnosis was established by the psychiatrist.

Features of the clinical presentation of CAD in patients with depression compared with patients without DD were assessed by the angina pectoris self-control diary (the number of angina pectoris episodes per week, the need for nitroglycerin per week) and by exercise tests (bicycle ergometry, six-minute walk test).To determine the functional class of angina pectoris, we used the classification developed by the Canadian Heart Association (L. Campeau, 1976) and additionally compared it with the results of bicycle ergometry (W). The life expectancy of patients was assessed from the moment of admission to the hospital for 5 years. Information on five-year survival was obtained via telephone interviews with patients and their relatives and the analysis of medical records. Patients who were followed up to a certain endpoint (5 years), as well as patients who dropped out of the follow-up for unknown reasons (contact with them was lost) were considered as censored patients.

The differences were considered statistically significant at $p < 0.05$. For normally distributed quantitative variables, the data were presented as $M \pm SD$, where M is the mean and SD is the standard deviation. In this case, the reliability was checked using the t-test; to compare two dependent samples, Student's t-test was used. For non-normally distributed variables, the data were presented as the median and the interquartile range (Me [Q_1; Q_3]) and described by the Mann – Whitney test. For the analysis of qualitative variables, contingency tables and the $\chi 2$ test were used. Patient survival was assessed using the Kaplan – Meier method and presented graphically; a log-rank test was used to assess the differences.

RESULTS

The groups were comparable by age and sex: in group 1, the average age was 57.3 ± 7.1 years, 41 men and 4 women; in group 2, the average age was 57.5 ± 7.3, 31 men and 3 women ($p > 0.5$). According to the anamnestic data and clinical, laboratory, and instrumental parameters, the patients of groups 1 and 2 did not differ: the duration of myocardial infarction was 30 [6; 96] vs 24 [7; 72] months (p = 0.4), history of hypertension – 108 [24; 180] vs 90 [36; 132] months (p = 0.6), number of smokers – 21 vs 15 people (p = 0.8), percutaneous coronary intervention – 28 vs 27 patients (p = 0.1), body mass index – 28.8 [25.5; 31] vs 28.9 [26; 31] kg / m2 (p = 0.8), ejection fraction – 60.6 ± 10.1 vs 61.2 ± 7.7% (p = 0.1), total cholesterol level – 5.6 ± 1.2 vs 5.5 ± 1.6 mmol / l (p = 0.08). Patients with and without DD also did not differ in the functional class of exertional angina: class II – 29 vs 26 patients (p = 0.2), class III – 16 vs 8 patients (p = 0.2). Patients received baseline treatment for stable angina pectoris and complied with the doctor's recommendations, which made it possible to maintain the target values of heart rate and blood pressure. The average heart rate per day was 64 [61; 67] vs 65 [62; 71] beats per min (p = 0.3), the average daily systolic blood pressure was 120 [110; 130] vs 121 [112; 124] mm Hg (p = 0.9), and the average daily

diastolic blood pressure was 76 mm Hg [70; 82] vs 75 mm Hg [73; 78] (p = 0.7).

When testing for the preliminary diagnosis of depression using HADS and BDI scales, the following results were obtained: HADS – 8 [8; 10] vs 4 [3; 6] points (p = 0.0000001), BDI – 22 [16; 26] vs 15 [14; 19] points in group 1 and group 2, respectively (p = 0.0001). Afterwards, the diagnosis of depression was verified by the psychiatrist.

The groups were comparable in terms of the functional class of angina, however, patients with DD complained of angina pectoris episodes significantly more often (10 [8; 14] vs 6 [4; 7] per week (p = 0.000004) and more often needed nitroglycerin (4 [0; 10] vs 0 [0; 4] tablets) (p = 0.001). Besides, patients with DD performed worse in the six-minute walk test: 350 [250; 400] vs 435 [350; 500] meters (p = 0.01). Bicycle ergometry showed a trend toward significantly lower exercise tolerance in CAD patients with DD compared with CAD patients without DD: 50 [25; 75] vs 75 [50; 75] W (p = 0.06). Therefore, the comorbidity of DD with CAD aggravates the course of the underlying disease.

The information on five-year survival was obtained via telephone interviews with patients and their relatives and the analysis of medical records. After 5 years of follow-up, 67 patients (84.8%) survived, 12 patients (15.1%) died. In the group of patients with CAD with DD, as opposed to CAD patients without depression, significantly fewer patients survived (35 (77.8%) vs 32 people (94.1%)), and more fatal outcomes from cardiovascular events (10 (22.2%) vs 2 (5.9%)) were noted (log-rank test p = 0.03) (Fig. 1).

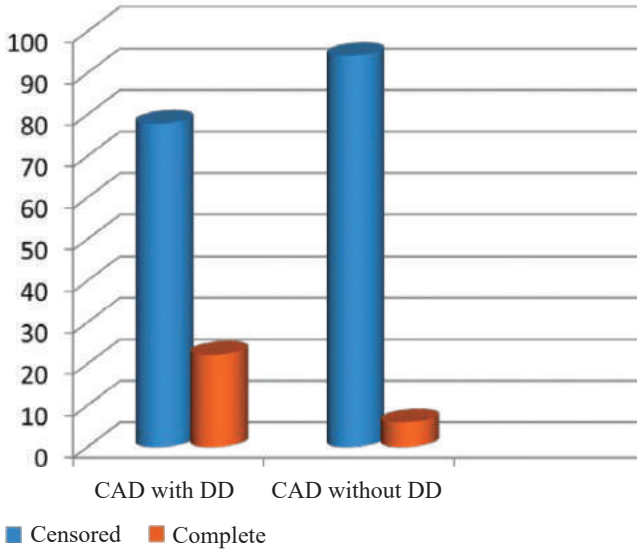

Fig.1. Five-year survival of patients

A significant difference was revealed in the Kaplan – Meier survival analysis; the data are presented in Figure 2. It was found that the survival rate was significantly lower in group 1 than in group 2: 69 [62; 72] vs 71 [68; 72] months (p = 0.04).

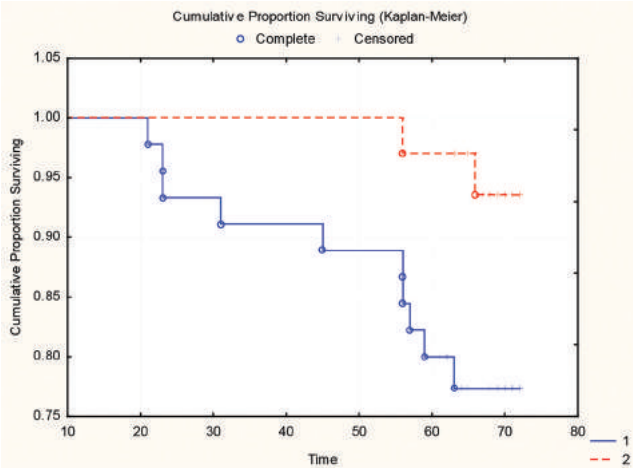

Fig. 2. Kaplan – Meier survival analysis in CAD patients with DD compared with CAD patients without DD

DISCUSSION

According to many large studies (MONICA, Cardiovascular Health Study, INTERHEART, ENRICHD, SADHART), depression can be considered as a predictor of cardiovascular events. Those studies included patients with acute coronary syndrome. We studied the clinical presentation of CAD in patients who experienced myocardial infarction (more than 6 months before) against the background of an affective disorder. Many psychometric scales and tests are currently used for DD screening. We used the most common and standardized questionnaires, such as BDI and HADS [13]. After testing, the patients were consulted by the psychiatrist, and the diagnosis of moderate DD was verified. In both groups, functional class II–III exertional angina was diagnosed, but according to self-control diaries, it was determined that patients with DD more often experienced episodes of angina and needed nitroglycerin. The aggravation of the clinical presentation of CAD in patients with DD was confirmed objectively: during bicycle ergometry, exercise tolerance was decreased, and patients covered a much shorter distance during the six-minute walk test. Similar results on the negative impact of DD on the course of CAD were described in previous studies [5, 14, 15].

Later, when analyzing the five-year survival rate using the Kaplan – Meier method, we found significant differences: the survival rate of CAD patients with

concomitant DD was significantly lower. The increase in mortality in CAD patients with DD is most likely associated with common pathogenetic mechanisms that exacerbate each other. Particularly, stress causes hyperactivation of the sympathetic adrenal system and autonomic dysfunction, which leads to development of life-threatening arrhythmias [16–18]. Moreover, DD results in an increased risk of thrombosis and, consequently, in CAD exacerbation [19]. Behavioral characteristics of patients with mental disorders play a crucial role in the disease prognosis: they do not adhere to a healthy lifestyle and show low compliance with CAD therapy, which significantly reduces their quality of life [14].

Therefore, DD significantly affects cardiovascular diseases: both the clinical course and the disease prognosis. For early detection of DD, according to the results of modern psychometric scales, patients should be timely referred to a psychiatrist for early prescription of antidepressants. Close observation of such patients by a joint team of a cardiologist and a psychiatrist will improve the survival of patients with CAD.

CONCLUSION

The aggravation of the clinical presentation of CAD and poor disease prognosis are typical of patients with CAD with associated depression. Our results confirm the need for timely verification and correction of DD. Affective disorders significantly reduce the long-term survival of patients with CAD.

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Expression of inhibitory receptors PD-1, CTLA-4, and Tim-3 by peripheral T cells during pregnancy

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ABSTRACT

Background. Inhibitory receptors and their ligands (also called checkpoint molecules) are important feedback regulators of the immune response. However, their role in immunological adaptation during pregnancy remains poorly understood.

The aim of the study was to evaluate the level of checkpoint molecule (PD-1, CTLA-4, Tim-3) expression in peripheral T cells in pregnant women compared with fertile non-pregnant women.

Materials and methods. The study included 36 women in the second half of pregnancy without pregnancy complications, 12 of whom had extragenital pathology. The control group consisted of 28 age-matched fertile non-pregnant women. The proportion of $CD8^+PD\text{-}1^+$, $CD8^+TIM\text{-}3^+$, $CD8^+PD\text{-}1^+TIM\text{-}3^+$, $CD4^+PD\text{-}1^+$, $CD4^+TIM\text{-}3^+$, and $CD4^+PD\text{-}1^+TIM\text{-}3^+$ was evaluated by flow cytometry using the corresponding monoclonal antibodies (BD Biosciences, USA).

Results. The proportion of CD4+Tim-3+ and CD8+PD-1+ T cells and CD4+ and CD8+ T lymphocytes co-expressing PD-1 and Tim-3 in the peripheral blood of pregnant women was statistically significantly higher than in non-pregnant women. An increase in CD4+Tim-3+ and CD8+PD-1+ T cells was observed both in pregnant women with and without extragenital pathology. However, pregnant women with extragenital pathology were characterized by a higher CD8+PD-1+ count and a smaller number of CD8+Tim-3+ cells, as well as by a lack of an increase in PD-1+Tim-3+ T cells typical of pregnant women. The number of comorbidities was directly correlated with the proportion of CD8+PD-1+ lymphocytes and inversely correlated with the proportion of CD8+Tim-3+ and CD4+ PD-1+Tim-3+ cells. In addition, the expression of checkpoint molecules was associated with gestational age (a direct correlation was found with the proportion of CD8+Tim-3+, CD4+PD-1+Tim-3+, and CD8+PD-1+Tim-3+ cells) and to a lesser extent – with the age of pregnant women (an inverse relationship was found with the proportion of CD8+Tim-3+ cells).

Conclusion. Pregnant women in the second half of pregnancy are characterized by increased expression of PD-1 and Tim-3 molecules in peripheral T cells. At the same time, concomitant extragenital pathology affects the expression of these molecules.

Keywords: T cells, pregnancy, inhibitory PD-1, TIM-3, CTLA-4 checkpoint molecules

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 study participants signed an informed consent. The study was approved by the local Ethics Committee at the Research Institute of Fundamental and Clinical Immunology (Protocol No.107 of 15.06.2018).

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Экспрессия ингибиторных рецепторов PD-1, CTLA-4 и Tim-3 периферическими Т-клетками при беременности

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РЕЗЮМЕ

Актуальность. Ингибиторные рецепторы и их лиганды (чек-поинт молекулы) являются негативными регуляторами иммунного ответа. Однако их роль в иммунной адаптации при беременности остается малоизученной.

Цель исследования – оценить уровень экспрессии чек-поинт молекул (PD-1, CTLA-4, Tim-3) на периферических Т-клетках у беременных в сравнении с фертильными небеременными женщинами.

Материалы и методы. В исследование были включены 36 женщин во второй половине беременности без гестационных осложнений, у 12 из которых имелась экстрагенитальная патология. Контрольную группу составили 28 сопоставимых по возрасту фертильных небеременных. Относительное содержание $CD8^+PD\text{-}1^+$, $CD8^+TIM\text{-}3^+$, $CD8^+PD\text{-}1^+TIM\text{-}3^+$, $CD4^+PD\text{-}1^+$, $CD4^+TIM\text{-}3^+$, $CD4^+PD\text{-}1^+TIM\text{-}3^+$ Т-клеток крови оценивали методом проточной цитометрии с использованием соответствующих моноклональных антител (BDBiosciences, США).

Результаты. Относительное содержание CD4+Tim-3+ и CD8+PD-1+ Т-клеток, а также CD4+ и CD8+ Т-лимфоцитов, коэкспрессирующих PD-1 и Tim-3 в периферической крови беременных, статистически значимо превышало аналогичные показатели у небеременных. Возрастание CD4+Tim-3+ и CD8+PD-1+ Т-клеток регистрировалось как у беременных с наличием, так и отсутствием экстрагенитальной патологии. Однако беременные с экстрагенитальной патологией отличались более высоким содержанием CD8+PD-1+ и меньшим количеством CD8+Tim-3+ клеток, а также отсутствием (характерного для беременных) возрастания PD-1+Tim-3+ Т-клеток. Количество сопутствующих патологий прямо коррелировало с долей CD8+PD-1+ лимфоцитов и обратно – с долей CD8+Tim-3+ и CD4+PD-1+Tim-3+ клеток. Кроме того, экспрессия чек-поинт молекул ассоциировалась со сроком гестации (прямая корреляция выявлялась с содержанием CD8+Tim-3+, CD4+PD-1+Tim-3+ и CD8+PD-1+Tim-3+ клеток) и в меньшей степени с возрастом беременных (обратная зависимость с долей CD8+Tim-3+ клеток).

Заключение. Беременные во второй половине гестации характеризуются повышенной экспрессией молекул PD-1 и Tim-3 на периферических Т-клетках. При этом сопутствующая экстрагенитальная патология влияет на характер экспрессии указанных молекул.

Ключевые слова: Т-клетки, беременность, ингибиторные PD-1, TIM-3, CTLA-4 чек-поинт молекулы

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

Источники финансирования. Исследование выполнено в рамках научной работы НИИФКИ (тема № 122011800353-4).

Соответствие принципам этики. Все участники исследования подписали информированное согласие. Исследование одобрено локальным этическим комитетом НИИФКИ (протокол № 107 от 15.06.2018).

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INTRODUCTION

Inhibitory receptors and their ligands, collectively referred to as inhibitory checkpoint molecules, belong to the category of signaling molecules. They mediate various immunosuppressive mechanisms and play an important role in suppressing the immune response and forming tolerance to autoantigens, and in pathology, they inhibit the immune response against tumors and viral infections [1]. The most studied inhibitory T cell receptors are cytotoxic T lymphocyte antigen-4 (CTLA-4), programmed cell death protein-1 (PD-1), and T cell immunoglobulin and mucin-domain containing-3 (TIM-3). Triggering signaling pathways during the interaction of these receptors with the corresponding ligands (CTLA4 / CD80/86, PD-1 / PD-L1, TIM-3 / Gal-9) leads to suppression of effector T cells, skews the Th1 / Th2 cytokine balance toward Th2 [2, 3], and, on the other hand, increases activity and expansion of regulatory T cells (Treg) [4]. In addition, increased expression and co-expression of checkpoint molecules characterize a dysfunctional state known as T cell exhaustion, reflecting a progressive decrease in the functional activity of T lymphocytes during the transition of effector T cells to memory cells [5, 6].

The study of checkpoint molecule expression during pregnancy is of particular interest, since successful bearing of a semi-allogeneic fetus requires significant restructuring of the immune system aimed at inducing tolerance to fetal antigens [7]. The molecular mechanisms of such rearrangement remain understudied. However, dynamic changes in the Th1 / Th2 cytokine balance, limited cytotoxic potential of CD8+ T cells, Treg induction, and the recently described signs of T cell depletion in uncomplicated pregnancy suggest an important role of checkpoint molecules in the immunological adaptation during gestation [8]. Recent studies in experimental animals and humans have shown that decidual T cells during pregnancy are characterized by increased expression and co-expression of CTLA-4, PD-1, and Tim-3, while antigen-presenting and stromal cells localized in the decidua highly express ligands of these receptors [8].

It has been shown that increased expression of inhibitory receptors by decidual T cells can be induced by HLA-C and HLA-G molecules on trophoblasts [9]. At the same time, data on the expression of inhibitory molecules on peripheral T cells and their role in pregnancy remain poorly understood and ambiguous. Meanwhile, it should be noted that fetal antigens that enter the lymph nodes and spleen with the bloodstream can activate T cells, so tolerance should be maintained not only at the local, but also at the systemic level.

Inducers of the expression of inhibitory receptors on circulating T cells can be interleukin (IL)-10, progesterone, and vascular endothelial growth factor (VEGF) [3, 10]. Our studies also showed that one of the modulators of the functions of activated T cells, which can enhance the expression of checkpoint molecules, is placental growth factor (PlGF), the concentration of which drastically increases during pregnancy [11].

Given these facts, we hypothesized that the expression of checkpoint molecules on peripheral T cells during pregnancy may also change, reflecting the restructuring of the immune system at the systemic level. The aim of this study was to test this hypothesis.

MATERIALS AND METHODS

The study included 36 pregnant women without pregnancy complications, who were examined at City Clinical Hospital No. 1, and 28 fertile non-pregnant women (Table 1). The study was carried out in accordance with the ethical principles of the Declaration of Helsinki developed by the World Medical Association “Ethical Principles for Medical Research Involving Human Subjects” amended in 2000 and “Rules of Clinical Practice in the Russian Federation” approved by the order of the Ministry of Health of the Russian Federation No. 266 of 19.06.2003.

The age of women in the group with uncomplicated pregnancy ranged from 18 to 45 years (median (*Me*) 27 years) and did not differ significantly from that in the group of fertile non-pregnant women (22 to 45 years, *Me* 31 years; $P_U = 0.11$). Gestational age ranged from 26 to 40 weeks with a median of 36 weeks. In 20 (55.6%) women, the pregnancy was the first, 16 (44.4%) women had repeat pregnancy. Concomitant extragenital pathology (arterial hypertension, including gestational hypertension; swelling during pregnancy; diabetes mellitus, including gestational diabetes; obesity; pathology of hemostasis;

thyroid disease, history of hypothyroidism; chronic kidney disease without exacerbations) was detected in 12 out of 36 (33 .3%) pregnant women. Diabetes mellitus was registered in 58.3% (7 / 12) of cases, arterial hypertension and obesity were observed in 33.3% (4 / 12) of women, pathology of hemostasis – in 25% (3 / 12) of patients, and a history of thyroid disease – in 16 .6% (3/12) of cases. Swelling was observed in 33.3% (4 / 12) of women and chronic pyelonephritis – in 8.3% (1/12) of cases. The presence of four pathologies simultaneously was recorded in one pregnant woman (8.3%), three pathologies – in four patients (33.3%), and two pathologies – in two pregnant women (16.7%). In the remaining 5 cases, the presence of one extragenital pathology was noted. At the time of the examination, concomitant chronic diseases were in remission. All pregnancies in this study were singleton gestations. None of the participants had active labor at the time of enrollment and blood sampling.

Peripheral blood mononuclear cells (MNCs) were isolated by standard centrifugation of heparinized venous whole blood in the ficoll – verografin density gradient (ρ = 1.078). Erythrocyte lysis was performed with the VersaLyse lysing solution (Beckman Coulter, France) according to the instructions. Relative counts of CD8+CTLA-4+, CD8+PD-1+, CD8+TIM-3+, CD8+PD-1+TIM-3+, CD4+CTLA-4+, CD4+PD-1+, CD4+TIM-3+, and CD4+PD-1+TIM-3+ were estimated by flow cytometry using anti-CD8 (FITC), anti-CD4 (FITC, PerCP), anti-CTLA-4 (PE), anti-PD-1 (APC), and anti-TIM-3 (PE, PerCP / Cy 5.5) monoclonal antibodies (BD Biosciences, USA). The study was carried out according to the generally accepted method using the parameters of forward and side light scatter and fluorescence in the FL-1 (FITC), FL-2 (PE), FL-3 (PerCP, PerCP/Cy 5.5, PE-Cy 5), and FL-4 (APC) channels (BD FACSCalibur, CellQuest Software, USA). We focused on the evaluation of PD-1-expressing T cells, their counts in CD4+ and CD8+ cell subsets were studied in all 36 pregnant and 28 fertile non-pregnant controls. In addition, the expression of CTLA-4 (18 pregnant women and 20 non-pregnant women) and Tim-3 (19 pregnant women and 26 non-pregnant women), as well as the co-expression of PD-1 and Tim-3 (14 pregnant women and 16 non-pregnant women) were evaluated.

Statistical data processing was performed using the Statistica 6.0 (StatSoft) and GraphPad Prism 5 (GraphPad Software, Inc.) software packages. The Mann – Whitney U test was used to assess the significance of differences between two independent groups. Statistical differences between dependent groups were analyzed by the paired Wilcoxon signed rank test. The Spearman's rank correlation coefficient (Rs) was used to evaluate correlations. The data were presented as the median and the interquartile range Me (Q_1–Q_3). The differences were considered statistically significant at $p < 0.05$ (two-tailed).

RESULTS

A comparative analysis of the relative counts of PD-1+ cells in CD4+ T lymphocytes did not reveal significant differences between the groups of pregnant and fertile non-pregnant women (Fig.). At the same time, the proportion of PD-1+ cells in CD8+ T lymphocytes in pregnant women was 2.4 times higher than in the group of non-pregnant women. The proportion of PD-1+ cells in CD4+ lymphocytes was significantly higher than in CD8+ lymphocytes. This was manifested through a pronounced trend in the non-pregnant group ($p_w = 0.09$) and a statistically significant difference in the pregnant group ($p_w = 0.004$). The study of CTLA-4 expression did not reveal any differences in the content of CD4+CTLA-4+ and CD8+CTLA-4+ T lymphocytes in the compared groups ($p_w = 0.16$ and $p_w = 0.19$; respectively). At the same time, evaluation of Tim-3+ cells revealed a 5-fold increase in the proportion of Tim-3+ cells in CD4+ T lymphocytes in pregnant women compared with the non-pregnant group.

The percentage of Tim-3+ cells among CD8+ T lymphocytes in the compared groups did not differ (Fig.). Given the increased expression of PD-1+ and Tim-3+ on T cells, it seemed important to study the co-expression of these molecules. As seen from the data in the Figure, the proportion of PD-1+Tim-3+ cells in CD4+ T lymphocytes in pregnant women was 5 times higher than in the control group. Similarly, the relative count of CD8+ cells co-expressing PD-1 and Tim-3 in pregnant women was 2.3 times higher than in non-pregnant women.

It should be noted that, despite the absence of late pregnancy complications, especially preeclampsia, women with uncomplicated pregnancy differed in the presence of concomitant extragenital pathologies, which were detected in every third pregnant woman (see Materials and methods section). To find out whether the increased expression of inhibitory receptors on T cells had been associated with comorbidity, at the next stage we compared the counts of T cells expressing checkpoint molecules in the groups of pregnant women with and without extragenital pathology (Table 1).

Table 1

Expression of inhibitory receptors in subsets of T cells in pregnant women with and without comorbidities, *Me* (Q_1–Q_3)

Parameter	Fertile non-pregnant women	Pregnant women without extragenital pathology	Pregnant women with extragenital pathology
Clinical parameters		*n* = 24	*n* = 12
Age, years		26 (20–32)	29 (26–33)
Gestational age, weeks		34 (32–37)	35 (32–37)
Gravidity, times		1 (1–3)	1.5 (1–2)
Parity, times		1 (1–1)	1 (1–2)
T cell subsets, %			
$CD4^+PD\text{-}1^+$	4.1 (3.0–5.9) *n* = 28	4.8 (3.0–7.4) *n* = 24	6.6 (2.5–9.6) *n* = 12
$CD4^+CTLA\text{-}4^+$	2.6 (1.3–4.0) *n* = 20	3.8 (2.0–4.0) *n* = 10	2.4 (2.1–2.8) *n* = 8
$CD4^+Tim\text{-}3^+$	1.4 (1.0–2.9) *n* = 24	6.5 (1.6–8.6)* *n* = 14	9.7 (6.9–13)* *n* = 5
$CD8^+PD\text{-}1^+$	2.9 (1.1–5.9) *n* = 26	5.8 (4.7–8.3)* *n* = 24	10 (6.9–15)*# *n* = 12
$CD8^+CTLA4^+$	2.0 (0.9–3.4) *n* = 20	1.2 (0.5–4.0) *n* = 10	3.4 (1.3–5.5) *n* = 8
$CD8^+Tim\text{-}3^+$	5.3 (2.5–9.2) *n* = 26	5.8 (2.3–19) *n* = 14	1.1 (0.6–1.9)*# *n* = 5
$CD4^+PD1^+Tim3^+$	0.35 (0.19–0.5) *n* = 16	2.1 (1.6–2.8)* *n* = 10	0.27 (0.18–1.5) *n* = 4
$CD8^+PD1^+Tim3^+$	1.5 (0.7–2.3) *n* = 16	3.9 (2.2–4.5)* *n* = 10	0.8 (0.6–3.5) *n* = 4

* $p_U < 0.05$ – statistically significant differences compared with non-pregnant women; # $p_U < 0.05$ – statistically significant differences between pregnant women with and without extragenital pathology.

There were no significant differences between the studied groups in the age, gestational age, gravidity, and parity. It can also be seen that an increase in the number of CD4+Tim3+ and CD8+PD-1+ cells typical of pregnancy was revealed in both groups. However, it was more pronounced in the group of pregnant women with extragenital pathology, as indicated by a higher count of CD8+PD-1+ cells ($p_U = 0.03$) and a trend toward a higher count of CD4+Tim-3+ cells ($p_U = 0.1$) compared with pregnant women without concomitant pathologies. At the same time, an increase in the count of PD-1+Tim3+ T cells was observed only in pregnant women without a concomitant disease and was not detected in the group of pregnant women with a comorbidity. Another feature of pregnant women with extragenital pathology was a decrease in the count of CD4+Tim-3+ cells, both in non-pregnant women and in pregnant women without a concomitant disease.

Considering that the number of extragenital pathologies in different women varied from 0 to 4, a relationship between the expression of checkpoint molecules and the number of concomitant pathologies per pregnant woman was studied (Table 2). The relative count of CD8+PD-1+ cells was directly correlated with the number of extragenital diseases, while the proportion of CD8+3Tim-3+ cells was inversely correlated with the number of comorbidities. Besides, there were inverse correlations between the proportion of PD-1+Tim-3+Tcells and the number of extragenital diseases (statistically significant for CD4+PD-1+Tim-3+ T cells and as a trend for CD8+PD -1+Tim-3+ T cells).

Table 2

Correlations (Rs) between the expression of checkpoint molecules and the number of concomitant diseases, age, and gestational age

Subset	Number of comorbidities	Age	Gestational age
CD4+PD-1+, *n* = 36	0.25 (0.14)	–0.08 (0.64)	0.19 (0.26)
CD4+CTLA-4+, *n* = 18	–0.19 (0.44)	0.04 (0.86)	0.06 (0.81)
CD4+Tim-3+, *n* = 19	0.34 (0.15)	–0.23 (0.33)	–0.34 (0.14)
CD8+PD-1+, *n* = 36	0.37(0.02)	–0.004 (0.86)	–0.23 (0.18)
CD8+CTLA-4+, *n* = 18	0.2 (0.42)	–0.04 (0.86)	–0.01 (0.95)
CD8+Tim-3+, *n* = 19	–0.58 (0.01)	–0.43 (0.06)	0.5 (0.03)
CD4+ PD-1+Tim-3+, *n* = 14	–0.57 (0.03)	0.05 (0.87)	0.54 (0.04)
CD8+ PD-1+Tim-3+, *n* = 14	–0.51 (0.06)	–0.42 (0.10)	0.61(0.02)

Note: statistical significance of the correlation is presented in the parentheses.

Since the pregnant women included in the study were characterized by a fairly wide age range (from 18 to 45 years), and the gestational age varied from 26 to 40 weeks, we also studied the association of these factors with the expression of checkpoint molecules by T cells. As can be seen from the data presented in Table 2, there were no significant correlations between the relative counts of T cells expressing PD-1, CTLA-4, and Tim-3 and age. However, there were strong trends toward inverse correlations between age and both CD8+ Tim-3+ cells and CD8+PD-1+Tim-3+ cells. At the same time, the relative counts of CD8+-Tim-3+ cells, CD4+PD-1+Tim-3+, and CD8+PD-1+Tim-3+ cells were directly correlated with gestational age.

DISCUSSION

Activation of immunosuppressive mechanisms can be observed from the moment of embryo implantation and throughout the entire pregnancy. However, the role of inhibitory receptors in the implementation of these mechanisms during pregnancy, especially at the systemic level, remains poorly understood.

The present study has shown that women in the second half of uncomplicated pregnancy are characterized by an increased count of circulating CD4+-Tim-3+ and CD8+PD-1+ T cells, as well as CD4+ and CD8+ T lymphocytes co-expressing PD-1 and Tim-3 compared with fertile non-pregnant women, which indicates an increase in the expression of inhibitory receptors on peripheral T cells during pregnancy. Increased expression of inhibitory receptors during pregnancy was described on decidual T cells. It was shown that CD4+PD-1+TIM-3+ T lymphocytes have Th2 phenotype [12], and activation of the PD-1/PD-L1 signaling pathway suppresses production of Th1 cytokines [13]. In turn, increased expression of checkpoint molecules on decidual CD8+ T cells is discussed as one of the mechanisms for reducing the cytotoxic potential of CD8+ lymphocytes [12, 14–16]. Thus, in *in vitro* studies, the interaction of TIM-3 and PD-1 with the corresponding ligands suppresses the cytotoxic activity of CD8+ T cells, which can provide tolerance to fetal antigens [17, 18]. R. Slutsky et al. described CD4+ and CD8+ T cells with the phenotype of effector memory T cells co-expressing PD-1 and Tim-3 in the decidua, and for the first time identified them as exhausted T cells [4]. However, earlier, T cells with a similar phenotype at the initial stages of pregnancy were described by a number of authors as T cells with regulatory (Th2) activity [2].

Few publications describe expression of checkpoint molecules on peripheral T cells. J. Zhao. et al. did not reveal an increase in Tim-3 in CD3+ T lymphocytes of pregnant women [19]. M. Meggyes et al. in their studies also found no differences between the count of CD8+Tim-3+ in pregnant and non-pregnant women, however, CD8+TIM-3+ cells in pregnant women in all trimesters produced less proinflammatory cytokines (TNF-α, IFN-γ) compared with CD8+TIM-3 cells in non-pregnant women [20].

Later, it was shown that the counts of CD4+ and CD8+ T cells expressing PD-1 in the third trimester of pregnancy also did not differ from those in non-pregnant women [21]. These results are consistent with our data on the absence of differences in the counts of CD4+PD-1+ and CD8+TIM-3+ cells between pregnant and non-pregnant women, however, these groups differed in the count of CD4+TIM-3+ and CD8+PD-1+ T cells, which was elevated during pregnancy, according to our data. At the same time, we studied for the first time T cells co-expressing TIM-3 and PD-1 and showed an increase in their relative counts in CD4+ and CD8+ T lymphocytes during pregnancy. The existing discrepancies with the data of the mentioned authors may be due to the differences in the cohorts of the examined pregnant women; additionally, the cytometric analysis in the previous studies was performed in cryopreserved cells.

The second important result of this study is the analysis of checkpoint molecule expression on T cells of pregnant women, depending on the presence or absence of concomitant extragenital pathology. The increase in the number of T cells expressing PD-1 and Tim-3 in both groups suggests that elevated expression of inhibitory receptors on peripheral T cells is a consequence of gestation rather than a comorbidity. On the one hand, this fragment of studies showed that the presence of concomitant pathology affects the pattern of inhibitory molecule expression, in particular, it is associated with a more pronounced increase in the number of CD8+PD-1+ cells (compared with pregnant women without concomitant pathology). On the other hand, comorbidity is associated with a lower content of CD8+Tim-3+ and the absence (characteristic of pregnant women without comorbidity) of an increase in the number of T cells co-expressing PD-1 and Tim-3 molecules. An additional confirmation of the association between comorbidity and the expression of checkpoint molecules is the revealed correlations between the number of comorbidities and the

expression of PD-1 and Tim-3 (a direct correlation with the proportion of CD8+PD-1+ and an inverse correlation with the proportion of CD8+Tim-3+ and CD4+PD-1+Tim-3+ cells).

According to the literature, concomitant extragenital pathologies are usually associated with chronic inflammation and can significantly complicate pregnancy and, in some cases, lead to maternal mortality [22, 23]. Our results demonstrate that concomitant extragenital pathology makes a contribution to it by expressing checkpoint molecules on T cells. At the same time, the multidirectional relationship between the number of comorbidities and subsets of CD8+PD-1+ (positive relationship) and CD8+Tim3+ cells (negative relationship) may indicate a different role of PD-1 and Tim-3 molecules in the regulation of CD8+ cell functions during pregnancy.

Moreover, in this work, it was shown for the first time that the expression of inhibitory receptors on circulating T cells of pregnant women is associated with the age of women and gestational age. Of the three analyzed inhibitory receptors, the relationship with age and gestational age was detected only for Tim-3 and was the most pronounced in relation to its expression on CD8+ cells. According to the data obtained, a higher count of Tim-3+ T cells was noted in younger pregnant women and at longer gestational ages.

CONCLUSION

The data obtained indicate increased expression of a number of inhibitory receptors on peripheral T cells during pregnancy and substantiate the relevance of further research on checkpoint molecules as potential biomarkers in pregnancy with complications.

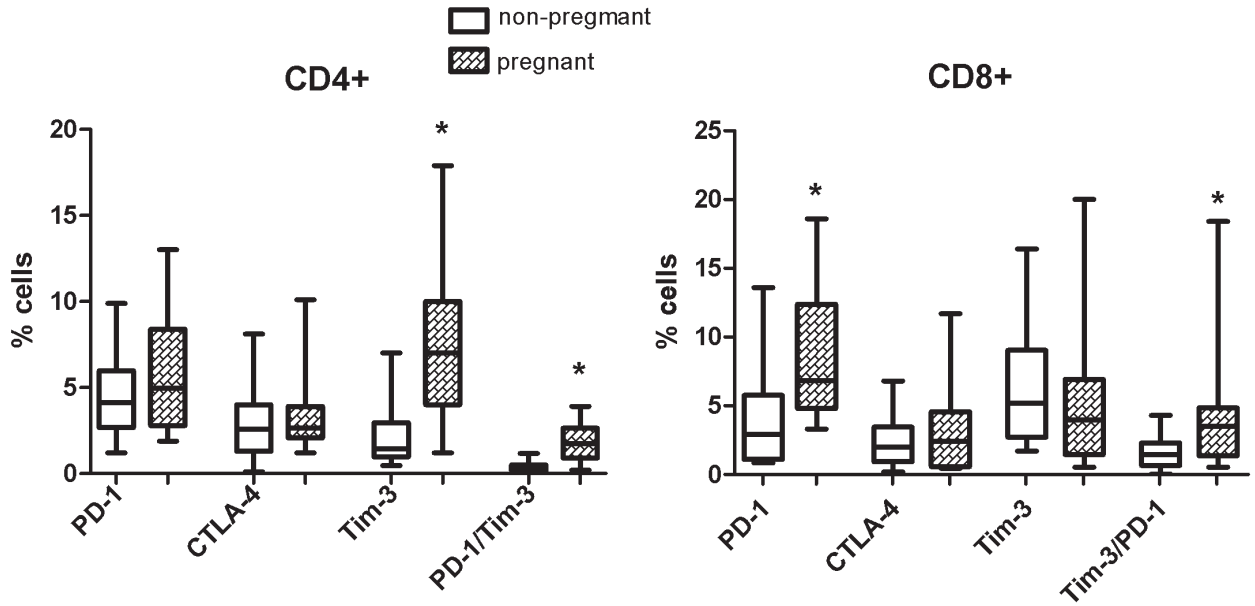

Figure. The content of T cells expressing inhibitory receptors in pregnant women with uncomplicated gestation and fertile non-pregnant women, *Me* (Q_1–Q_3)

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Authors contribution

Smetanenko E.A. – selection of patients, analysis of clinical data, drafting of the article. Batorov E.V., Tikhonova M.A. – preparation of samples, analysis of checkpoint expression on the flow cytometer. Leplina O. Yu. – analysis and interpretation of the data. Khonina N.A. – critical revision of the manuscript for important intellectual content, drafting of the article. Pasman N.M. – conception, drafting of the article. Chernykh E.R. – final approval of the article for publication.

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sPD-1/sPD-L1 proteins in non-small cell lung cancer and esophageal squamous cell carcinoma

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ABSTRACT

Background. Implementation of immunotherapy in clinical oncological practice has significantly improved the results of cancer treatment. It resulted in the need for seeking new markers to assess the effectiveness of therapy and the disease prognosis.

Aim. To analyze the content of soluble forms of PD-1 and PD-L1 immune checkpoint proteins in the blood serum of patients with non-small cell lung cancer and esophageal squamous cell carcinoma and their association with clinical and morphological characteristics of the disease and the disease prognosis.

Materials and methods. The study included tumor samples obtained from 43 patients with non-small cell lung cancer and 21 patients with esophageal squamous cell carcinoma. The concentration of sPD-L1 and sPD-1 in the blood serum was determined using enzyme-linked immunosorbent assay (ELISA). The Mann – Whitney test was used to determine statistically significant differences in independent groups. A correlation analysis was performed using the Spearman's rank correlation coefficient. Overall survival was analyzed by constructing survival curves using the Kaplan – Meier method and a Cox proportional hazards model. The differences were considered statistically significant at $p < 0.05$.

Results. The study showed that sPD-1 and sPD-L1 were found in the blood serum of both cancer patients and healthy donors, and their concentrations did not differ significantly. It was shown that the high concentration of sPD-L1 in the blood serum of patients with non-small cell lung cancer was significantly associated with the late stage of the disease and was an independent unfavorable prognostic factor. It should be noted that for patients with esophageal cancer, an unfavorable prognostic marker was the high concentration of the soluble form of PD-1 protein, and not PD-L1 ligand, as in case of lung cancer.

Conclusion. The content of sPD-1 and sPD-L1 in the blood serum can have different prognostic significance for various types of cancer, and further studies are required to confirm their clinical usability.

Keywords: sPD-1, sPD-L1, immunotherapy, non-small cell lung cancer, esophageal cancer, prognosis

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 N.N. Blokhin National Medical Research Center of Oncology.

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Белки sPD-1/sPD-L1 при немелкоклеточном раке легкого и плоскоклеточном раке пищевода

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РЕЗЮМЕ

Введение. Активное внедрение иммунотерапии в клиническую онкологическую практику позволило значительно улучшить результаты лекарственного лечения опухолей. Это привело к необходимости поиска новых маркеров, с помощью которых можно оценить эффективность проводимой терапии и прогноз заболевания.

Цель исследования – анализ содержания растворимых форм белков контрольных точек иммунитета sPD-1 и PD-L1 в сыворотке крови больных немелкоклеточным раком легкого и плоскоклеточным раком пищевода, а также их ассоциации с клинико-морфологическими характеристиками заболевания и прогнозом.

Материалы и методы. В исследование включены образцы опухолей от 43 пациентов с немелкоклеточным раком легкого и 21 пациента с плоскоклеточным раком пищевода. Концентрацию sPD-L1 и sPD-1 определяли в сыворотке крови с помощью иммуноферментного анализа. Для определения статистически значимых различий в независимых группах использовали критерий Манна – Уитни. Корреляционный анализ проводили с помощью определения коэффициента ранговой корреляции Спирмена. Анализ общей выживаемости – путем построения кривых дожития по методу Каплана – Мейера и с использованием модели пропорциональных рисков Кокса. Статистически достоверными считались различия при $p < 0,05$.

Результаты. Показано, что sPD-1и sPD-L1 обнаруживаются в сыворотке крови как у пациентов с онкологическими заболеваниями, так и здоровых доноров, и их концентрации значимо не отличаются. Показано, что высокая концентрация sPD-L1 в сыворотке крови больных немелкоклеточным раком легкого значимо ассоциирована с поздней стадией заболевания и является независимым неблагоприятным прогностическим фактором. Необходимо отметить, что для пациентов с раком пищевода неблагоприятным прогностическим маркером является высокое содержание растворимой формы рецептора PD-1, а не его лиганда PD-L1, как для рака легкого.

Выводы. Содержание в сыворотке крови sPD-1и sPD-L1 может иметь различное прогностическое значение для злокачественных опухолей различных нозологий, и необходимость его анализа для клинического применения требует дальнейшего изучения.

Ключевые слова: sPD-1, sPD-L1, иммунотерапия, немелкоклеточный рак легкого, рак пищевода, прогноз

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

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INTRODUCTION

Currently, immunohistochemistry is considered to be a gold standard in molecular diagnosis, which is used as the main method for making decisions on immunotherapy. However, available methods for predicting a patient's response to therapy or detection of residual disease using molecular biology research are insufficient. At the same time, the field of application of immunotherapy is constantly expanding, and there is an urgent need for new diagnostic markers or methods of their use that will allow to improve the results of cancer treatment.

According to their functional characteristics, immune checkpoints, which are the targets of immunotherapy, can be divided into two groups: molecules activating the immune response and those inhibiting it. The most well-described checkpoint molecules are PD-1 / PD-L1 and CTLA-4 that belong to the second group. The PD-1 / PD-L1 interaction promotes tumor escape from immune surveillance by suppressing T cell activity. High tissue expression of these proteins may be associated with a poor prognosis for various types of tumors.

In recent years, a large number of new receptors and their ligands involved in immune regulation have been identified and described. In addition to immune checkpoints on the cell surface, soluble forms of these proteins have been identified in the bloodstream. The presence of these proteins in biological fluids is due to proteolysis, alternative splicing, and their presence on the surface of exosomes [1–3]. It has been shown that as a result of proteolysis or alternative splicing, soluble forms of PD-1 and PD-L1 (sPD-1 and sPD-L1, respectively) can be generated [4]. sPD-L1 and sPD-1 can be detected and quantified in the bloodstream of patients with various solid tumors [5–7], which opens prospects for the development of methods for minimally invasive diagnosis and therapy monitoring. However, the role of sPD-L1 and sPD-1 in the pathogenesis of malignant tumors has not been clearly defined. In this regard, the aim of this study was to analyze the content of sPD-1 and sPD-L1 in the blood serum of patients with non-small cell lung cancer (NSCLC) and esophageal squamous cell carcinoma (ESCC) and their association with clinical and morphological characteristics of the disease and the disease prognosis.

MATERIALS AND METHODS

The study included 43 patients with NSCLC and 21 patients with ESCC, as well as 9 healthy donors who were examined and treated at the N.N.Blokhin National Medical Research Center of Oncology. All procedures involving patients and healthy donors, performed during the study, comply with the ethical principles of the local Ethics Committee and the 1964 Declaration of Helsinki and its subsequent amendments or comparable ethical standards. An informed consent was obtained from each individual included in the study. The clinical diagnosis in all patients was confirmed by the data of the morphological study of the tumor according to the WHO Classification of Tumors of the Digestive System (2019) and Tumors of the Lung (2021). The description of the studied samples is presented in Tables 1 and 2.

Table 1

Clinical and morphological characteristics of NSCLC patients

Parameter	Number of cases (%)
Age	
≤61	22 (51%)
>61	21 (49%)
Gender	
Male	37 (86%)
Female	6 (14%)
Histology	
Adenocarcinoma	18 (42%)
Squamous cell lung cancer	25 (58%)
Stage	
I–II	25 (58%)
III–IV	18 (42%)
Localization	
Central	26 (60%)
Peripheral	17 (40%)
Tumor size (T)	
T1–T2	28 (65%)
T3–T4	15 (35%)
Nodal status (N)	
N0	17 (40%)
N+	26 (60%)
Grade (G)	
G1–G2	27 (63%)
G3	16 (37%)

Table 2

Clinical and morphological characteristics of ESCC patients

Parameter	Number of cases (%)
Age	
≤62	11 (52%)
>62	10 (48%)
Gender	
Male	18 (86%)
Female	3 (14%)
Stage	
I–II	9 (43%)
III–IV	12 (57%)
Tumor size (T)	
T1–T2	6 (29%)
T3–T4	15 (71%)
Nodal status (N)	
N0	10 (48%)
N+	11 (52%)
Grade (G)	
G1–G2	15 (24%)
G3	6 (76%)

The levels of sPD-L1 and sPD-1 were determined in the blood serum obtained according to the standard method before the initiation of specific treatment using PD-L1 Human ELISA and PD-1 Human ELISA kits (Affimetrix, eBioscience, USA) in accordance with the manufacturer's instructions. The measurements were carried out on the automated ELISA analyzer BEP 2000 Advance (Siemens Healthcare Diagnostics, Germany). The concentrations of the markers were expressed in picograms (pg) per 1 ml of the blood serum.

The obtained data were processed using the GraphPad Prizm 9.0 software. To compare the variables and analyze their relationships, we used the nonparametric Mann – Whitney test and the Spearman's rank correlation coefficient. To analyze the overall survival, patients were divided into 2 comparison groups depending on the median content of sPD-1 and sPD-L1 in the blood serum. Overall survival was analyzed by constructing survival curves using the Kaplan – Meier method. Statistical significance of differences was compared using the log rank test. To assess the potential impact of various risk factors on survival, a multivariate analysis was additionally performed using a nonparametric Cox proportional hazards model. The differences and correlations were considered statistically significant at $p < 0.05$.

RESULTS

This study is devoted to the analysis of the content of soluble forms of sPD-1 and sPD-L1 checkpoint proteins in the blood serum of patients with NSCLC and ESCC and their association with the clinical and morphological characteristics of patients and the prognostic value. At the first stage of the study, the diagnostic potential of the studied proteins was assessed. The median concentrations of sPD-1 and sPD-L1 in the blood serum of healthy donors were 30.9 (28.2–42.8) pg / ml and 0.59 (0.40–1.5) pg / ml, respectively, in the group of patients with NSCLC – 34.3 (27.4–45.4) pg / ml and 0.95 (0.33–2.08) pg / ml, respectively, and in the group of patients with ESCC – 30.9 (26.0–53.9) and 0.956 (0–2.45) pg / ml, respectively. The statistical analysis showed that the content of soluble forms of sPD-1 and sPD-L1 did not differ significantly between healthy donors and patients with cancer, therefore, it was concluded that these proteins cannot be used as diagnostic markers. Further, the association between the levels of sPD-1 and sPD-L1 in the blood serum of patients with NSCLC and ESCC and the clinical and morphological characteristics of the diseases was analyzed. The results are presented in Tables 3 and 4.

Table 3

Association between serum sPD-L1 and sPD-1 levels and clinical and morphological characteristics of NSCLC patients

Parameter	sPD-1, pg / ml			sPD-L1, pg / ml		
	Me	(Q_1–Q_3)	*p*	*Me*	(Q_1–Q_3)	*p*
Age						
≤61	33.81	(27.22–51.69)	0.833	0.965	(0.401–1.835)	0.824
>61	34.60	(27.27–38.63)		1.090	(0.211–2.758)	
Gender						
Male	34.19	(27.17–45.57)	0.458	0.965	(0.464–2.143)	0.738
Female	35.25	(31.85–54.65)		0.776	(0.062–4.252)	
Histology						
Adenocarcinoma	34.47	(27.05–47.58)	0.765	0.903	(0.464–2.481)	0.513
Squamous cell lung cancer	34.19	(27.88–42.22)		0.965	(0.106–2.020)	
Stage						
I–II	33.44	(27.71–41.97)	0.966	0.715	(0.000–1.649)	0.037*
III–IV	34.98	(26.56–47.18)		1.463	(0.652–3.067)	

Table 3 (continued)

Parameter	sPD-1, pg / ml			sPD-L1, pg / ml		
	Me	(Q_1–Q_3)	*p*	*Me*	(Q_1–Q_3)	*p*
Localization						
Central	34.26	27.31–47.18	0.820	0.965	0.306–2.019	0.698
Peripheral	34.60	27.37–41.97		1.090	0.274–2.143	
Tumor size (T)						
T1–T2	33.88	(27.66–38.16)	0.701	0.965	(0.083–1.927)	0.372
T3–T4	35.63	(23.92–51.95)		1.214	(0.464–2.944)	
Nodal status (N)						
N0	35.56	(30.74–56.66)	0.153	0.715	(0.042–1.835)	0.419
N+	30.57	(26.31–40.41)		1.028	(0.464–2.298)	
Grade (G)						
G1–G2	34.33	(27.65–51.60)	0.394	1.214	(0.211–2.575)	0.513
G3	32.96	(24.66–36.84)		0.840	(0.464–1.494)	

The analysis showed that the increased concentration of sPD-L1 in the blood serum of patients with NSCLC was significantly associated with the late stage of the disease. No associations with other tumor characteristics were found for NSCLC, although it should be noted that there was a trend toward a decrease in the sPD-1 concentration in the blood serum of patients in the presence of regional metastasis.

For ESCC, it was found that in the group of patients with T1–T2 tumors, the concentration of sPD-L1 was significantly higher than in the group of patients with T3–T4 tumors (Table 4).

Table 4

Association between serum concentrations of sPD-L1 and sPD-1 and clinical and morphological characteristics of ESCC patients

Parameter	sPD-1, pg / ml			sPD-L1, pg / ml		
	Me	(Q_1–Q_3)	*p*	*Me*	(Q_1–Q_3)	*p*
Age						
≤62	30.98	(29.62–37.15)	0.545	0.965	(0.000–2.821)	0.685
>62	29.08	(24.27–59.24)		0.840	(0.000–2.174)	
Gender						
Male	30.98	(25.29–51.32)	0.406	0.778	(0.000–2.451)	0.836
Female	33.57	(30.23–72.88)		1.090	(0.000–2.944)	
Stage						
I–II	32.28	(29.92–53.90)	0.496	1.463	(0.295–2.698)	0.230
III–IV	29.52	(24.92–63.95)		0.274	(0.000–2.111)	
Tumor size (T)						
T1–T2	53.90	(33.04–78.70)	0.010*	1.772	(0.818–2.852)	0.202
T3–T4	29.62	(24.73–32,28)		0.464	(0.000–2.451)	
Nodal status (N)						
N0	32.92	(29.23–59.24)	0.306	1.277	(0.443–2.574)	0.194
N+	30.23	(24.73–37.15)		0.083	(0.000–2.451)	
Grade (G)						
G1–G2	30.98	(25.56–36.25)	0.436	1.028	(0.000–2.729)	0.473
G3	48.75	(26.09–80.21)		0.464	(0.000–1.772)	

PROGNOSTIC VALUE OF SPD-L1 / SPD-1 IN PATIENTS WITH NSCLC AND ESCC

Depending on the concentration of soluble forms of the studied proteins, for the analysis of survival rates, the patients were divided into 2 groups: with high and low levels of sPD-1 / sPD-L1 relative to the median. Patient survival graphs are shown in the Figure.

The results of the study showed that the increased concentration of sPD-L1 in NSCLC was significantly associated with a poor disease prognosis. For sPD-L1 in ESCC, a similar pattern was observed, but the data did not reach statistical significance. The concentration of sPD-1 in the blood serum is not a prognostic marker in patients with both NSCLC and ESCC. However, it should be noted that

in patients with a high content of sPD-1 in the blood serum, a trend toward an unfavorable prognosis was noted.

Next, a univariate and multivariate statistical analysis of the prognostic value of the studied markers was carried out. The results are presented in Table 5.

Table 5

Statistical analysis of the prognostic value of sPD-L1 / sPD-1 in patients with NSCLC and ESCC

Parameter	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p*	HR	95% CI	*p*
sPD-1 NSCLC (high / low)	0.885	(0.285–2.749)	0.831	1.020	(0.985–1.053)	0.253
sPD-L1 NSCLC (high / low)	3.937	(1.257–12.34)	0.026*	1.214	(0.983–1.464)	0.046*
sPD-1 ESCC (high / low)	2.199	(0.731–6.613)	0.139	1.045	(1.014–1.082)	0.006*
sPD-L1 ESCC (high / low)	1.998	(0.647–6.164)	0.199	1.269	(1.002–1.650)	0.051

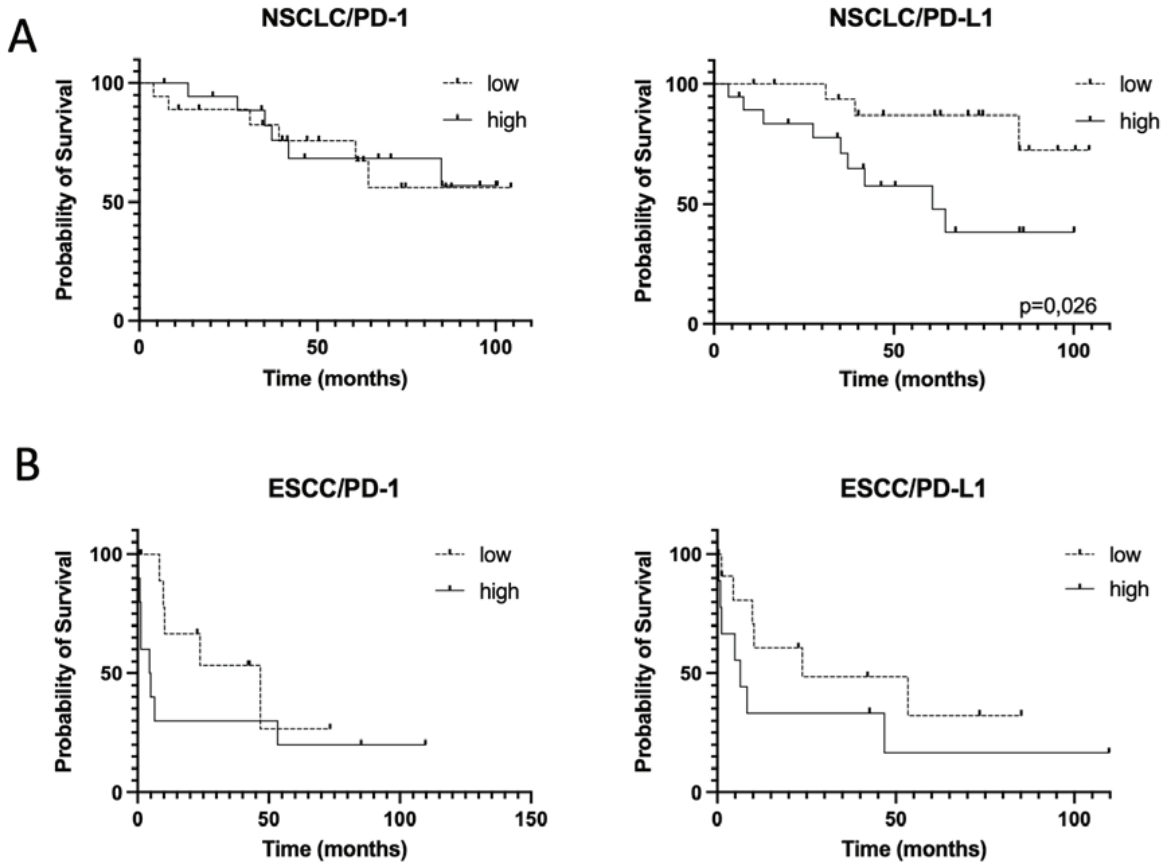

Figure. Analysis of the overall survival in NSCLC and ESCC patients depending on serum concentrations of sPD-L1 and sPD-1

Cox's regression analysis showed that high levels of sPD-1 in ESCC and high levels of sPD-L1 in NSCLC were independent prognostic factors associated with reduced survival. Thus, a high content of the soluble form of the sPD-L1 ligand in the blood serum of patients with NSCLC is an unfavorable prognostic factor, while for patients with ESCC, a high content of the soluble form of the sPD-1 receptor, rather than its ligand, is an unfavorable prognostic marker.

At the final stage of the study, a correlation analysis of the content of sPD-1 / sPD-L1 in the blood serum of patients with NSCLC and ESCC was carried out. The analysis did not reveal a correlation between the content of the studied proteins in ESCC ($r = 0.119$; $p = 0.609$), however, in NSCLC, the content of sPD-1 significantly correlated with the content of sPD-L1 $r = 0.331$; $p = 0.03$). It may indicate that the mechanisms of interaction of these proteins may be tissue independent.

DISCUSSION

Despite the rapid development of immunotherapy in the treatment of cancer, the frequency of objective responses to drugs of this class for patients with ESCC and NSCLC is insufficient. In this regard, the identification and validation of new biomarkers of the effectiveness of immunotherapy drugs is currently extremely relevant. The results of modern studies demonstrate that the assessment of PD-L1 expression is a key factor for evaluating the effectiveness of treatment with immune checkpoint inhibitors in patients with malignant tumors of various localizations. However, the analysis of tissue expression of PD-L1 is not unified and differs depending on the drug used. Thus, there is an evaluation scale that takes into account only tumor cells, while another evaluation method takes into account the expression of PD-L1 on the surface of both tumor and immune cells of the stroma. In addition, currently, when methods for minimally invasive diagnosis and neoadjuvant chemotherapy are being developed, it is of great importance to have a possibility to evaluate the effectiveness of prescribed drugs and the response to ongoing treatment using soluble blood markers [8].

Currently, anti-PD-1/PD-L1 immunotherapy is the preferred second-line, and in some cases, first-line therapy for NSCLC [9]. From the literature data, it is known that sPD-L1 in the blood can be considered as a marker of the effectiveness of treating patients with NSCLC with immune checkpoint inhibitors. It was shown that a high level of sPD-L1 two months after treatment with nivolumab was associated with a poor response to ongoing therapy. It should be noted that, according to the results of this study, the concentration of sPD-L1 did not correlate with the level of tissue expression of this protein [10]. The PD-1 / PD-L1 checkpoint inhibitors nivolumab and pembrolizumab are both FDA and EMA approved for the treatment of esophageal tumors, but the use and efficacy of these drugs, similar to NSCLC, depend on PD-L1 expression in the tumor. There is emerging evidence that sPD-L1 levels also have a predictive potential for evaluating the effectiveness of anti-PD-1/PD-L1 monotherapy in cases of ESCC [8], namely, a higher plasma concentration of sPD-L1 before treatment is a predictor of increased effectiveness of this type of therapy.

The present study analyzed the concentrations of soluble sPD-1 and sPD-L1 in the blood serum of patients with NSCLC and ESCC. The obtained results showed that an increased level of sPD-L1 in the blood serum of patients with NSCLC was associated with a later stage of the disease and a poor disease prognosis. The results obtained are consistent with the literature data [11–13]. Moreover, D. Jovanovic et al. demonstrated that a higher level of sPD-L1 in the blood is typical of patients with NSCLC, compared with lung tumors of other types [13]. It should be noted that the study of the sPD-L1 content in the blood of patients with lung cancer is relevant not only in the context of immunotherapy, but also in the study of the effectiveness of tyrosine kinase inhibitors [14]. In our study, the level of sPD-1 in NSCLC was not prognostically significant, although literature data show that an increase in the concentration of this protein is associated with a rise in overall and disease-free survival rates in patients with advanced NSCLC with *EGFR* mutations treated with erlotinib [15].

The analysis of the association of sPD-1 level with clinical and morphological characteristics of esophageal cancer showed that a higher concentration of this protein is characteristic of T1–T2 tumors. At the same time, it should be noted that our study showed for the first time that sPD-1 is an independent statistically significant marker of a poor ESCC prognosis. High serum levels of PD-L1 in patients with esophageal tumors were also associated with a poor prognosis, but the data did not reach statistical significance. This is consistent with a number of published studies [16, 17]. The obtained results together with the published data indicate that additional studies with larger cohorts are needed making it possible to use sPD-1 and sPD-L1 in clinical practice.

CONCLUSION

The results of the study indicate that currently there is no unequivocal opinion regarding the clinical and prognostic value of both tissue expression of the key immune checkpoints PD-1 / PD-L1 and their soluble forms. It is also worth noting that most studies have revealed the absence of a correlation between the tissue expression of these proteins and their presence in a soluble form in biological fluids, which certainly indicates the need for further study of their interaction. With further accumulation of data, their spread will decrease, which will ultimately lead to the possibility of their application in clinical practice.

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Authors contribution

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Association of polymorphic variants of *GRIN2A* and *GRIN2B* genes with alcohol and tobacco abuse in patients with schizophrenia

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ABSTRACT

Aim. To compare the frequency of genotypes for polymorphic variants of *GRIN2A* and *GRIN2B* genes in patients with schizophrenia and addictive behavior (alcohol / tobacco abuse) and in patients with schizophrenia without addictive behavior in the Slavic population of the Tomsk region.

Materials and methods. The study included 219 inpatients with the established diagnosis of schizophrenia who received treatment in the clinics of Mental Health Research Institute and Tomsk Clinical Psychiatric Hospital. A history of alcohol / tobacco abuse was identified during a clinical interview and objective data collection. DNA was isolated from peripheral blood leukocytes by standard phenol – chloroform extraction.

15 single nucleotide polymorphisms (SNPs) in the *GRIN2A* gene and 9 polymorphisms in the *GRIN2B* gene were selected for genotyping. Allelic variants were determined by real-time polymerase chain reaction (PCR) with specific primers. The SPSS 17.0 software package was used for statistical data processing. The distribution of genotype frequency was assessed using the Pearson's $\chi 2$ test with the Yates' correction and the Fisher's exact test.

Results. Significant differences in the allele frequency for the rs9788936 polymorphism in the *GRIN2A* gene ($\chi 2 = 4.23$, $p = 0.04$) and for the rs10845838 polymorphism in the *GRIN2B* gene ($\chi 2 = 4.27$, $p = 0.04$) were revealed between the groups of patients with and without alcohol abuse. It was found that the polymorphic variant rs8049651 of the *GRIN2A* gene had a clear association ($F = 8.06$, $p = 0.029$) with the development of tobacco addiction in patients with schizophrenia.

Conclusion. The study identified the association between alcohol abuse and the rs9788936 polymorphism in the *GRIN2A* gene and the rs10845838 polymorphism in the *GRIN2B* gene in patients with schizophrenia. The association between the rs8049651 and rs7190619 polymorphisms in the *GRIN2A* gene and the development of tobacco abuse in patients with schizophrenia was revealed.

Keywords: schizophrenia, genetics, single nucleotide polymorphisms, smoking, alcohol addiction, glutamate

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 included in the treatment and control groups signed an informed consent to participate in the study. The study was approved by the Ethics Committee at the Mental Health Research Institute, Tomsk NRMC (Protocol No. 142 of 14.05.2021).

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Ассоциации полиморфных вариантов генов *GRIN2A* и *GRIN2B* со злоупотреблением алкоголем и табаком у больных шизофренией

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РЕЗЮМЕ

Цель. Сравнить частоты генотипов полиморфных вариантов генов *GRIN2A* и *GRIN2B* в группах больных шизофренией с аддиктивным поведением (злоупотребление алкоголем и курение табака) и без них в славянской популяции Томской области.

Материалы и методы. Обследованы 219 пациентов с установленным диагнозом «шизофрения», проходивших стационарное лечение в клиниках НИИ психического здоровья Томского НИМЦ и Томской клинической психиатрической больницы. Наличие злоупотребления алкоголем и курения в анамнезе выявлялось в процессе клинического интервью и сбора объективных сведений. ДНК выделяли из лейкоцитов периферической крови стандартным фенол-хлороформным методом.

Для генотипирования было выбрано 15 SNP в гене *GRIN2A* и 9 полиморфизмов в гене *GRIN2B*. Определение аллельных вариантов проводили методом real-time PCR со специфическими праймерами. Для статистической обработки данных использовался пакет программ SPSS 17.0. Распределение частот генотипов оценивалось при помощи критерия χ2 Пирсона с поправкой Йетса и точного теста Фишера.

Результаты. Выявлены статистически значимые различия в частотах аллелей полиморфизма rs9788936 в гене *GRIN2A* ($\chi^2 = 4{,}23$; $p = 0{,}04$), а также полиморфного варианта rs10845838 в гене *GRIN2B* ($\chi^2 = 4{,}27$; $p = 0{,}04$) в группах пациентов, злоупотребляющих алкоголем, и непьющих. Было установлено, что полиморфный вариант rs8049651 гена *GRIN2A* имеет четкую ассоциацию ($F = 8{,}06$; $p = 0{,}029$) с формированием зависимости от табака у больных шизофренией.

Заключение. Показаны ассоциации злоупотребления алкоголем с полиморфным вариантом rs9788936 в гене *GRIN2A* и полиморфным вариантом rs10845838 в гене *GRIN2B* у пациентов с шизофренией, а также ассоциация полиморфных вариантов rs8049651 и rs7190619 гена *GRIN2A* с формированием табачной зависимости у больных шизофренией.

Ключевые слова: шизофрения, генетика, однонуклеотидные полиморфизмы, курение, алкоголизм, глутамат

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

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Соответствие принципам этики. Все лица, входившие в основную и контрольную группы, дали информированное согласие на участие в исследовании. Исследование одобрено этическим комитетом НИИ психического здоровья Томского НИМЦ (протокол № 142 от 14.05.2021).

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INTRODUCTION

Psychoactive substance abuse is quite common in patients with schizophrenia. Thus, the risk of developing mental disorders associated with alcohol abuse in this cohort is three times higher than in the general population [1]. A recent meta-analysis showed that the lifetime prevalence of alcohol-related disorders in this group of patients is 24.3% [2]. Symptoms of alcohol abuse are often present before the onset of a psychotic disorder [3]. It does not matter which of these disorders manifests earlier, as their comorbidity is characterized by poor prognosis, increased risk of relapses, and instability of remissions [4]. In individuals with schizophrenia, alcohol abuse is associated with depression, a high risk of suicide, poor adherence to antipsychotic medications, chronic physical illness, homelessness, high levels of aggression, and frequent hospitalizations [5]. Alcohol abuse in patients with schizophrenia often contributes to the development of a hostile attribution bias, which is closely associated with delusions, delusional thinking, and the ability to recognize emotions [6].

Currently, there are a number of theories that attempt to explain high comorbidity of schizophrenia and alcohol abuse. First of all, this is the diathesis – stress model proposed in 1970 to describe the contribution of predisposed vulnerability and life experiences to the development of the disorder [7]. The alternative is the self-medication hypothesis, which suggests that individuals with schizophrenia use psychoactive substances to relieve symptoms or as an attempt to reduce adverse events of antipsychotic treatment. However, the latter has not been confirmed by research; among young people with a first episode psychosis, substance use disorder often developed before treatment [8]. There is also a unifying hypothesis that comorbidity of schizophrenia and substance abuse may be associated with dysregulation of the reward pathway in the brain (primary addiction hypothesis or reward deficiency syndrome), which was studied using functional magnetic resonance imaging (fMRI) [9].

The idea that early brain development disruption may lead to the onset of schizophrenia in late adolescence or early adulthood is one of the most promising theories. It is notable that in animal models of schizophrenia (based on neonatal ventral hippocampal damage), experimental rats consumed more psychoactive substances than the control group [10]. These data indicate a high probability of a common biological and, possibly, genetic substrate in schizophrenia and alcohol-related disorders.

The prevalence of tobacco smoking among patients with schizophrenia is 2–3 times higher than in the general population in Western countries [11]. Recent epidemiological, biological, and clinical data indicate a correlation between smoking, severity of positive symptoms, and the frequency of suicidal tendencies in patients with schizophrenia [12].

Some studies show that there are also common pathogenetic pathways in schizophrenia and tobacco addiction. A group of authors led by S. Leonard [13] found that various polymorphic patterns in the promoter region of the nicotinic acetylcholine receptor α7-subunit gene (*CHRNA7* or α7), leading to a decrease in the transcription of this gene, are significantly more frequent in patients with schizophrenia compared with healthy individuals.

It was established that negative symptoms of schizophrenia are associated with NMDA receptor hypofunction [14]. Exposure to nicotine during systematic tobacco smoking increases the density of NMDA receptors in the hippocampus and at the same time enhances glutamatergic activity through the activation of presynaptic nicotinic acetylcholine receptors [15]. It leads to stimulation of the release and metabolism of dopamine, one of the fundamental neurotransmitters in the pathogenesis of schizophrenia. Also, nicotinic acetylcholine receptors modulate activity in the frontal and parietal lobes of the cerebral hemispheres, thereby participating in excitation and motor activity, as well as in glutamatergic neurotransmission [16, 17].

Glutamate is the main excitatory neurotransmitter of the neocortex [18]. The imbalance between glutamate and its metabolite, the inhibitory neurotransmitter GABA, is involved in the pathogenesis of a number of mental and neurodegenerative diseases [19, 20]. The main physiological function of glutamate is participation in the intracellular regulation of Ca^{2+} ions. Accumulation of glutamate in the extracellular space and, as a result, entry of high levels of calcium ions into the cell through NMDA receptors underlie the phenomenon of excitotoxicity [21].

Glutamatergic activity plays a role in brain development, synaptic plasticity, mood disorders, and schizophrenia spectrum disorders [22, 23]. Proteins encoded by the *GRIN2A* and *GRIN2B* genes are subunits of the NMDA receptor, a member of the ionotropic glutamate receptor superfamily. The GRIN2B protein also acts as a receptor agonist binding site. In turn, NMDA receptors are involved in the calcium-mediated component of excitatory synaptic transmission in the central nervous system.

Based on these data, we have formulated a hypothesis that polymorphic variants of the *GRIN2A* and *GRIN2B* genes may be associated with the development of addictive behavior in patients with schizophrenia.

The aim of this study was to compare the frequency of genotypes for polymorphic variants of *GRIN2A* and *GRIN2B* genes in patients with schizophrenia with comorbid addictive behavior (alcohol abuse and tobacco use) and in patients with schizophrenia without addictive behavior in the Slavic population of the Tomsk region.

MATERIALS AND METHODS

The study involved 219 patients with an established diagnosis of schizophrenia who received inpatient treatment at the clinics of the Mental Health Research Institute of Tomsk NRMC and in the Tomsk Regional Psychiatric Hospital. Alcohol abuse and smoking history were identified during a clinical interview and objective data collection.

The study was carried out in accordance with ethical standards of the Declaration of Helsinki developed by the World Medical Association "Ethical Principles for Medical Research Involving Human Subjects" as amended in 2000 and the "Rules of Clinical Practice in the Russian Federation" approved by Order of the Ministry of Healthcare of the Russian Federation No. 266 of 19.06.2003. The main inclusion criteria for patients were a verified diagnosis of schizophrenia according to ICD-10 (International Classification of Diseases, Tenth Revision), age 18–65 years, a signed informed consent from the patient, Caucasian race, and permanent residence in Western Siberia. Exclusion criteria were acute and chronic infectious, inflammatory, autoimmune, and somatic symptom diseases in the acute phase, as well as dependence on opioids, cannabis, sedatives, hypnotics, cocaine, and other stimulants, including caffeine, hallucinogens, and inhalants.

The sample size was 219 people: 144 men (65.8%) and 75 women (34.2%). The study included persons aged 18–65 years, the average age was 38.9 ± 13.4 years.

The majority of the examined patients – 114 (52.1%) – had disease duration of more than 10 years. The disorder duration of 5–10 years was observed in 44 patients (20.1%), duration of 1–5 years – in 47 patients (21.5%), and less than 1 year – in 8 patients (3.7%). The age of disease onset and duration could not be established in three people. The duration of alcohol and tobacco abuse corresponded to that of the disease.

Daily chlorpromazine equivalent (CPZeq) dose of all antipsychotics was 536 [240; 762.5] mg; the median duration of treatment was 11 [4; 21] years. During the initial examination of patients using the PANSS, we obtained the following results (Table 1).

Table 1

PANSS parameters in patients of the sample

Scale	*n*	*Me* [Q_1; Q_3]
Positive symptoms	219	21 [18; 26]
Negative symptoms	219	26 [22; 31]
General psychopathological symptoms	219	52 [42; 63]
Total	219	99 [85; 118]

DNA was isolated from peripheral blood leukocytes using standard phenol – chloroform extraction. A total of 15 single nucleotide polymorphisms (SNPs) in the *GRIN2A* gene and 9 SNPs in the *GRIN2B* gene were selected for genotyping. Allelic variants were determined by real-time PCR with specific primers using the SNP Genotyping Assay kits on StepOnePlus real-time PCR system (USA). SPSS 17.0 software was used for statistical data processing. Genotype frequency distribution was assessed using the Pearson's $\chi 2$ test with the Yates's correction and the Fisher's exact test (*F*).

RESULTS

Statistically significant differences were found in the allele frequency of the rs9788936 polymorphism in the *GRIN2A* gene ($\chi^2 = 4.23$; $p = 0.04$) and the rs10845838 polymorphism in the *GRIN2B* gene ($\chi^2 = 4.27$; $p = 0.04$) in the group of patients with comorbid alcohol abuse and in the group of patients without alcohol abuse (Table 2). The G allele of the rs9788936 polymorphism (odds ratio (OR) = 0.47; 95% confidence interval (CI): 0.22–0.98) and the A allele of the rs10845838 polymorphism (OR = 0.60; 95% CI: 0.37–0.98) have a protective effect. At the same time, the A allele of the rs9788936 polymorphism (OR = 2.15; 95% CI: 1.02–4.51) and the G allele of the rs10845838 polymorphism (OR = 1.64; 95% CI: 1.02–2.72) predispose to alcohol abuse.

It was also found that the rs8049651 polymorphic variant of the *GRIN2A* gene had a clear association ($F = 8.06$; $p = 0.029$) with tobacco addiction development in patients with schizophrenia (Table 3). In terms of statistical significance, the AG genotype was less common in the group of smokers (OR = 0.48; 95%

CI: 0.26–0.87) compared with the group of non-smokers. Also, the analysis of the allele distribution showed that their frequencies in the rs7190619 polymorphic variant of the *GRIN2A* gene were significantly different in the groups of smokers and non-smokers ($\chi^2 = 4.71$; $p = 0.03$). The OR for the A allele was 0.49 [95% CI: 0.25–0.94], for the G allele it was 2.05 [95% CI: 1.06–3.94].

Table 2

Frequency distribution for genotypes and alleles of *GRIN2A* and *GRIN2B* gene polymorphisms in persons with schizophrenia with alcohol abuse and in persons with schizophrenia without alcohol abuse, abs., %

SNP	Genotypes / Alleles	Alcohol abuse	No alcohol abuse	OR	95% CI	*F*/χ2	*p*
GRIN2A							
rs9788936	AA	34 (79.1%)	102 (63.0%)	0.45	0.20–1.00	*F* = 4.32	0.18
	AG	9 (20.9%)	55 (34.0%)	1.94	0.87–4.34		
	GG	0 (0%)	5 (3.0%)	–	–		
	A	77 (89.5%)	259 (79.9%)	2.15	1.02–4.51	$\chi^2 = 4.23$	0.04*
	G	9 (10.5%)	65 (20.1%)	0.47	0.22–0.98		
rs11866328	AA	4 (9.3%)	19 (11.4%)	1.26	0.41–3.92	*F* = 0.12	0.969
	AC	15 (34.9%)	90 (54.2%)	2.21	1.10–4.44		
	CC	24 (55.8%)	57 (34.3%)	0.41	0.21–0.82		
	A	23 (26.7%)	128 (38.6%)	0.58	0.34–0.99	$\chi^2 = 28.9$	< 0.001*
	C	63 (73.3%)	204 (61.4%)	1.72	1.02–2.91		
GRIN2B							
rs10845838	AA	8 (19.0 %)	19 (11.7%)	0.56	0.23–1.39	$\chi^2 = 4.3$	0.12
	AG	22 (52.4%)	70 (42.9%)	0.68	0.35–1.35		
	GG	12 (28.6%)	74 (45.4%)	2.08	0.99–4.34		
	A	38 (45.2%)	108 (33.5%)	1.64	1.005–2.67	$\chi^2 = 4.27$	0.04*
	G	46 (55.8%)	214 (66.5%)	0.61	0.38–0.995		

* p value < 0.05 (here and in Table 3).

Table 3

SNP	Genotypes / Alleles	Smokers	Non-smokers	OR	95% CI	*F*/χ2	*p*
rs7190619	AA	0 (0%)	3 (6.0%)	–	–	*F* = 7.29	0.051
	AG	25 (19.4%)	12 (24.0%)	0.76	0.35–1.66		
	GG	104 (70.6%)	35 (70.6%)	1.78	0.85–3.76		
	A	25 (9.7%)	18 (18.0%)	0.49	0.25–0.94	$\chi^2 = 4.71$	0.03*
	G	233 (90.3%)	82 (82.0%)	2.05	1.06–3.94		
rs8049651	AA	19 (12.2%)	4 (6.7%)	1.94	0.63–5.96	*F* = 8.06	0.029*
	AG	65 (41.7%)	36 (60.0%)	0.48	0.26–0.87		
	GG	72 (46.1%)	20 (33.3%)	1.71	0.92–3.19		
	A	103 (33.0%)	44 (36.7%)	0.85	0.55–1.32	$\chi^2 = 0.52$	0.47
	G	209 (67.0%)	76 (64.3%)	1.18	0.76–1.82		

DISCUSSION

In this study, we analyzed the associations of alcohol and tobacco abuse with 17 polymorphic variants of two genes involved in glutamate metabolism. The results led to the conclusion that two polymorphisms (rs8049651 and rs7190619) in the *GRIN2A* gene contribute to the development of tobacco addiction in patients with schizophrenia. Polymorphic variants rs9788936 and rs11866328 of the *GRIN2A* gene and rs10845838 of the *GRIN2B* gene demonstrated a strong association with alcohol abuse in both groups. In addition, the rs2072450 polymorphism in the *GRIN2A* gene may be associated with disruptions in aversion learning, and its allelic state may be one of the risk factors for alcohol addiction development [24].

The rs2058878 and rs2300272 polymorphisms of the *GRIN2B* gene may serve as an indicator of the ef-

fectiveness of acamprosate in the treatment of alcohol-related disorders [25], since researchers demonstrated an association of allelic variants of these polymorphisms with the duration of alcohol abstinence in patients.

The role of heredity in the pathogenesis and clinical presentation of schizophrenia is undeniable, and the associations we found also reflect this association. The influence of genetic factors and glutamatergic neurotransmission on the development of pathological behavioral patterns in patients with schizophrenia is a poorly studied issue in modern biological psychiatry.

The majority of genetic studies on schizophrenia are associated with its key symptoms (positive, negative, and cognitive), while a few works dedicated to comorbidity of schizophrenia and addictive disorders cannot fully cover the issue yet. Further research in this area, combining both genetic and clinical approaches, may define the role of glutamate in the development of addictive behavior in patients with schizophrenia.

CONCLUSION

This study showed associations of alcohol abuse with the rs9788936 polymorphic variant in the *GRIN2A* gene and the rs10845838 polymorphic variant in the *GRIN2B* gene in patients with schizophrenia. The study also demonstrated that rs8049651 and rs7190619 polymorphic variants of the *GRIN2A* gene have a clear association with the development of tobacco addiction in patients with schizophrenia.

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Authors contribution

Tiguntsev V.V. – collection of clinical and biological material, analysis and interpretation of the data, drafting of the manuscript. Gerasimova V.I. – collection of clinical material, drafting of the manuscript. Kornetova E.G. – conception and design, drafting of the manuscript, final approval of the manuscript for publication. Fedorenko O.Yu. – conception and design. Semke A.V. – substantiation of the manuscript. Kornetov A.N. – critical revision of the manuscript for important intellectual content.

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Insulin-like growth factors and their carrier proteins in kidneys of rats with experimental diabetes, malignant tumor, and their combination

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ABSTRACT

Persistent hyperglycemia resulting from diabetes mellitus causes microvascular lesions and long-term diabetic complications, such as nephropathy.

The aim of the study was to analyze the levels of insulin-like growth factors (IGFs), their carrier proteins (IGFBP), and markers of kidney tissue damage (IL-18, L-FABP, cystatin C, NGAL, and KIM-1) in male rats with diabetes mellitus, tumor growth, and their combination.

Materials and methods. The study included white outbred male rats (n = 32) weighing 180–220 g. The animals were divided into four groups (n = 8 each): group 1 – intact animals; controls (2) – animals with diabetes mellitus; controls (3) – animals with Guerin carcinoma; experimental group (4) – animals with Guerin carcinoma against the background of diabetes mellitus. Levels of IGF-1, IGF-2, IGFBP-1, IGFBP-2 and markers of acute kidney injury (IL-18, L-FABP, cystatin C, NGAL, and KIM-1) were determined in the kidney homogenates using enzyme-linked immunosorbent assay.

Results. Increased levels of acute kidney injury markers were found in the kidneys of male rats with diabetes mellitus alone and in combination with Guerin carcinoma. In the animals with diabetes mellitus, the levels of IGF-1, IGFBP-1, and IGFBP-2 were decreased on average by 1.3 times, and the level of IGF-2 was increased by 2.1 times compared with the values in the intact male rats. The elevation of IGF-2 / IGF-1 on average by 2.8 times indicated increasing hypoglycemia in the kidney tissue of the animals with diabetes mellitus and in the experimental group with diabetes mellitus and Guerin carcinoma. In the kidney tissues of the rats with Guerin carcinoma, IGF-1 and IGF-2 were elevated on average by 1.5 times, and IGFBP-2 was decreased by 1.7 times. In the animals with malignant tumors growing against the background of diabetes mellitus, IGF-2 and IGFBP-1 were increased by 2.3 and 1.7 times, respectively, and the levels of IGF-1 and IGFBP-2 were similar to those in the intact animals.

Conclusion. The study demonstrated abnormalities in the metabolic profile of the kidneys in male rats with experimental diabetes mellitus, Guerin carcinoma, and their combination.

Keywords: diabetes mellitus, Guerin carcinoma, markers of acute kidney injury, IGF, IGFBP

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Инсулиноподобные факторы роста и их белки-переносчики в почках крыс при экспериментальном диабете, злокачественном росте и их сочетании

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РЕЗЮМЕ

Устойчивая гипергликемия в результате сахарного диабета вызывает повреждение микрососудов и долгосрочные диабетические осложнения, такие как нефропатия.

Целью настоящего исследования явилось изучение уровня инсулиноподобных факторов роста (IGF), их белков-переносчиков (IGFBP) и маркеров повреждения (IL-18, L-FABP, цистатина С, NGAL, КИМ-1) в ткани почек самцов крыс при сахарном диабете, опухолевом росте и их сочетании.

Материалы и методы. В исследование включены самцы белых беспородных крыс (n = 32) массой 180–220 г, разделены на четыре группы по 8 особей в каждой. Группа 1–интактные животные, контрольная группа (2) –животные с сахарным диабетом, контрольная группа (3) – животные с карциномой Герена, основная группа (4) – животные с карциномой Герена на фоне сахарного диабета. В гомогенатах почек методом иммуноферментного анализа определяли IGF-1, IGF-2, IGFBP-1, IGFBP-2 и маркеры острого повреждения почек: IL-18, L-FABP, цистатин С, NGAL, KIM-1.

Результаты. При сахарном диабете в самостоятельном варианте и сочетанном с ростом карциномы Герена у самцов крыс в почках установлено повышение уровня маркеров острого повреждения почек. При развитии сахарного диабета уровень IGF-1, IGFBP-1 и IGFBP-2 был снижен в среднем в 1,3 раза, а уровень IGF-2 повышен в 2,1 раза относительно показателя у интактных самцов. Повышение IGF-2/IGF-1в среднем в 2,8 раза свидетельствовало о нарастании гипогликемии ткани почек животных при сахарном диабете и в группе с сахарным диабетом и опухолью Герена. При опухоли Герена в ткани почек самцов уровень IGF-1 и IGF-2 был повышен в среднем в 1,5 раза, а уровень IGFBP-2 снижен в 1,7 раза. При сочетанном развитии злокачественной опухоли на фоне сахарного диабета содержание IGF-2 и IGFBP-1 было повышено в 2,3 и 1,7 раза соответственно, а IGF-1 и IGFBP-2 не отличались от показателей у интактных животных.

Заключение. Обнаружены нарушения метаболического состояния ткани почек самцов при развитии сахарного диабета, опухоли Герена и их сочетания.

Ключевые слова: сахарный диабет, карцинома Герена, маркеры острого повреждения почек, IGF, IGFBP

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

The growing incidence of diabetes mellitus (DM) and chronic kidney disease (CKD) worldwide has prompted research efforts to overcome the growing prevalence of diabetic nephropathy (DN), which has become a global disaster due to the limited effectiveness of existing treatments [1]. Persistent hyperglycemia resulting from DM causes microvascular damage and long-term diabetic complications, such as nephropathy, also known as diabetic kidney disease (DKD) [2]. The kidneys play an important role in maintaining blood glucose homeostasis. In normal conditions, about 180 g of glucose is filtered through the glomeruli every day and almost completely reabsorbed by the renal tubules, of which about 90% is reabsorbed by the proximal tubules of the kidneys [3]. Both DM and CKD are known to be associated with aging. The incidence of DM in people over 65 years more than doubles that in people over 20 years [1], and aging is a key factor explaining the loss of nephrons and leading to CKD [4].

DM is believed to be an inducer of accelerated cellular aging and is associated with cardiovascular and kidney diseases due to high glucose levels [5]. However, tissue-specific aging remains poorly understood. DM is the leading cause of end-stage renal disease worldwide, especially in the elderly [6]. Accelerated kidney aging in DM is associated with multiple stressors, such as accumulation of advanced glycation end products, hypertension, oxidative stress, and inflammation [7].

IGF, a peptide growth factor secreted by the collecting duct of the adult kidney, binds to IGF1R and phosphorylates insulin receptor substrate proteins, thereby initiating downstream pathways, including PI3K-Akt-mTOR, to participate in the regulation of cell proliferation and apoptosis [8]. IGF-1 infusion improves hemodynamic parameters, such as renal plasma flow, inulin clearance, and renal vascular resistance in fasted rats. Studies have shown that IGF signaling is significantly involved in kidney development and various kidney diseases [9]. IGF-1 decreases after ischemic injury, and treatment with exogenous IGF-1 accelerates recovery by limiting cell apoptosis and promoting cell proliferation [10]. These findings were further supported by a study showing that administration of rhIGF-1 2 hours after injury suppressed the inflammatory response in the kidneys and increased EGF levels. IGF-1 also promotes tubular regeneration after acute kidney injury (AKI) by transactivating EGFR [7]. In addition to ligands, receptors, insulin, and IGF, there is a family of high affinity insulin-like growth factor binding proteins (IGFBPs). These proteins primarily counteract IGF function and can serve as independent biomarkers [11].

The DKD-affected kidney is believed to be particularly prone to hypoxic medullary injury [12]. The use of biomarkers of AKI for detection and assessment of its severity is expanding, and combined analyzes of several biomarkers increase their sensitivity and specificity [13]. However, although elevated markers in young and stable patients with intact kidneys are a strong sign of AKI, they are less predictable in elderly patients with comorbidities, especially with DM and pre-existing renal failure [13]. A study [14] measured serum and urine KIM-1 levels in addition to liver fatty acid binding protein (L-FABP), another marker of proximal renal tubular damage, in DM patients to clarify the relationship between these parameters. Experimental studies *in vivo* can reveal characteristics of cancer development in the presence of comorbid diseases [15].

The aim of this study was to analyze the levels of IGF, their carrier proteins (IGFBP), and markers of kidney tissue damage (IL-18, L-FABP, cystatin C, NGAL, and KIM-1) in the kidneys of male rats with DM, tumor growth, and their combination.

MATERIALS AND METHODS

The study included white outbred male rats weighing 180–220 g obtained from the Research Center for Biomedical Technologies of FMBA (Andreevka branch, Moscow Region). The animals were kept under natural light conditions with free access to water and food. The animals were used in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Directive 86/609/EEC), the International Guiding Principles for Biomedical Research Involving Animals, and the order of the Ministry of Health of Russia No. 267 of 19.06.2003 “On the approval of the rules of laboratory practice”.

The animals were divided into four groups, with 8 rats in each: intact animals (1); two control groups: with alloxan-induced diabetes (2) and Guerin carcinoma (3); experimental group (4) with Guerin carcinoma growing in presence of alloxan-induced diabetes. Experimental diabetes was reproduced by an intraperitoneal alloxan injection (150 mg / kg of body weight). Blood levels of glucose were monitored daily for one week. The blood glucose levels in intact animals

were 5.2 ± 0.3 mmol / l, in rats with induced DM – 27.3 ± 2.6 mmol /l, and in the experimental group – 25.4 ± 2.2 mmol / l. The rats from groups 3 and 4 after 1 week of persistent hyperglycemia received subcutaneous injections (downward from the corner of the right shoulder blade) of 0.5 ml suspension of Guerin carcinoma cells diluted 1 : 5 in saline. The experimental animals were monitored daily; subcutaneous tumor growth could be recorded 3 days after the injections of Guerin carcinoma cell suspension. Examinations carried out after 10 days of the malignant tumor growth corresponded to the exponential phase of Guerin carcinoma growth. 10 days after, the animals were decapitated using a guillotine. The levels of IGF-1, IGF-2 IGFBP-1, and IGFBP-2, as well as AKI markers (IL-18, L-FABP, cystatin C, NGAL, and KIM-1) were measured by ELISA in kidney homogenates in all animals (Cusabio, China).

The results were statistically processed using the Statistica 13.0 software package. The results were presented as the mean and the standard error of the mean $M \pm SE$. Normality of data distribution was checked by the Shapiro – Wilk test. The significance of differences between the independent variables was assessed using the Mann – Whitney test. The differences were considered significant at $p < 0.05$.

RESULTS

The results of the analysis of IGFs and their carrier proteins in the kidney tissue of male rats with DM, Guerin carcinoma, and Guerin carcinoma growing in the presence of DM are presented in Table 1.

Table 1

Levels of IGF and IGFBP (ng / g of tissue) in the kidneys of male rats, $M \pm SE$

Parameter	IGF-1	IGF-2	IGF-2/IGF-1	IGFBP-1	IGFBP-2
Group 1 (intact), $n = 8$	125.1 ± 10.8	66.6±5.9	0.5 ± 0.06	63.0 ± 6.7	456.79 ± 54.2
Group 2 (DM), $n = 8$	93.5 ± 8.4[1]	140.5±13.2[1]	1.5 ± 0.14[1]	46.2 ± 4.1[1]	349.3 ± 31.5[1]
Group 3 (Guerin carcinoma), $n = 8$	197.6 ± 16.3[1,2]	93.8 ± 9.5[1,2]	0.5 ± 0.05[2]	56.1 ± 5.8	274.3 ± 26.9[1,2]
Group 4 (DM + Guerin carcinoma), $n = 8$	120.6 ± 11.8[2]	152.1 ± 13.7[1]	1.3 ± 0.15[1]	105.6 ± 11.2[1,2]	372.0 ± 33.8

Note: statistically significant ($p < 0.05$) compared with: 1 – levels in intact animals; 2- levels in group 2 (here and in Table 2).

The levels of IGF-1, IGFBP-1, and IGFBP-2 in the animals with DM were decreased on average by 1.3 times ($p < 0.05$) compared with the values in the intact males, while IGF-2, on the contrary, was increased by 2.1 times. IGF-1 and IGF-2 in the kidney tissues of the male rats with Guerin carcinoma were increased by 1.6 and 1.4 times ($p < 0.05$), respectively, IGFBP-2 was decreased by 1.7 times ($p < 0.05$), and IGFBP-1 did not differ significantly from the levels in the intact males. In the animals with combined DM and Guerin carcinoma, the levels of IGF-1 and IGFBP-2 in the kidney tissues did not differ significantly from those in the intact males, and IGF-2 and IGFBP-1 were increased by 2.3 and 1.7 times ($p < 0.05$), respectively.

Clinical application of IGF-2 measurement in the diagnosis of non-islet cell tumor hypoglycemia was shown. Recent advances in understanding the pathophysiology of IGF-2 in cancer revealed new clinical potential of its use [16]. An increase in the IGF-2 / IGF-1 ratio indicated increasing tissue hypoglycemia.

The tumor tissue was not the subject of the study, nevertheless, we considered it reasonable to calculate the IGF-2 / IGF-1 ratio in the kidney tissue of the animals. IGF-2 / IGF-1 in the kidney tissue increased only in the male rats with DM and with Guerin carcinoma growing in presence of DM by 3 and 2.6 times, respectively. This indicated an increase in kidney tissue hypoglycemia in the animals precisely in conditions associated with an increase in the blood glucose level.

The bioavailability of the IGF-1 / IGFBP-1 and IGF-2 / IGFBP-1 ratios is also worth noting. IGF-1 / IGFBP-1 in the kidney tissue was elevated only in the animals with Guerin carcinoma (3.5 ± 0.4 vs. 2.0 ± 0.1). The IGF-2 / IGFBP-1 ratio was increased in all studied processes: in DM from 1.1 ± 0.09 to 3.0 ± 0.2; in Guerin carcinoma from 1.1 ± 0.09 to 1.7 ± 0.08; in DM + Guerin carcinoma from 1.1 ± 0.09 to 1.4 ± 0.07 ($p < 0.05$).

We studied AKI markers in the kidney tissue of rats with DM, Guerin carcinoma, and Guerin carcinoma growing in the presence of DM (Table 2).

The level of all markers, except for cystatin C, were increased in DM, compared with the values in the intact male frats: IL-18 – by 1.6 times ($p < 0.05$), L-FABP – by 1.9 times ($p < 0.05$), NGAL – by 2.3 times, and KIM-1 – by 1.6 times ($p < 0.05$). The male rats with Guerin carcinoma were characterized by elevated levels of some markers, compared with the intact animals: IL-18 – by 1.8 times ($p < 0.05$), cystatin C – by 1.3 times ($p < 0.05$), and KIM-1 – by 1.3 times

($p < 0.05$). In the kidney tissue of the male rats with Guerin carcinoma growing in the presence of DM, the level of IL-18 was increased by 1.5 times ($p < 0.05$), L-FABP – by 1.4 times ($p < 0.05$), cystatin C – by 1.8 times ($p < 0.05$), NGAL – by 2 times ($p < 0.05$), and KIM-1 – by 1.4 times ($p < 0.05$).

Table 2

Levels of AKI markers in the kidneys of male rats

Parameter	IL-18 (pg / g of tissue)	L-FABP (pg / g of tissue)	cystatin C (ng / g of tissue)	NGAL (ng / g of tissue)	KIM-1 (pg / g of tissue)
Group 1 (intact), $n = 8$	5,415.1 ± 398.6	2,319.4 ± 251.7	960.8 ± 83.5	0.12 ± 0.02	679.1 ± 58.4
Group 2 (DM), $n = 8$	8,520.4 ± 611.8[1]	4,365.2 ± 369.5[1]	828.4 ± 77.1	0.28 ± 0.03	1,058.5 ± 84.9[1]
Group 3 (Guerin carcinoma), $n = 8$	9,536.3 ± 842.5[1]	2,883.6 ± 334.6[2]	1,267.9 ± 113.5[1,2]	0.13 ± 0.015[2]	904.9 ± 76.3[1]
Group 4 (DM + Guerin carcinoma), $n = 8$	8,253.4 ± 731.2[1]	3,298.7 ± 248.3[1,2]	1,717.5 ± 99.4[1,2]	0.24 ± 0.028[1]	935.5 ± 81.7[1]

DISCUSSION

DM is an increasingly dangerous public health problem both due to its high prevalence and incidence and poor outcomes of vascular complications, such as DKD. Acute hyperglycemia occurs in diabetic ketoacidosis, and hyperglycemia can cause a series of metabolic disorders. Does it mean that rapidly elevated blood glucose may also lead to "acute hyperglycemic renal toxicity" [3]?

IGFs are essential for normal pre- and postnatal kidney development. IGF-1 mediates many growth hormone effects, and both excess and deficiency of growth hormone are associated with impaired renal function. IGFs affect renal hemodynamics both directly and indirectly by interacting with the renin – angiotensin system. In addition to IGF ligands, the IGF system includes IGF-1, IGF-2 / mannose-6-phosphate, and insulin receptors, as well as a family of 6 high affinity IGFBP that modulate the IGF effect. Dysregulation of the IGF system causes a number of kidney diseases.

Our results are consistent with the results from a number of studies showing that abnormal IGF levels are found in diabetic nephropathy and chronic renal failure [9]. In addition, IGF-1 can induce proliferation and differentiation of renal tubular epithelial cells and modulate immune cells, reducing the production of proinflammatory cytokines [17]. Obviously, decreased IGF-1 levels in the kidney tissues could lead to an increase in the production of proinflammatory cytokines and greater susceptibility of the organ to their action. However, our study did not reveal a decrease in the level of IGF-1 in the kidney tissues (both in the control and in the experimental groups), although elevated IL-18 levels were found in all studied tissue samples.

IL-18 is a proinflammatory cytokine produced by the proximal tubular epithelium after the action of nephrotoxic factors. Interleukins are important mediators of the immune response in the innate and adaptive immunity. All cytokines are freely filtered and then reabsorbed and metabolized in the proximal tubules; therefore, an increase in the level of IL-18 indicates damage to these tubules [18]. The determination of IL-18 in urine allows for identification of renal damage caused by ischemia at the earliest stage. IGF-1 controls the anti-apoptotic Bcl-2 protein, which is the main mechanism of protection and survival of the renal epithelium during injury, and Bcl-2 is regulated by IGF-1 at the post-transcriptional level [19].

In contrast to IGF-1, the levels of IGF-2 were increased in all kidney tissue samples, although in the animals with DM, i.e. in the experimental and control groups, these changes were more pronounced, compared with the rats with the independent growth of Guerin carcinoma. IGF-2 is a 7.5 kDa mitogenic peptide hormone expressed by the liver and many other tissues. It is three times more abundant in serum than IGF-1, but our understanding of its physiological and pathological roles is insufficient compared with IGF-1. Expression of the IGF-2 gene is strictly regulated. Its overexpression is observed in many cancers and associated with a poor prognosis. Elevated serum IGF-2 levels are also associated with an increased risk of developing various cancers [16].

In this study, IGF-2 / IGF-1 demonstrated increasing hypoglycemia in the kidney tissue of animals precisely in conditions associated with an increase in blood glucose levels, i.e. in the experimental group and in the control group with DM. The increased IGF-2 / IGFBP-1 ratio showed, on the one hand, an increase in the bioavailability of IGF-2 in all studied pathological processes, and, on the other hand, that this factor is pathognomonic in both malignant growth and DM. There are many common risk factors

for DM and cancer [20]. Obviously, IGF-2 is one of such pathognomonic factors.

In recent decades, new methods for studying kidney diseases have been proposed, such as tubular enzymes and new AKI biomarkers. Very promising new AKI biomarkers have been termed "renal troponins" and have suggested early detection of kidney disease.

Numerous studies on urinary neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), cystatin C (CysC), liver fatty acid binding protein (L-FABP), and interleukin (IL)-18 have been carried out. Elevated levels of NGAL, a biomarker of distal tubular segments, may indicate that these segments are affected by inflammation. After ischemic or nephrotoxic AKI, intrarenal NGAL is dramatically upregulated at the transcriptional and protein levels. In contrast, unaltered KIM-1, a proximal tubular biomarker, may reflect improved cortical oxygenation [12].

Biomarkers can be localized in certain nephron segments. A recent study [21] suggested that IL-18, NGAL, L-FABP, and KIM-1 can help characterize the function of glomeruli or tubules; glomerular, tubular or interstitial damage, inflammation. At the same time, NGAL levels can be elevated in sepsis, CKD or urinary tract infections, and no specific threshold values were noted for it [22]. The levels of KIM-1 can be elevated in chronic proteinuria and inflammatory diseases [23]. L-FABP can be closely associated with anemia in patients without DM [24].

CysC is produced by nucleated cells at a constant rate, filtered by glomerular cells, and almost completely reabsorbed and catabolized (but not secreted) in the proximal tubules. Over the past decade, CysC was revealed to be a stronger death risk predictor in older people than creatinine [25].

L-FABP is a 14 kDa protein from a large fatty acid-binding protein superfamily. It is predominantly localized in the proximal tubules [25]. The described studies make it obvious that the kidneys of males with DM, Guerin carcinoma, and the combined pathology were subject to any kind of damaging effects, be it hyperglycemia or stress associated with tumor growth. The L-FABP protein levels were increased only in the samples of the animals with DM, alone or with concomitant disease. CysC, on the contrary, increased in the kidney tissues of the animals with Guerin carcinoma, alone or with concomitant disease. The IL-18 and NGAL levels were increased in the kidney tissues of the male rats with both pathological processes. Thus, male rats with different pathologies showed glomerular, tubular or interstitial damage. The glomerular filtration was affected only in the animals with independently growing tumors (control group) or in combination with DM (experimental group). Similar results associated with kidney ischemia and cancer were earlier obtained in the experiment and in clinical practice [26, 27].

CONCLUSION

DM as a concomitant process in malignant growth increases the levels of IGF-2, IGF-1 / IGF-2, and IGFBP-2 in kidney samples of nonlinear male rats with a rise in the local level of AKI markers. Perhaps the reason for it should be sought in the predictive role of sex hormones.

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Differentiation and subpopulation composition of VEGFR2$^+$ cells in the blood and bone marrow in ischemic cardiomyopathy

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ABSTRACT

Aim. To identify disturbances of differentiation and subpopulation composition of VEGFR2+ cells in the blood and bone marrow associated with the features of the cytokine profile in the blood and bone marrow in patients with coronary artery disease (CAD) with and without ischemic cardiomyopathy (ICM).

Materials and methods. The study included 74 patients with CAD with and without ICM (30 and 44 people, respectively) and 18 healthy donors. In all patients with CAD, peripheral blood sampling was performed immediately before coronary artery bypass grafting, and bone marrow samples were taken during the surgery via a sternal incision. In the healthy donors, only peripheral blood sampling was performed. In the bone marrow and blood samples, the number of VEGFR2$^+$ cells (CD14$^+$VEGFR2$^+$ cells) and their immunophenotypes CD14^{++}CD16$^-$VEGFR2$^+$, CD14^{++}CD16$^+$VEGFR2$^+$, CD14$^+$CD16^{++}VEGFR2$^+$, and CD14$^+$CD16$^-$VEGFR2$^+$ was determined by flow cytometry. Using enzyme-linked immunosorbent assay, the levels of VEGF-A, TNFα, M-CSF, and IL-13, as well as the content of MCP-1 (only in the blood) and the M-CSF / IL-13 ratio (only in the bone marrow) were determined.

Results. The content of CD14$^+$VEGFR2$^+$ cells in the blood of CAD patients with and without ICM was higher than normal values due to the greater number of CD14^{++}CD16$^-$VEGFR2$^+$, CD14^{++}CD16$^+$VEGFR2$^+$, and CD14$^+$CD16^{++}VEGFR2$^+$. In the bone marrow of the patients with ICM, the content of CD14^{++}CD16$^-$VEGFR2$^+$, CD14$^+$CD16^{++}VEGFR2$^+$, and CD14$^+$CD16$^-$VEGFR2$^+$ was lower than in patients with CAD without ICM, and the number of CD14^{++}CD16$^+$VEGFR2$^+$ cells corresponded to that in the controls. Regardless of the presence of ICM in CAD, a high concentration of TNFα and normal levels of VEGF-A and IL-13 were observed in the blood. In CAD without ICM, an excess of MCP-1 and deficiency of M-CSF were revealed in the blood. In the bone marrow, the levels of VEGF-A, TNFα, M-CSF, and IL-13 were comparable between the groups of patients against the background of a decrease in the M-CSF / IL-13 ratio in the patients with ICM.

Conclusion. Unlike CAD without cardiomyopathy, in ICM, no excess of VEGFR2$^+$ cells and MCP-1 in the blood is observed, which hinders active migration of CD14$^+$CD16^{++}VEGFR2$^+$ cells from the myeloid tissue, and a decrease in the M-CSF / IL-13 ratio in the bone marrow disrupts differentiation of other forms of VEGFR2$^+$ cells, preventing vascular repair.

Keywords: endothelial progenitor cells, monocytes, bone marrow, cytokines, vascular repair, ischemic cardiomyopathy, coronary artery disease

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Дифференциация и субпопуляционный состав VEGFR2+ моноцитов крови и костного мозга при ишемической кардиомиопатии

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РЕЗЮМЕ

Цель: установить нарушения дифференцировки и субпопуляционного состава VEGFR2$^+$ моноцитов в крови и костном мозге во взаимосвязи с особенностями цитокинового профиля крови и костного мозга у больных ишемической болезнью сердца (ИБС), страдающих и не страдающих ишемической кардиомиопатией (ИКМП).

Материалы и методы. В исследование вошли 74 больных ИБС, страдающих и не страдающих ИКМП (30 и 44 человека соответственно), и 18 здоровых доноров. У всех больных ИБС забор периферической крови производился непосредственно перед операцией коронарного шунтирования, а костного мозга – из разреза грудины во время операции. У здоровых доноров забирали только периферическую кровь. В костном мозге и крови методом проточной цитофлуориметрии определяли численность VEGFR2$^+$ моноцитов (CD14$^+$VEGFR2$^+$ клеток) и их иммунофенотипов CD14^{++}CD16$^-$VEGFR2$^+$, CD14^{++}CD16$^+$VEGFR2$^+$, CD14$^+$CD16^{++}VEGFR2$^+$, CD14$^+$CD16$^-$VEGFR2$^+$, методом иммуноферментного анализа регистрировали концентрацию VEGF-A, TNFα, M-CSF, IL-13, а также содержание MCP-1 (только в крови) и соотношение M-CSF/IL-13 (только в костном мозге).

Результаты. Содержание CD14$^+$VEGFR2$^+$ клеток в крови у больных ИБС без кардиомиопатии и с ИКМП было выше нормы из-за большей численности CD14^{++}CD16$^-$VEGFR2$^+$, CD14^{++}CD16$^+$VEGFR2$^+$ и CD14$^+$CD16^{++}VEGFR2$^+$ форм. В костном мозге у больных ИКМП содержание CD14^{++}CD16$^-$VEGFR2$^+$, CD14$^+$CD16^{++}VEGFR2$^+$ и CD14$^+$CD16$^-$VEGFR2$^+$ форм было ниже, чем у больных ИБС без кардиомиопатии, а количество CD14^{++}CD16$^+$VEGFR2$^+$ клеток соответствовало их числу в группе сравнения. Вне зависимости от наличия ИКМП при ИБС в крови отмечалась высокая концентрация TNFα, нормальный уровень VEGF-A и IL-13; при ИБС без кардиомиопатии – избыток MCP-1 и дефицит M-CSF в крови. В костном мозге концентрация VEGF-A, TNFα, M-CSF, IL-13 была сопоставимой между группами больных на фоне снижения M-CSF/IL-13 у пациентов с ИКМП.

Заключение. В отличие от ИБС без кардиомиопатии при ИКМП не формируется избыток VEGFR2$^+$ моноцитов и MCP-1 в крови, что затрудняет активную миграцию CD14$^+$CD16^{++}VEGFR2$^+$ клеток из миелоидной ткани, а снижение M-CSF/IL-13 в костном мозге нарушает дифференцировку остальных форм VEGFR2$^+$ моноцитов, препятствуя репарации сосудов.

Ключевые слова: моноциты, прогениторные эндотелиальные клетки, репарация сосудов, костный мозг, цитокины, ишемическая кардиомиопатия**,** ишемическая болезнь сердца

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INTRODUCTION

Ischemic cardiomyopathy (ICM) is a complicated form of chronic coronary artery disease (CAD) and one of the most common causes of death from cardiovascular diseases worldwide [1]. ICM is characterized by dilatation of heart chambers, hypertrophy (mainly of the left ventricle), and a decrease in the heart pumping function with the formation of chronic heart failure [2]. It is believed that ICM development is based on impaired myocardial contractile function due to coronary microvascular dysfunction, which is a trigger for widespread myocardial ischemia and hibernation, necrosis, and apoptosis of cardiomyocytes, followed by fibrosis and ventricular remodeling [1, 2]. Endothelium plays an important role in the physiology and pathology of the cardiovascular system, modulating vascular tone, hemocoagulation, fluid and solute exchange, as well as inflammation and angiogenesis [3–5].

At the same time, endothelial dysfunction is considered mainly as an imbalance of vasoconstrictor and vasodilator stimuli, and due attention is not paid to endothelial regeneration in the literature. Meanwhile, impaired endothelial repair may be an important pathogenetic factor of ICM, which substantiates the study of the mechanisms of differentiation and migration of endothelial progenitor cells (EPC) in this pathology.

EPCs are mainly present in the bone marrow, maturing from hematopoietic stem cells, but they can also be isolated from peripheral blood and blood vessel walls [6, 7]. Endothelial cells, including EPCs, express the type 2 vascular endothelial growth factor receptor (VEGFR2) at all stages of differentiation. VEGF (VEGFR2) is associated with tyrosine kinase and is the main activator of angiogenesis, because phosphorylation of its Y1175 domain activates proliferation, and phosphorylation of Y951 and Y1214 domains activates cell migration [8]. It has been established that blood mononuclear cells *in vitro* can acquire endothelial markers and endotheliocyte morphology under the influence of proangiogenic stimuli [7], which indicates their mixed phenotype and suggests the relevance of studying monocytic VEGFR2$^+$ cells (CD14$^+$VEGFR2$^+$ cells) belonging to early EPCs, with weak expression of CD34 [9, 10]. The literature describes several subpopulations of CD14$^+$VEGFR2$^+$ cells with different phenotypes (classical CD14^{++}CD16$^-$, intermediate CD14^{++}CD16$^+$, non-classical CD14$^+$CD16^{++}, and transitional CD14$^+$CD16$^-$) in healthy donors [11], however, there is no information about changes in the ratio of CD14$^+$VEGFR2$^+$ cell subpopulations in cardiovascular pathology.

Normally, EPCs are a very small population of blood cells, but in case of damage or hypoxia, they are mobilized from the bone marrow under the action of cytokines and are attracted to the damaged area [12]. In a mouse model of angiogenesis, S.K. Chauhan et al. (2015) identified mononuclear cells expressing protein tyrosine kinase 7 and VEGFR2 (as descendants of a macrophage and monocyte subpopulation

located in the perivascular region of *de novo* forming vessels), as well as expressing pericyte markers and secreting angiopoietin-1 and other proangiogenic factors [13].

The process of EPC mobilization is activated by several cytokines: vascular endothelial growth factor (VEGF), hypoxia-inducible factor (HIF)-1, interleukin (IL)-6 [14, 15], monocyte chemotactic protein (MCP)-1 [15, 16], tumor necrosis factor α (TNFα) [6], and macrophage colony-stimulating factor (M-CSF) [17]. At the same time, it is possible that an increase in the number of EPCs in the blood may also be associated with the intensification of their differentiation in myeloid tissue. M-CSF, IL-6, and TNFα can be involved in EPC maturation as proinflammatory cytokines stimulating maturation of monocytic cells [18], while VEGF can contribute to this process as a cytokine interacting with specific receptors on the EPC membrane [10, 13]. In addition, anti-inflammatory cytokines can have a negative effect on myelopoiesis [19], such as IL-13 produced by regulatory T cells (Treg), which induces secretion of IL-10 by macrophages.

Information about the effect of cytokines on EPC differentiation and the content of monocyte progenitor cells of $CD14^+VEGFR2^+$ endotheliocytes in the bone marrow or blood of patients with ICM is not present in the literature. Changes of the subpopulation belonging of EPC to various immunophenotypes of monocytes in CAD, complicated and uncomplicated by ICM, have not been described. Meanwhile, identifying deviations of the above-mentioned parameters characteristic of ICM and understanding the role of cytokines in the differentiation and migration of EPCs in CAD could be the key to successful treatment of ICM.

The aim of the study was to identify the disturbances of differentiation and subpopulation composition of $VEGFR2^+$ monocytes in the blood and bone marrow associated with the features of blood and bone marrow cytokine profile in CAD patients with and without ICM.

MATERIALS AND METHODS

A single-stage, controlled (case-control), single-center, observational study was carried out involving 74 CAD patients with NYHA functional class II–IV angina pectoris and class I–III circulatory insufficiency, with ICM (27 men and 3 women, age 61.0 [56.0; 64.0] years) and without ICM (36 men and 8 women, age 64.0 [59.5; 68.0] years), with a history of myocardial infarction. The control group consisted of 18 healthy donors matched by sex and age with the cohorts of patients.

The diagnostic criteria for ICM were: left ventricular ejection fraction ≤ 40%, a history of acute myocardial infarction or revascularization, ≥ 75% stenosis of the left main or proximal part of the left anterior descending artery, or ≥ 75% stenosis of two or more epicardial vessels [20]. The patients did not have significant differences in the functional class of angina pectoris and heart failure, the incidence of hypertension (stage III), gastrointestinal tract, and lung diseases. However, ICM patients had a higher frequency of chronic cerebrovascular accidents (90 vs 59.09%, $p = 0.023$), and CAD patients without cardiomyopathy – of type 2 diabetes mellitus (31.82 vs 6.67%, $p = 0.046$). The exclusion criteria were the presence of autoimmune diseases, severe allergic reaction, cancer, hypoplastic, B12- or folate deficiency anemia, leukemia and other hematological diseases and syndromes, chronic infections (viral hepatitis, syphilis, HIV infection), treatment with iron-containing drugs, erythropoietin or immunosuppressive therapy before surgery, the presence of acute infectious diseases less than 3 weeks before surgery, as well as patient's refusal to enroll in the study.

The patients underwent coronary artery bypass grafting in combination with the left ventricular reconstruction using cardiopulmonary bypass at the Cardiovascular Surgery Department of the Cardiology Research Institute of Tomsk National Research Medical Center. At the preoperative stage, CAD patients in both study groups received similar drug treatment: antianginal therapy with long-acting nitrates, beta1-blockers, Ca^{2+}-channel blockers, hemostasis correction with platelet antiaggregants, and lipid metabolism correction with statins. Premedication and induction of general anesthesia in patients in both study groups was carried out in a similar way using sedatives and anesthetics, narcotic analgesics and muscle relaxants (diazepam, ketamine, fentanyl, promedol, pipecuronium) in comparable doses.

In all CAD patients with and without ICM, 5 ml of peripheral blood was collected from the cubital vein immediately before the surgery, with subsequent stabilization with heparin (25 U / ml). During the surgery, after gaining access to the heart via median sternotomy and before establishing cardiopulmonary bypass, red bone marrow was taken via a sternal incision in the amount of 2 ml in a test tube with the addition of heparin (25 U / ml).

The relative count of $CD14^+VEGFR2^+$ cells in the total population of monocytes (taking cells positive for CD14 as 100%), $CD14^+VEGFR2^+$ cell

distribution among monocyte subpopulations (classical $CD14^{++}CD16^{-}$, intermediate $CD14^{++}CD16^{+}$, non-classical $CD14^{+}CD16^{++}$, transitional $CD14^{+}CD16^{-}$ monocytes), and the proportion of $CD14^{++}CD16^{-}VEGFR2^{+}$, $CD14^{++}CD16^{+}VEGFR2^{+}$, $CD14^{+}CD16^{++}VEGFR2^{+}$, and $CD14^{+}CD16^{-}VEGFR2^{+}$ cells in the total number of $CD14^{+}VEGFR2^{+}$ cells (taking the number of cells positive for CD14 and VEGFR2 as 100%) were determined in the blood and bone marrow samples of patients of both study groups and in the blood of healthy donors by laser flow cytometry at the Central Research Laboratory of Siberian State Medical University.

Monoclonal antibodies CD14-FITC, CD16-PE, VEGFR2 (KDR, CD309)-Alexa Fluor 647 (BD Biosciences, USA) were used to identify subpopulations of the studied blood cells. The absolute count of the above cell subpopulations was assessed based on their proportion and the absolute count of monocytes in the bone marrow, determining the total number of myelokaryocytes with standard hematological methods using the Goryaev's chamber grid and the proportion of monocytic cells (monocytes and promonocytes) by the microscopic examination of bone marrow smears and with account of the absolute count of monocytes in the blood, which was recorded from the clinical patients' records.

Blood plasma and myeloplasma (bone marrow supernatant) of the patients were obtained by centrifugation of the corresponding biomaterial for 15 min at 2,000 g, preserved, and stored at –80 °C. The concentrations of TNFα, MCP-1, IL-13, M-CSF, and vascular endothelial growth factor A (VEGF-A) were determined using commercial enzyme immunoassay kits TNF alpha IFA-BEST and MSR-1-IFA-BEST (Vector-BEST, Novosibirsk), Human IL-13 Platinum ELISA kit (eBioscience, Austria), RayBio Human M-CSF ELISA Kit (RayBiotech, USA), and Human VEGF-A ELISA Kit (Cloud-Clone-Corp., USA) at the Pathophysiology Division of Siberian State Medical University.

The median and the interquartile range $Me\ [Q_1; Q_3]$ were calculated for statistical description of the study results. Nonparametric methods of statistical analysis were used due to the small number of samples and non-normality of data distribution. The Mann – Whitney U-test with the Benjamini–Hochberg correction for multiple comparison was used in order to test the null hypothesis when comparing independent variables. The Spearman's rank correlation coefficient was calculated to analyze the associations. The results were considered statistically significant at a p level of less than 0.05. A statistical analysis of the data was performed using Statistica 10.0 software package.

RESULTS

According to the results of the study, the total count of monocytes of all subpopulations in the blood of CAD patients, regardless of the presence of ICM, corresponded to the normal values (Table 1). At the same time, the number of $CD14^{+}VEGFR2^{+}$ cells was increased only in the CAD patients without ICM and significantly differed from the physiological values of this parameter in the ICM patients (Table 1).

Table 1

The content of monocytes, cytokines, and $CD14^{+}VEGFR2^{+}$ cells in the blood in CAD patients with and without ICM, $Me\ [Q_1; Q_3]$

Parameters	Healthy donors	CAD patients without ICM	CAD patients with ICM
Content of monocytes, $\times 10^9$ / l	0.58 [0.40; 0.66]	0.57 [0.50; 0.76] $p_c = 0.616$	0.67 [0.57; 0.73] $p_c = 0.189$ $p = 0.464$
Content of $CD14^{+}VEGFR2^{+}$cells, %	1.91 [0.75; 3.92]	4.98 [3.86; 10.12] $p_c = 0.006$	2.13 [0.95; 2.66] $p_c = 0.665$ $p = 0.002$
Content of $CD14^{+}VEGFR2^{+}$cells, $\times 10^6$ / l	15.25 [5.07; 22.70]	26.01 [24.24; 34.31] *pc = 0.049*	13.16 [8.51; 15.97] $p_c = 0.685$ $p = 0.024$
VEGF-A, pg / ml	6.50 [1.75; 13.25]	7.00 [4.50; 15.25] $p_c = 0.721$	7.00 [6.00; 13.00] $p_c = 0.415$ $p = 0.811$
TNFα, pg / ml	0.64 [0.04; 0.83]	1.16 [0.90; 1.82] $p_c = 0.012$	2.08 [1.04; 3.60] $p_c = 0.009$ $p = 0.247$
MCP-1, pg / ml	175.0 [145.0; 185.0]	225.0 [182.0; 280.0] $p_c = 0.027$	205.0 [170.0; 260.0] $p_c = 0.104$ $p = 0.660$

Table 1 (continued)

Parameters	Healthy donors	CAD patients without ICM	CAD patients with ICM
IL-13, (pg / ml)	0.50 [0.40; 0.75]	0.60 [0.41; 0.82] $p_c = 0.683$	0.82 [0.40; 0.95] $p_c = 0.420$
M-CSF, (pg / ml)	2.50 [1.60; 4.40]	0.40 [0.12; 2.37] $p_c = 0.046$	2.00 [1.21; 3.24] $p_c = 0.177$ $p = 0.097$

Note: the level of statistical significance of differences in the parameters compared with the content of cytokines and (or) cells in healthy donors – p_c, in patients with CAD – p (here and in Table 2).

The subpopulation composition of $CD14^+VEGFR2^+$ cells, depending on the intensity of CD14 and CD16 expression on peripheral blood monocytes in the ICM patients, fully corresponded to that in healthy donors (Fig. 1). At the same time, the CAD patients without ICM had an increased content of non-classical $CD14^+CD16^{++}VEGFR2^+$ monocytes relative to the norm and a clear trend ($p = 0.072$) toward an increase in the number of classical $CD14^{++}CD16^-VEGFR2^+$ cells in the blood. The content of $VEGFR2^+$ monocytes of all immunophenotypes was higher in this category of patients than in ICM patients (except for equal values for transitional $CD14^+CD16^-VEGFR2^+$ cells, Fig. 1).

In the bone marrow, the total content of monocytic cells and the relative and absolute count of $CD14^+VEGFR2^+$ monocytes were comparable between the groups of CAD patients, however, there were characteristic features of the subpopulation composition of these cells (Table 2, Fig. 2). Thus, the number of classical $CD14^{++}CD16^-VEGFR2^+$, non-classical $CD14^+CD16^{++}VEGFR2^+$, and transitional $CD14^+CD16^-VEGFR2^+$ cells in the bone marrow of CAD patients without ICM exceeded their content in ICM patient. It is worth noting that the number of intermediate $CD14^{++}CD16^+VEGFR2^+$ monocytes in the bone marrow in CAD patients in both groups was comparable, while in the blood it was different: in ICM patients it was 2.4 times less than in CAD patients without ICM (Fig. 2).

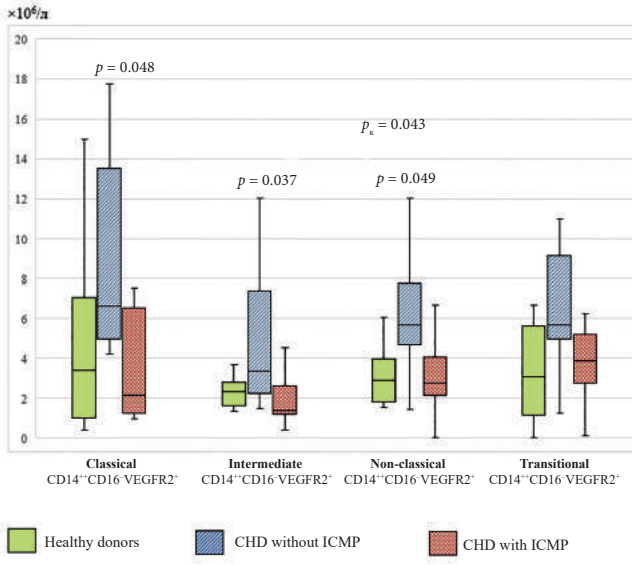

Fig. 1. Subpopulation composition of CD14+VEGFR2+ blood monocytes in patients with CAD with or without ICM: p_c – the level of statistical significance of differences in the parameters compared with the content of cells in healthy donors, p – in patients with CAD without ICM

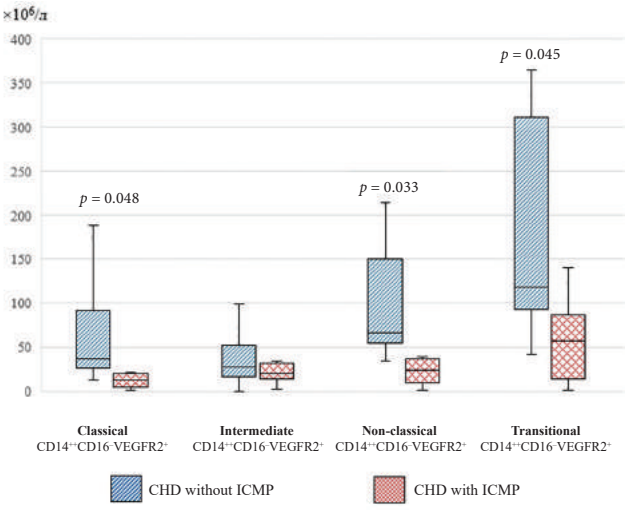

Fig. 2. Subpopulation composition of CD14+VEGFR2+ bone marrow monocytes in the patients with CAD with or without ICM: p – level of statistical significance of differences in the parameters compared with the content of cells in the patients with CAD without ICM

Table 2

The content of monocytes, cytokines, and $CD14^+VEGFR2^+$ cells in the bone marrow in CAD patients with and without ICM, *Me* [Q_1; Q_3]

Parameters	CAD patients without ICM	CAD patients with ICM
Content of monocytes, $\times 10^9$ / l	1.05 [0.84; 1.57]	0.89 [0.71; 1.40] $p = 0.525$
Content of $CD14^+VEGFR2^+$cells, %	27.85 [22.57; 42.88]	28.30 [11.98; 36.21] $p = 0.637$
Content of $CD14^+VEGFR2^+$cells, $\times 10^6$ / l	343.90 [208.75; 638.10]	223.90 [38.20; 546.10] $p = 0.325$
VEGF-A, pg / ml	18.50 [12.10; 25.30]	21.00 [16.00; 28.50] $p = 0.344$
TNFα, pg / ml	10.80 [9.90; 21.84]	18.06 [14.15; 19.40] $p = 0.517$
IL-13, pg / ml	1.00 [0.80; 1.23]	1.22 [0.80; 2.41] $p = 0.874$
M-CSF, pg / ml	7.16 [3.45; 16.33]	3.22 [1.20; 8.04] $p = 0.792$
M-CSF / IL-13	9.00 [2.13; 22.09]	1.02 [0.41; 2.00] $p = 0.047$

A correlation analysis of the parameters of $VEGFR2^+$ composition in the blood revealed two positive correlations in the CAD patients without ICM: between the absolute count of $CD14^+VEGFR2^+$ monocytes and the number of classical $CD14^{++}CD16^-VEGFR2^+$ cells ($r_s = 0.86$; $p < 0.01$) and between the number of transitional $CD14^+CD16^-VEGFR2^+$ monocytes and the number of non-classical $CD14^+CD16^{++}VEGFR2^+$ cells ($r_s = 0.93$; $p < 0.01$). We also found three positive correlations in ICM patients: between the absolute count of $CD14^+VEGFR2^+$ monocytes and the number of classical $CD14^{++}CD16^-VEGFR2^+$ ($r_s = 0.74$; $p < 0.01$), non-classical $CD14^+CD16^{++}VEGFR2^+$ ($r_s = 0.79$; $p < 0.01$), and transitional $CD14^+CD16^-VEGFR2^+$ monocytes ($r_s = 0.81$; $p < 0.01$). A similar correlation pattern was revealed in the bone marrow of ICM patients with a positive correlation between the number of classical $CD14^{++}CD16^-VEGFR2^+$ monocytes and the number of non-classical $CD14^+CD16^{++}VEGFR2^+$ cells ($r_s = 0.90$; $p < 0.05$). In the bone marrow of the CAD patients without ICM, a directly proportional relationship was identified between the absolute count of $CD14^+VEGFR2^+$ monocytes and their non-classical subpopulation ($r_s = 0.71$; $p < 0.05$).

Regardless of the presence of ICM, the concentration of VEGF-A and IL-13 in the blood of the CAD patients varied within physiological values, and the content of TNFα exceeded the normal values. At the same time, the CAD patients without ICM had an excess of MCP-1 and a deficiency of M-CSF compared with the ICM patients, in whom the content of these cytokines remained similar to that of healthy donors (Table 1). The concentrations of VEGF-A, IL-13, TNFα, and M-CSF in the CAD patients with and without ICM were comparable, characterized by a trend toward higher TNFα values and lower M-CSF values in the bone marrow in the ICM patients. At the same time, reciprocal changes in the M-CSF and IL-13 levels in the bone marrow were noted in many patients during the visual analysis of the data. It prompted the calculation of the M-CSF / IL-13 ratio, which turned out to be 9 times higher in the CAD patients without ICM than in the ICM patients (Table 2).

DISCUSSION

According to the obtained data, in the CAD patients without ICM, there is an almost twofold increase in the content of $CD14^+VEGFR2^+$ monocytes in the blood, both relative to the normal values and ICM patients (Table 1). This phenomenon in CAD patients without ICM can be considered as a compensatory reaction of the body in the conditions of atherogenesis. Accumulation of macrophages loaded with lipids in atheroma is accompanied by NADPH oxidase activation in them, followed by generation of reactive oxygen species, as well as by secretion of matrix metalloproteinases types 2 and 9 by macrophages, damaging the elements of the extracellular matrix and the basement membrane of blood vessels, which leads to destruction of the vascular endothelium [21].

At the same time, endothelial NO synthase (eNOS) activation due to vascular damage, ischemia, and hypoxia induces the release of proangiogenic factors

(HIF-1α, VEGF, etc.) from the endothelium and tissue cells, as well as EPC migration and proliferation [22]. Since $CD14^+VEGFR2^+$ monocytes are early EPCs that can stimulate vascular cell differentiation in a paracrine fashion and prevent their apoptosis by secreting VEGF, angiopoietin-1, and other proangiogenic factors [10, 13], the physiological level of $CD14^+VEGFR2^+$ monocytes in the blood of ICM patients (Table 1) with a verified atherosclerotic process can be considered as the absence of a compensatory reaction of the body aimed at repairing vessels in case of damage.

The analysis of the subpopulation composition of $CD14^+VEGFR2^+$ blood monocytes demonstrates that their transitional $CD14^+CD16^-VEGFR2^+$ immunophenotype is not involved in the increase in the total $CD14^+VEGFR2^+$ count in the blood of the CAD patients without ICM (Fig. 1). This is explained by the fact that transitional $CD14^+CD16^-$ cells are immature forms of monocytes concentrated mainly in the bone marrow, while their content in the blood is about 6% [23], so $CD14^+CD16^-VEGFR2^+$ cells do not enter the bloodstream in large numbers. However, despite the small number of this monocyte population in the blood, transitional $CD14^+CD16^-VEGFR2^+$ monocytes account for 25–35% of all $CD14^+VEGFR2^+$ cells in healthy donors and in CAD patients (Fig. 1). This may indicate a significant proangiogenic role of transitional $CD14^+CD16^-VEGFR2^+$ cells as younger representatives of monocytic cells in the blood, which are less subject to modulation of their own numbers in the bloodstream.

Mature forms of $CD14^+VEGFR2^+$ monocytes are most susceptible to quantitative changes in the blood. Thus, according to the data obtained, the increase in their total blood content in the CAD patients relative to those in the ICM patients occurs due to almost 3-fold accumulation of classical $CD14^{++}CD16^-VEGFR2^+$ and intermediate $CD14^{++}CD16^+VEGFR2^+$ monocytes with a 2-fold increase in the number of non-classical $CD14^+CD16^{++}VEGFR2^+$ cells. However, the number of the latter exceeds that even in healthy donors (Fig. 1). Therefore, the content of non-classical $CD14^+CD16^{++}VEGFR2^+$ cells in the blood is a parameter that varies the least and most accurately reflects the enhancement of vascular repair in cardiovascular pathology.

We have previously shown that a distinctive feature of ICM patients is the deficiency of non-classical monocytes in the blood (regardless of the VEGFR2 expression on their membrane), which are able to eliminate immune complexes and dead cells from the vascular intima surface and protect it from damage [24]. Due to the fact that intermediate monocytes are characterized by the highest expression of VEGFR2 and VEGFR1 and the highest proportion of $VEGFR2^+$ cells compared with classical and non-classical cells (8.25, 5.00 and 2.80% of the corresponding population of monocytes) [11], and, probably, it is the intermediate forms that have high proangiogenic activity, the trend toward a decrease in this cell subpopulation in the blood in ICM patients relative to the normal values is of great interest (Table 1). Therefore, it can be assumed that in ICM, there is no compensatory activation of vascular repair in atherosclerosis manifested by $CD14^+VEGFR2^+$ cell accumulation in the blood, endothelium clearance from immune complexes decreases with a non-classical monocyte deficiency, and there is a trend toward a lack of physiological regeneration in the vascular endothelium with the participation of intermediate $CD14^{++}CD16^+VEGFR2^+$ monocytes.

The reason for the absence of an increase in the number of $CD14^+VEGFR2^+$ cells in the blood in ICM is probably the peculiarities of the blood cytokine profile. With an equivalent surplus of TNFα in the cohorts of patients and physiological VEGF-A and IL-13 levels in the blood in patients of both groups, there was an excess of MCP-1 and a deficiency of M-CSF in the blood of the CAD patients without ICM, while the concentration of these cytokines in the ICM patients corresponded to the normal values (Table 1). Therefore, an increase in the content of $CD14^+VEGFR2^+$ monocytes in the blood in the CAD patients with ICM is associated with the MCP-1 accumulation and does not depend on the M-CSF plasma concentration. It is known that under physiological conditions, differentiation of monocytes and macrophages is determined by the levels of M-CSF, which is constitutively synthesized by stromal progenitor cells, fibroblasts, and macrophages. However, under conditions of inflammation, the colony-stimulating factor of granulocytes and macrophages (GM-CSF) becomes more important in the regulation of hematopoiesis and monocytopoiesis [25]. In addition, in the presence of M-CSF or GM-CSF, monocyte precursors in the bone marrow actively proliferate and differentiate, after which mature monocytes become refractory to these growth stimuli [26], and they need the M-CSF receptor, first of all, for regulation of macrophage differentiation [27]. Therefore, it is probably not M-CSF, but MCP-1, a pro-inflammatory cytokine and the most powerful chemoattractant for monocytes and macrophages [21,

24], that is involved in the migration of $CD14^+VEGFR2^+$ cells from the myeloid tissue into the blood in CAD patients without ICM.

A comparable number of $CD14^+VEGFR2^+$ cells in the bone marrow of CAD patients with and without ICM (Table 2) with differences in their count in the blood (Table 1) allows to tentatively conclude that only the migration of $CD14^+VEGFR2^+$ monocytes into the bloodstream is impaired in ICM without changes in their differentiation in the myeloid tissue. Meanwhile, when analyzing the subpopulation composition of $CD14^+VEGFR2^+$ cells, it becomes obvious that this phenomenon is observed only in intermediate $CD14^{++}CD16^+VEGFR2^+$ monocytes, the number of which in the blood turned out to be comparable between the groups of patients against the background of their increased values in the blood of CAD patients without ICM (Fig. 1, 2).

As discussed above, the high MCP-1 plasma concentration in these patients (Table 1) allows $CD14^+VEGFR2^+$ cells to actively migrate from the bone marrow into the blood, which is not seen in ICM patients. However, the bone marrow contains a large number of hematopoietic and stromal stem cells and progenitor cells, which are in a microenvironment abundant in factors at different stages of differentiation and become different subsets of cells [12]. The content of classical $CD14^{++}CD16^-VEGFR2^+$ and non-classical $CD14^+CD16^{++}VEGFR2^+$ monocytes in the myeloid tissue in the CAD patients without ICM was almost 3 times higher than in the ICM patients, and the number of intermediate $CD14^{++}CD16^+VEGFR2^+$ monocytes was 2 times higher (Fig. 2). Consequently, differentiation of these three subpopulations of $CD14^+VEGFR2^+$ monocytes is impaired in ICM, which explains the trend toward a decrease in the total number of $CD14^+VEGFR2^+$ cells in the bone marrow in the ICM patients.

The comparative analysis of the VEGF-A, TNFα, IL-13, and M-CSF concentrations in the myeloid tissue in the CAD patients did not reveal any differences between the groups of patients, which does not allow to select a specific cytokine responsible for impaired differentiation of $CD14^+VEGFR2^+$ cells in the bone marrow in ICM. At the same time, a clear trend toward elevated M-CSF values in the CAD patients without ICM with reciprocal (in many patients) IL-13 changes in the bone marrow made it possible to calculate the ratio of these cytokines and reveal a 9-fold decrease in the M-CSF / IL- 13 ratio in the ICM patients compared with CAD patients without ICM. This is important because these cytokines have different effects on monocytopoiesis.

Stimulation of the receptor for M-CSF (CSF-1R), the expression of which increases by 10 times in "the colony-forming unit of macrophages – monoblast – promonocyte – monocyte – macrophage" cell series, induces an early reaction of monocytic cells in the form of increased protein synthesis and cytoskeleton actin network rearrangement, later – macrophages differentiation and proliferation with trophic and growth-stimulating properties. During myelopoiesis, a proliferative response to M-CSF is possible precisely in myeloblasts due to increased activity of ζ-type protein kinase C, which stimulates the Erk1 / 2 pathway. This enzyme is absent in promonocytes, and CSF-1R stimulation only leads to their differentiation with the participation of the PI3K / Akt pathway, which also ensures the survival of monocytes / macrophages [28, 29].

Along with this, Treg-derived IL-13, acting on monocytes, has a differentiation potential (induces maturation of monocytes into alternative macrophages and secretion of immunosuppressive IL-10 by them) [19], but its proliferative and anti-apoptotic effects on monocyte cells have not been described. In monocytes, IL-13 activates STAT 1, 3, 5, 6 via Jak2 and Tyk2 kinases, inhibits synthesis of proinflammatory cytokines, including IL-1β, IL-6, IL-8, and TNFα, and downregulates the surface expression of Fc receptor for IgG (i.e. CD16 molecules) [30]. The latter probably explains the reduced content of non-classical $CD14^+CD16^{++}VEGFR2^+$ monocytes in the bone marrow in the ICM patients compared with the CAD patients without ICM (Table 2). The decrease in the number of classical $CD14^{++}CD16^-VEGFR2^+$ and transitional $CD14^+CD16^-VEGFR2^+$ cells that do not express CD16 molecules in the ICM patients (Table 2) is rather associated with both insufficient proliferation and reduced survival of monocytic cells in the bone marrow at low M-CSF / IL-13 ratio. The high M-CSF / IL-13 ratio in the CAD patients without ICM indicates the predominance of proliferative, anti-apoptotic, and differentiation M-CSF signals in monocyte progenitors over the IL-13-mediated stimulus that blocks maturation of the most differentiated $CD16^+$ forms of $VEGFR2^+$ monocytes. In ICM, these signals in monocytic cells are balanced (the M-CSF / IL-13 ratio is close to 1, Table 2).

The results of the correlation analysis for the parameters of the $VEGFR2^+$ subpopulation composition demonstrate that in the CAD patients, the content of $CD14^+VEGFR2^+$ cells in the bone marrow is

determined by the number of non-classical $CD14^{+}CD16^{++}VEGFR2^{+}$ cells and in the blood – by the number of classical $CD14^{++}CD16^{-}VEGFR2^{+}$ monocytes as the most numerous subpopulation of blood monocytes in these patients. In the blood of the ICM patients, the population of transitional $CD14^{+}CD16^{-}VEGFR2^{+}$ monocytes is the most numerous, determining, along with classical $CD14^{++}CD16^{-}VEGFR2^{+}$ and non-classical $CD14^{+}CD16^{++}VEGFR2^{+}$ cells, the total number of $CD14^{+}VEGFR2^{+}$ cells both in the blood and the bone marrow. At the same time, the ratio between $CD14^{+}VEGFR2^{+}$ cell subpopulations in the ICM patients is equivalent in both tissues (Fig. 1, 2), and in the CAD patients without ICM, the dominant populations are transitional CD14+CD16-VEGFR2+ monocytes in the bone marrow and classical CD14++CD16-VEGFR2+ monocytes in the blood.

Taken together, this means that in ICM, there is no further differentiation of $CD14^{+}VEGFR2^{+}$ cells and their subpopulation composition in the blood corresponds to that in the bone marrow, where non-classical $CD14^{+}CD16^{++}VEGFR2^{+}$ monocytes differentiate from classical $CD14^{++}CD16^{-}VEGFR2^{+}$ cells (according to the identified correlations, see above). In CAD without ICM, the formation of non-classical $CD14^{+}CD16^{++}VEGFR2^{+}$ cells occurs not only in the bone marrow, but probably continues in the blood from transitional $CD14^{+}CD16^{-}VEGFR2^{+}$ monocytes (according to the correlation). Apparently, the formation of classical $CD14^{++}CD16^{-}VEGFR2^{+}$ and transitional $CD14^{+}CD16^{-}VEGFR2^{+}$ cells occurs constitutively to a greater extent, while the non-classical $CD14^{+}CD16^{++}VEGFR2^{+}$ generation is inducible. It is worth noting that the content of intermediate $CD14^{++}CD16^{+}VEGFR2^{+}$ monocytes did not correlate either with the numbers of their other subpopulations or with the total number of $CD14^{+}VEGFR2^{+}$ cells. It can be explained by a combination of differentiation of these monocytes with their parallel homing into the vascular wall as a subpopulation of $CD14^{+}VEGFR2^{+}$ cells, which have the maximum $VEGFR2^{+}$ expression on monocytes [11]. It was shown that EPCs are involved in the angiogenesis directly by incorporation into the vascular wall in the area of vascular growth and indirectly by secretion of proangiogenic factors [6].

CONCLUSION

ICM development is apparently associated with insufficient vascular repair due to the absence of the compensatory response of the body manifested through an increase in the number of $CD14^{+}VEGFR2^{+}$ cells during the atherogenesis. The content of $CD14^{+}VEGFR2^{+}$ cells in the blood during ICM is reduced compared with their number in the CAD patients without ICM due to the lower number of classical $CD14^{++}CD16^{-}VEGFR2^{+}$, intermediate $CD14^{++}CD16^{+}VEGFR2^{+}$, and non-classical $CD14^{+}CD16^{++}VEGFR2^{+}$ monocytes. At the same time, in the bone marrow of the ICM patients, due to the low M-CSF / IL-13 ratio, the differentiation of their precursors (transitional $CD14^{+}CD16^{-}VEGFR2^{+}$ cells) and, as a result, classical $CD14^{++}CD16^{-}VEGFR2^{+}$ and non-classical $CD14^{+}CD16^{++}VEGFR2^{+}$ cells is reduced. The formation of the latter in ICM is not activated from transitional $CD14^{+}CD16^{-}VEGFR2^{+}$ cells either in the bone marrow or in the blood, which, on the contrary, is typical of CAD patients without ICM.

The maturation of intermediate $CD14^{++}CD16^{+}VEGFR2^{+}$ cells in the myeloid tissue is not affected, and their lower content in the blood is due to the absence of active migration of these cells from the bone marrow at the physiological concentration of MCP-1 in the blood, which is increased in CAD patients without ICM. At the same time, the VEGF-A and TNFα concentrations in the blood and bone marrow probably do not affect the number of $CD14^{+}VEGFR2^{+}$ cells and their subpopulations. Consequently, they differentiate and migrate from the myeloid tissue, like monocytic cells during inflammation, and the hypoxia mediator VEGF-A controls these processes to a lesser extent (due to the absence of an increase in its concentration in the blood and bone marrow). Knowledge about such patterns of EPC generation and migration will help to induce this process in ICM patients and develop a new treatment method for this severe heart disease in the future.

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Chumakova S.P. – research design, review of literature, statistical processing of research results and their interpretation, drafting and design of the manuscript. Urazova O.I. – research design, coordination of laboratory research, interpretation of the results, editing of the manuscript. Shipulin V.M. – consulting on research planning and interpretation of the clinical aspects of the results. Denisenko O.A. – preparation of biomaterial samples, review of literature. Kononova T.E. – carrying out of the enzyme immunoassay, preparation of images. Nevskaya K.V. – carrying out of flow cytometry. Andreev S.L. – collection of patients' data, provision of clinical material, intraoperative bone marrow sampling.

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Clinical possibilities of HER2-positive breast cancer diagnosis using alternative scaffold proteins

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ABSRTACT

HER2-positive breast cancer occurs in 15–20% of breast cancer patients and is associated primarily with a poor prognosis of the disease and the need for highly specific targeted therapy. Despite the clinical importance of determining HER2/neu, traditional diagnostic methods have their disadvantages and require the study of new additional research techniques.

The information presented in this review makes it possible to consider current trends in the radionuclide diagnosis of HER2-positive breast cancer using the latest class of alternative scaffold proteins and to consider various aspects of their use in clinical practice.

Keywords: breast cancer, radionuclide diagnosis, alternative scaffold proteins, HER2/neu

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Клинические возможности диагностики HER2-позитивного рака молочной железы с применением альтернативных каркасных белков

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РЕЗЮМЕ

HER2-позитивный рак молочной железы (РМЖ) встречается у 15–20% пациенток с РМЖ и ассоциируется прежде всего с неблагоприятным прогнозом заболевания и необходимостью назначения высокоспецифичной таргетной терапии. Несмотря на клиническую важность определения рецептора эпидермального фактора роста 2-го типа, существующие диагностические методики являются несовершенными и требуют изучения новых дополнительных методов исследования.

Представленные в обзоре данные позволяют рассмотреть современные тенденции в радионуклидной диагностике HER2-позитивного РМЖ с применением новейшего класса «нацеливающих» модулей (альтернативных каркасных белков), а также демонстрируют различные аспекты их использования в клинической практике.

Ключевые слова: рак молочной железы, радионуклидная диагностика, альтернативные каркасные белки, DARPinG3, HER2/neu

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

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INTRODUCTION

Human epidermal growth factor receptor 2 (HER2 / neu) is one of the most well-described molecular targets on the surface of tumor cells belonging to the receptor tyrosine kinase family, the epidermal growth factor receptor subfamily [1]. The highest expression of HER2 / neu is most often observed in patients with breast cancer (15–20% of cases) and associated with an unfavorable prognosis of the disease and its aggressive course [2]. In addition, according to clinical recommendations, overexpression of HER2 / neu requires targeted therapy, including mandatory prescription of such drugs as trastuzumab (herceptin), lapatinib, pertuzumab, and trastuzumab emtanzine (T-DM1) [3, 4].

Targeted therapy is highly specific and determines the need for careful selection of candidates for the treatment. Currently, several FDA-approved (U.S. Food and Drug Association) techniques are used to determine the status of HER2 / neu, which include

immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH). According to the American Society of Clinical Oncology (ASCO), category 0 and 1+ cases are considered negative, and category 3+ cases are considered positive (as of 2018). Controversial category 2+ cases require FISH; amplification is considered positive if an average number of copies for the *ERBB2* gene and an average number of chromosome 17 centromeres in a cell are more than 2.2 [5].

An obvious disadvantage of IHC, despite availability and relative cheapness, is significant influence of many factors on the results, including the method of sample preparation (the duration of fixation and the fixator used), the characteristics of the antibodies used, the qualifications of the staff, and the interpretation of the results (mainly category 2+ cases) [6]. FISH remains a very reliable method for assessing amplification of the *ERBB2* gene, however, it takes nine times longer (36 vs 4 hours) and costs several times more compared with standard IHC. Moreover, FISH requires expensive equipment for detecting and recognizing signals and highly qualified staff for interpreting results [7].

One of the main disadvantages of the study is a need for invasive manipulations to obtain material for diagnosis, as well as inability to simultaneously assess tumor spread in a patient and analyze molecular characteristics of tumor sites before specific treatment [8]. The latter is especially relevant in terms of possible differences in HER2 / neu expression in a primary tumor and regional lymph nodes and distant metastases, which can occur in 6–48% of cases, according to various studies [9].

The problem of intratumoral heterogeneity, which occurs in 40% of breast cancer cases and is characterized by lower rates of relapse-free survival and effective targeted therapy with trastuzumab also remains unresolved. All this dictates the need for development of a new additional diagnostic technique to optimize the diagnosis in breast cancer patients [10].

CLINICAL STUDIES ON THE DIAGNOSIS OF HER2-POSITIVE BREAST CANCER USING ALTERNATIVE SCAFFOLD PROTEINS

In recent years, targeted radionuclide methods have been studied to identify malignancies. They have a number of significant advantages, such as non-invasiveness with a possibility of repeated studies; runtime assessment of marker expression with ongoing treatment; simultaneous visualization of the whole patient's body with an assessment of HER / neu expression in the primary tumor and metastatic sites [11], as well as improvement of diagnostic equipment, manifested through development of devices combining modules for radionuclide studies and anatomical visualization of metastatic sites (computed tomography and magnetic resonance imaging) [12].

Over the past decade, a new class of "targeting" modules called alternative scaffold proteins or scaffolds has become very popular. It meets all the requirements for optimal radionuclide delivery to tumor cells [13]. The advantages of targeted molecules include significantly smaller sizes compared with a standard antibody, which increases penetration of the substance into the tumor; stable structure; additional functionalization and expression in the bacterial system, which contributes to low production costs; high thermal stability, contributing to long-term storage at room temperature, as well as a possibility of direct chemical synthesis [14]. Currently, 3 scaffolds have been clinically tested in the diagnosis of HER2-positive breast cancer: affibodies, ADAPTs, and DARPins.

Affibodies. Affibody molecules are three tightly packed alpha helices stabilized by a hydrophobic core. They consist of 58 amino acids with a molecular weight of 6–7 kDa and have a small size. The largest number of studies on affibodies are based on their high affinity to the HER2 / neu receptors [15]. In the phase I clinical trial of ^{111}In-ABY-025 (^{111}In, half-life of 2.8 days), including patients with locally advanced and metastatic breast cancer (5 patients with HER2 / neu overexpression and 2 patients without receptor expression), J. Sorensen et al. demonstrated the safety of ^{111}In-ABY-025 affibody molecule and the possibility of differentiating a primary tumor and metastatic sites depending on the HER2 / neu status [16]. However, this study demonstrated a limited ability to visualize small tumor sites in HER2-positive patients, which was probably due to the low resolution of single-photon emission computed tomography (SPECT / CT). The obtained data determined the beginning of the study on ^{68}Ga-ABY-025 (^{68}Ga, half-life of 68 minutes) already for positron emission computed tomography (PET / CT).

The phase I clinical trial of ^{68}Ga-ABY-025 demonstrated the safety of the affibody molecule in 8 patients with metastatic breast cancer and stressed the importance of the dosage. So, using 78 μg of the protein resulted in statistically higher accumulation of ^{68}Ga-ABY-025 in the liver and kidneys compared with accumulation resulting from the use of 427 μg of

protein [17]. The study of ^{68}Ga-ABY-025 by J. Sorensen et al. involving 16 patients with metastatic breast cancer (12 patients with overexpression of HER2 / neu; 4 patients without it) showed not only the possibility of visualizing metastases to regional lymph nodes and distant organs and tissues in all cases, but also the possibility of their exact differentiation depending on the HER2 / neu expression [18].

One of the findings in the study was a clinical observation of a breast cancer patient with a HER2-negative breast tumor and liver metastasis with HER2 / neu overexpression, which were detected by ^{68}Ga-ABY-025 and confirmed by immunohistochemistry of the biopsy material from the identified tumor sites (Fig. 1).

In an additional analysis of ^{111}In-ABY-025 and ^{68}Ga-ABY-025 involving 23 patients with metastatic breast cancer, D. Sandberg et al. also determined that the spleen was the best reference organ, while the tumor-to-spleen ratio reached 100% accuracy in the differentiation of tumor nodules depending on the HER2 / neu status 4 hours after the injection according to PET data and 24 hours after the injection according to SPECT [19].

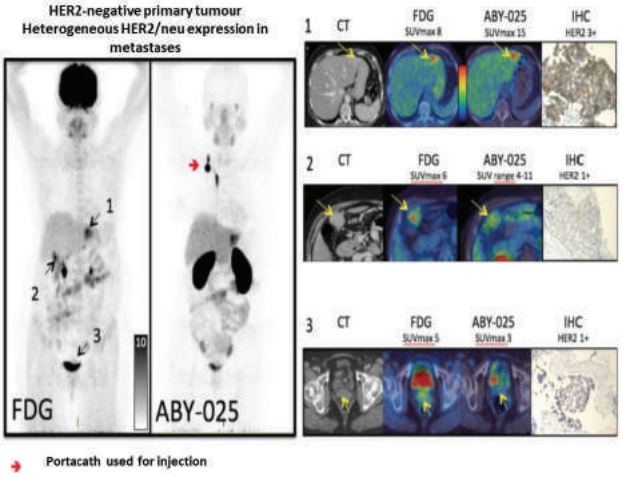

Fig 1. HER2-negative primary breast tumor patient with HER2-positive metastases in left liver lobe according to PET / CT findings using ^{68}Ga-ABY-025

ADAPTs (ABD-Derived Affinity Proteins). The molecules were developed using a 46-amino acid framework derived from an albumin binding domain (ABD), which spontaneously folds into a three-spiral structure and is independent of disulfide bridges [20]. The HER2 / neu-specific ADAPT6 molecule was chosen because of its high affinity (1 nM) and rapid removal from the bloodstream due to weak binding to albumin, which was reflected by the results of preclinical studies [21].

In the phase I clinical trial of ^{99m}Tc-ADAPT6 (^{99m}Tc, half-life of 6.01 hours), including 22 breast cancer patients with different HER2 / neu expression in the primary tumor, three protein dosages were studied: 250, 500, and 1,000 μg. According to the results, ^{99m}Tc-ADAPT6 demonstrated good tolerability and the absence of changes in vital organs. The best difference between HER2-posivive and HER2-negative tumors was observed 2 hours after the injection of the protein at a dose of 500 μg with an average tumor-to-background ratio of 37±19 for HER2-positive tumors compared with 5±2 for HER2-negative tumors ($p < 0.05$, Mann – Whitney test). The difference between the groups in other time points was unreliable. The tumor-to-background ratio for HER2-positive breast tumors was significantly higher in patients who received 500 μg of the protein compared with the doses of 250 and 1,000 μg ($p < 0.05$, Mann – Whitney test). In addition, according to the study, a relatively low effective dose was determined for the patient when injecting 500 and 1,000 μg of the protein – 0.009 ± 0.002 and 0.010 ± 0.003 mSv / MBq, respectively, which was comparable with the data obtained in the study of other scaffold proteins (Fig. 2) [22].

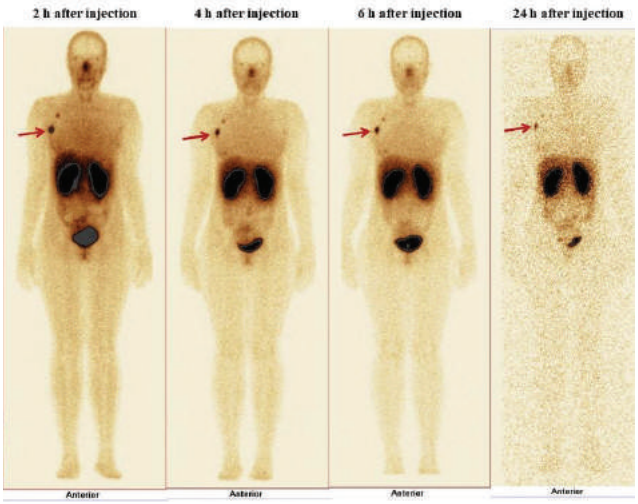

Fig. 2. Anterior projection of planar scintigraphy of the HER2-positive breast cancer patient 2, 4, 6, and 24 hours after the injection of 500 mμ ^{99m}Tc-ADAPT6 (arrows indicate tumor in the right breast)

In this study, one of the breast cancer patients was included with a HER2-positive tumor according to IHC of the biopsy material, however, after the ^{99m}Tc-ADAPT6 injection, a low tumor-to-background ratio

was revealed (1.33 2 hours after the injection of the protein at a dose of 500 μg). After IHC revision, the tumor status was changed to 2+, and FISH showed no amplification of the *ERBB2* gene. According to the results, the HER2-status of the breast tumor was changed to "negative", and targeted therapy was canceled (Fig. 3) [23].

Another clinical example of the use of ^{99m}Tc-ADAPT6 at a dose of 500 μg is detection of additional metastatic sites in the patient with HER2-positive breast cancer projected at the right 5th rib along the mid-clavicular line and at 8th–9th thoracic vertebrae. The revealed changes were not diagnosed by a standard bone scan and computed tomography of the chest, but were confirmed by magnetic resonance imaging (MRI) data (Fig.4) [24].

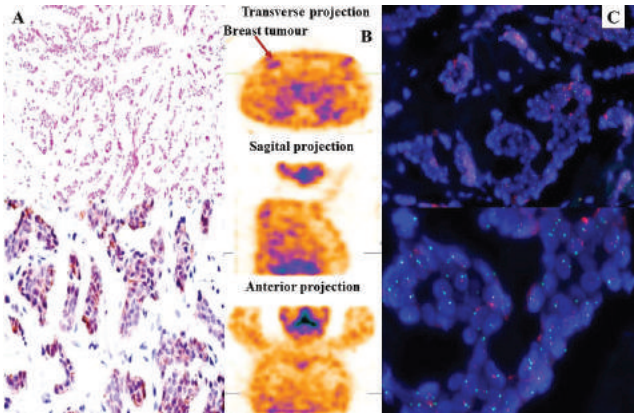

Fig. 3. Results of IHC, FISH, and radionuclide study with ^{99m}Tc-ADAPT6 at a dose of 500 μg in the breast cancer patient: *A* – IHC of the biopsy material (HER2/neu 2+ expression); *B* – radionuclide study with ^{99m}Tc-ADAPT6 (arrows indicate tumor in the right breast); *C* – FISH with negative amplification of the *ERBB2* gene in the biopsy material

DARPins (Designed Ankyrin Repeat Proteins) are representatives of scaffold proteins which were designed on the basis of ankyrin proteins. Ankyrins are involved in the attachment of membrane proteins to the cytoskeleton. The framework of DARPins can include 4–6 ankyrin domains, each of which contains 33 amino acids; the domains are organized as two antiparallel alpha helices with a beta turn between them [25]. Since the molecular weight of one module is slightly more than 3.5 kDa, and DARPins consist of 4-6 modules, their molecular weight ranges from 14 to 21 kDa and is about one tenth the size of a conventional antibody (IgG) or one third the size of Fab [26].

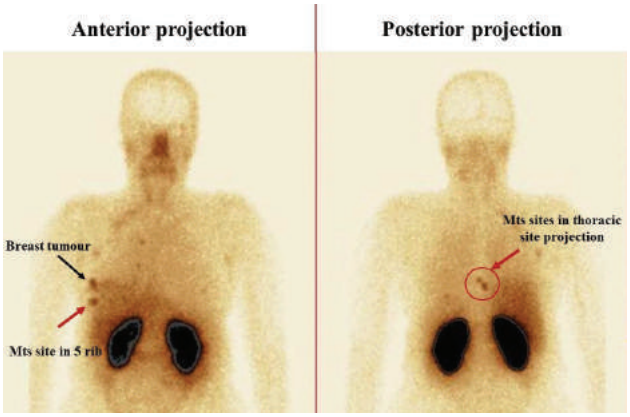

Fig. 4. Planar scintigraphy of the HER2-positive breast cancer patient 2 hours after the ^{99m}Tc-ADAPT6 injection (anterior and posterior projection): visualization of pathologic accumulation in the primary breast tumor projection (indicated by the black arrow); metastatic sites projected at the 5th rib (right) and Th 8–9 (indicated by the red arrow)

The phase I clinical trial of ^{99m}Tc-DARPinG3 was performed on 28 breast cancer patients with different HER2 / neu expression using three doses of the protein: 1,000, 2,000, and 3,000 μg. Whole-body planar scintigraphy and SPECT of the chest were performed in all patients 2, 4, 6, and 24 hours after the injection of the protein. The results of the study showed the absence of toxic effects of ^{99m}Tc-DARPinG3 over the entire follow-up period, its rapid excretion with the blood flow, as well as a relatively low effective dose on the patient when injecting the protein at a dose of 1,000, 2,000, and 3,000 μg (0.011 ± 0.001, 0.012 ± 0.006, and 0.012 ± 0.003 mSv / MBq, respectively) (Fig.5). The best tumor-to-background ratio was observed in patients with HER2 / neu overexpression 2 and 4 hours after the injection of the protein at a dose of 1,000 and 2,000 μg; and 2, 4, and 6 hours after the injection of the protein at a dose of 3,000 μg ($p < 0.05$, Mann – Whitney test). At the same time, the most effective dose of ^{99m}Tc-DARPinG3, allowing to visualize liver metastasis, was 3,000 μg [27].

One of the patients with HER2-negative breast cancer (HER2 /neu 1+ according to IHC of the biopsy material) included in the study showed a high tumor-to-background ratio of 12.5 (4 hours after the administration of the protein at a dose of 2,000 μg). FISH of the biopsy material revealed amplification of the *ERBB2* gene in 35% of tumor cells, and IHC data determined overexpression of HER2 / neu. As a result, the patient's tumor status was changed to "positive", and targeted therapy was added to the planned systemic treatment (Fig. 6).

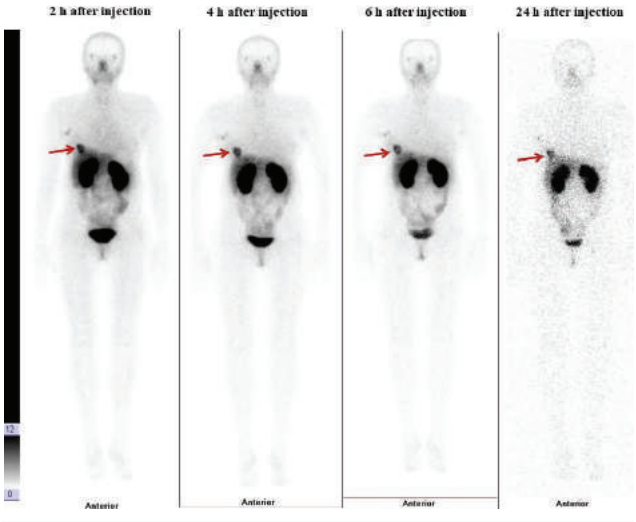

Fig. 5. Anterior projection of planar scintigraphy of the HER2-positive breast cancer patient 2, 4, 6, and 24 hours after the injection of ^{99m}Tc-DARPinG3 at a dose of 3,000 mμ (arrows indicate tumor in the right breast)

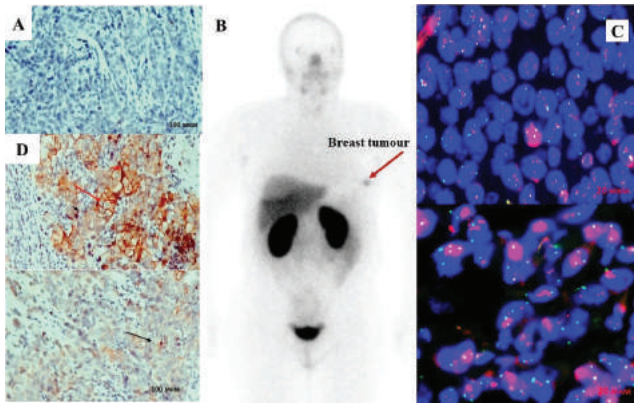

Fig. 6. Results of IHC, FISH, and radionuclide study with ^{99m}Tc-DARPinG3 injected at a dose of 2,000 μg in the breast cancer patient: *A* – IHC of the biopsy material showing negative HER2 / neu expression; *B* – radionuclide study with ^{99m}Tc-DARPinG3 (left breast tumor is indicated by the arrow); *C* – FISH results with *ERBB2* amplification in 35 % of tumor cells (operative material); *D* – IHC of the biopsy material with HER2 / neu overexpression

A similar example was observed in the patient with a HER2-negative breast tumor according to IHC, who also had a high tumor-to-background ratio after the ^{99m}Tc-DARPinG3 injection (the ratio of 14.4 4 hours after the injection of 2,000 μg of the protein). According to the results of FISH, amplification of the *ERBB2* gene was revealed, and the tumor status was changed to "positive" (Fig. 7) [28].

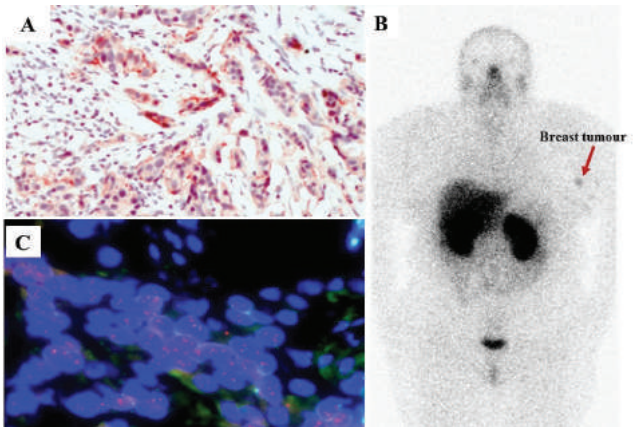

Fig. 7. Results of IHC, FISH, and radionuclide study with ^{99m}Tc-DARPinG3 injected at a dose of 2,000 μg in the breast cancer patient: *A* – IHC of the biopsy material showing negative HER2 / neu expression; *B* – radionuclide study with ^{99m}Tc-DARPinG3 (left breast tumor is indicated by the arrow); C– FISH results with *ERBB2* amplification in the biopsy material

DISCUSSION

HER2-positive breast cancer belongs to cancer subtypes with the most unfavorable disease prognosis, requiring highly specific targeted treatment. Unfortunately, the currently used IHC and FISH are not optimal and cannot solve all the problems. One of the options for optimizing the diagnostic algorithm for HER2-positive breast cancer detection is radionuclide imaging methods using alternative scaffold proteins labeled with various radionuclides.

CONCLUSION

The results of clinical studies on radiopharmaceuticals based on labeled affibody molecules, ADAPTs, and DARPins for SPECT and PET demonstrated in this review allow to consider various aspects of their application in clinical practice which are not available in conventional diagnostic methods. In particular, the possibility of simultaneous assessment of tumor spread and detection of molecular characteristics for identified tumor sites is the most relevant. The data obtained during the performed clinical studies undoubtedly indicate the importance of this research method and the need for its further study.

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Authors contribution

Bragina O.D., Chernov V.I., Deyev S.M., Tolmachev V.M. – conception and design, analysis and interpretation of the data; substantiation of the manuscript and critical revision of the manuscript for important intellectual content; final approval of the manuscript for publication.

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Diagnostic radiology methods for assessing coronary artery bypass graft viability

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ABSTRACT

The review describes available modern radiological methods which are currently applied for a detailed and comprehensive anatomical and functional assessment of the viability of various coronary artery bypass grafts. In addition, it presents some aspects of the implementation of these methods and clinical interpretation of the results.

Keywords: coronary artery bypass grafting, arterial and venous conduits, diagnostic radiology methods

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Лучевые методы диагностики в оценке состоятельности коронарных шунтов

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РЕЗЮМЕ

В обзоре представлено описание лучевых инструментов и методов, доступных в настоящее время, для получения тщательной и полной анатомической и функциональной оценки состоятельности различных коронарных шунтов, а также некоторые детали выполнения и клинической интерпретации результатов этих исследований.

Ключевые слова: аортокоронарное шунтирование, артериальные и венозные кондуиты, лучевые методы диагностики

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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

Coronary artery disease (CAD) is one of the leading causes of morbidity and mortality worldwide, affecting nearly 20% of people over 65 years [1] and accounting for 370,000 deaths each year [2]. The clinical and prognostic benefits of coronary artery bypass grafting (CABG) are well accepted, because the surgical method allows to achieve complete revascularization in patients with multivessel CAD and is performed in over 1 million patients annually [3]. Although the 5-year survival after CABG is 75–80% [4, 5], nearly 20% of patients develop congestive heart failure (CHF) within 2 years after the surgery [6]. CABG provides excellent short- and intermediate-term results, but a long-term outcome is highly dependent on the patency of the vascular conduits used.

Venous grafts are more susceptible to the development of pathological changes due to the fact that the structure of the vessel itself is not designed for high blood pressure. Impaired vascular tone, vessel dilation, and slowed blood flow can eventually cause thrombosis of the graft. Over time, the venous conduit can adapt to the arterial blood flow, but becomes susceptible to atherosclerotic damage [7, 8]. The use of arteries similar in their anatomical structure to coronary vessels gives a much better result, but arteries also differ from each other in their biological characteristics.

Currently, internal thoracic (mammary) arteries (ITA) are increasingly used in CABG as the most viable ones having a number of advantages. The impressive long-term patency of the left ITA as a graft to the left anterior descending coronary artery (LITA – LAD) combined with proven long-term viability determined that this conduit has become the gold standard for CABG. However, the use of arterial conduits has expanded beyond ITAs and included the right gastroepiploic artery (GEA), the inferior epigastric artery (IEA), and the radial artery (RA).

INTERNAL THORACIC ARTERY

Based on its long-term patency rates, the ITA has been shown to be an excellent conduit for myocardial revascularization compared with the saphenous vein graft (SVG). The incidence of atherosclerosis in the ITA is low even in patients with severe CAD who underwent CABG [9].

The survival benefits associated with the use of the LITA as a bypass to the LAD were established in the Cleveland Clinic almost 30 years ago [10]. The improved outcome using the ITA is almost certainly due to its superior long-term patency rate of more than 90% within 10 years after surgery [10]. The excellent viability of the ITA can probably be explained by its peculiar morphological features.

The ITA has a discontinuous internal elastic lamina, a relatively thin media and lacks a significant muscular component, which explains its reduced tendency for spasm and development of atherosclerosis [11]. Moreover, compared with all other arterial and venous conduits, it shows increased production of anti-inflammatory and vasoactive molecules [12, 13]. Indeed, endothelial cells of the ITA release more prostacyclin [12] and nitric oxide [13] than those in SVG.

The highest patency rates were documented when the ITA (either *in situ* or as a Y or free graft) was sutured to the left coronary artery and its branches [14]. Poor rates were registered when the ITA was used for right coronary artery bypass (probably due to size discrepancy and disease progression, or to a smaller amount of viable myocardium) [14].

Bilateral use of natural ITA bifurcations (BITA) for myocardial revascularization should be highlighted separately. Only one randomized

trial compared outcomes between single ITA and BITA grafting. In the Arterial Revascularization Trial (ART), the primary endpoint – 10-year survival and an interim analysis at 1 year (a "safety" endpoint), reported excellent outcomes with both strategies. Mortality, stroke, myocardial infarction, and repeat revascularization accounted for less than 2.5% in the compared groups [14].

In addition to the ART study, a systematic review of matched cohorts of almost 15,000 CABG patients who received BITA grafts, initiated more than a decade ago, reported significant reduction in the hazard ratio (HR) for mortality [3]. In the past 2 years, two independent meta-analyses have supported this finding, not only in larger cohorts of patients, but also with longer-term follow-up [15, 16].

The major concern with the use of BITA grafts is the increased risk of sternal wound complications and mediastinitis. One of the largest meta-analyses on this issue showed that adding a second ITA to the ITA–LAD bypass graft significantly increases the incidence of sternal complications, and this risk is even higher in diabetes mellitus and in patients with pulmonary diseases [17]. In the ART study, the incidence of sternal wound complications increased from 0.6% in the single ITA group to 1.9% in the BITA group [14].

However, the incidence of serious wound problems can be significantly reduced by judicious patient selection and the choice of harvesting technique. Consideration should be given to avoiding BITA in patients with certain risk factors, especially if they occur simultaneously (diabetes mellitus, obesity, respiratory problems), and in patients receiving steroids or immunosuppressive therapy. Moreover, two systematic reviews both reported that ITA skeletonization, rather than a pedicled harvesting technique, significantly reduces deep sternal wound infections, even in patients with diabetes [17, 18].

RADIAL ARTERY

Introduced in coronary surgery in the 1970s [19], the radial artery (RA) was "rediscovered" in the early 1990s [20]. Concerns over vasospasm due to the muscular nature of the RA wall have been reduced after the demonstration of progressive morphofunctional remodeling of the artery toward an elastomuscular profile after implantation in the coronary circulation [21]. The largest angiographic studies report a RA patency rate of 80 to 90% after 7–10 years of follow-up [22]. According to other authors, 2 years after surgery, the frequency of occlusions and stenosis of *a. radialis* grafts was 35 and 15%, respectively [23].

The severity of stenosis of the target vessel is a key factor in determining RA patency. There is general agreement that the RA should be used only to bypass a vessel with > 70% stenosis, and there is evidence that a 90% stenosis limit ensures an even better RA patency rate, especially in the right coronary artery [24, 25]. The site of proximal anastomosis and the harvesting technique (open and endoscopic) do not affect RA patency rates, whereas skeletonization of the artery can lead to better patency [26].

The RA is an arterial conduit for which there is the most evidence of benefit derived from computed tomography (CT). Results of the RA use have been compared with either SVG or the right internal thoracic artery (RITA) in 4 randomized controlled trials (RCT) [25, 27–29]. A number of meta-analyses that pooled data from these RCTs and large observational studies to compare the RA and the SVG [30–34] with median follow-up time extending beyond the first postoperative year reported significant benefits in terms of graft patency for the RA. The only meta-analysis that included clinical outcomes revealed reduced cardiac death, myocardial infarction, and repeat coronary procedures in addition to a better RA graft patency in the late postoperative period [33].

The RAPCO (Radial Artery Patency and Clinical Outcomes) study revealed no differences in the patency of RA and RITA, but only a slight trend toward better survival without cardiac events for the RA during 6 years of follow-up [29]. The only comparative meta-analysis with clinical endpoints reported comparable mortality, but at the same time reduced cardiac events (myocardial infarction, heart failure, ischemia) for the RA [31]. However, a meta-analysis of angiographic studies showed that the use of RITA was associated with nonsignificant reduction of the absolute risk (by 27%) for late graft occlusion when compared with the RA [34].

Compared with the RITA, the RA appears to be the preferred choice in patients at risk for post-operative sternal complications (diabetes, obesity, chronic obstructive pulmonary disease). Indeed, harvesting of RA is extremely safe and well tolerated even by seriously ill patients [31] and (unlike RITA) does not affect sternal vascularization and healing [35, 36]. Furthermore, a recent Radial Artery Patency Study (RAPS) focusing only on diabetic patients reported a very strong protective effect against graft occlusion with the use of RA [37], making the use of this conduit in diabetics particularly attractive.

Most researchers consider the absence of natural blood supply to the vascular wall through the *vasa vasorum* as one of the main disadvantages of radial grafts. M. Gaudino et al. [38] showed the propensity of radial grafts to spasm. In addition, there is a pronounced proliferative reaction of the vascular wall, leading to stenosis and occlusion of the grafts in the first year after surgery, and the administration of calcium antagonists to prevent *a. radialis* spasm did not improve graft patency in the first year after CABG [39].

GASTROEPIPLOIC ARTERY

J. Pym et al. [40] and H. Suma et al. [41] first independently reported systematic use of the gastroepiploic artery graft for CABG in 1987. Since then, GEA grafts have been widely applied in clinical practice. Very few CABG candidates have contraindications to GEA harvesting [42]; the conduit has a low incidence of severe atherosclerosis [43] and good flow capacity [44].

The biological and physiological profile of the GEA has now been extensively studied [45], and the use of this artery does not increase perioperative risk [46]. The incidence of atherosclerosis in the GEA is rare, but somewhat more common than in ITA [43]. The most favorable target for the *in situ* GEA graft is the distal right coronary artery, but the conduit can also be used for the distal circumflex system. Subocclusive (> 90%) stenosis of the target coronary artery is essential to maximize patency rates and avoid spasm and eventual failure due to chronic competitive coronary flow. This issue is formally recognized in the 2011 ACCF / AHA Guideline for Coronary Artery Bypass, which contraindicates RCA arterial bypass (Class III) in patients with less than critical (i.e., > 90%) stenosis of the native vessel [47].

In one of the largest series of publications on GEA, the cumulative patency rate of the artery was 97.1% at 1 month, 92.3% at 1 year, 85.5% at 5 years, 80.9% at 7 years, and 66.5% at 10 years after surgery [48]. This relatively low patency rate at late periods has improved by using a skeletonized GEA graft only to target vessels with > 90% stenosis. Using this approach, T. Suzuki et al. [49] have reported 97.8%, 94.7%, and 90.2% cumulative patency rates in the early post-operative period and at 5 and 8 years after surgery, respectively.

A number of studies have established that the use of GEA, instead of SVG, to graft the right coronary artery in patients with BITA to the left coronary artery leads to a significant increase in late survival [50, 51]. However, other studies have not confirmed this finding, and a recent meta-analysis of contrast-enhanced coronary angiographies comparing all conduits used in CABG surgery has found that the GEA is associated with the highest risk of functional and complete graft occlusion [34]. Of note, the majority of publications report the use of GEA as a pedicled, rather than a skeletonized graft; skeletonized harvesting of the artery has been shown to significantly improve its patency [49].

MORPHOLOGICAL AND PATHOPHYSIOLOGICAL FACTORS AFFECTING LONG-TERM GRAFT PATENCY

Flow competition is a significant factor affecting arterial morphology and conduit patency. The string sign is an atrophic change in the arterial conduit. This phenomenon occurs due to competitive flow in bypass grafting of only mildly stenosed coronary arteries (CA). It has been shown that the diameter of ITA graft decreases if the stenosis of the native CA is less than critical [52]. In the GEA, patency rates seem to be reduced due to competitive flow. H. Suma et al. [53] reported that the 10-year patency rate of the GEA was 62.5%, and that anastomosis to a less critically stenosed CA was one of the major causes of late graft occlusion. The relationship between the SVG patency and native CA stenosis has also been controversial, but competitive flow may be a negligible factor in SVG graft patency.

Wall shear stress is believed to play an important role in the development of atherosclero-

sis. T. Shimizu et al. [54] found that shear rates of the ITA were higher compared with the SVG, suggesting that these differences might contribute to the development of degenerative graft disease and affect long-term conduit patency. Indeed, endothelial cells in the ITA release more prostacyclin [12] and nitric oxide [55] than those in the SVG. The effect of flow competition on wall shear stress in a coronary artery bypass conduit is unknown. Greater flow rates and smaller vessel diameter increase wall shear stress.

G. Tinica et al. [56] conducted a study in order to identify morphological and pathophysiological factors associated with long-term patency of grafts used in CABG. The results of CT evaluation of the patency of 340 grafts in 127 patients at 139.78 ± 36.64 months post-CABG were analyzed. Graft patency varied according to the vessel type and target territory. The maximum patency rate was obtained with the RA (80.65%) for the right coronary territory, RITA (92.86%) for the anterolateral territory, and SVG (82.54%) for the circumflex territory.

The LITA – LAD graft occluded in 13 (7.93%) cases, in 7 of them – due to competitive flow. The influence of graft length on patency rates after indexing on height was not significant. The degree of stenosis of the native (bypassed) vessel influenced arterial graft patency rates with an occlusion odds ratio (OR) of 3.02 when anastomosed to target vessels with < 90% stenosis. Target vessel caliber also influenced patency rates with occlusion OR of 2.63 for SVG [95% confidence interval (CI) 1.32–2.98, $p = 0.0041$] and 2.31 for arterial grafts [95% CI 1.53–3.19, $p = 0.0001$] when anastomosed to ≤ 1.5 mm target vessels.

Little is known regarding the transit time flow measurement (TTFM) variables in grafts anastomosed to vessels with chronic total occlusion (CTO). In the study by H. Oshima et al. [57], the TTFM cut-off values were established for detecting graft failure in bypass grafts anastomosed to chronically totally occluded arteries in order to clarify the relationship between early graft failure and the grade of collateral circulation / regional wall motion in the CTO area. A multivariate regression analysis and receiver operating characteristic (ROC) analysis revealed the following predictors of early graft failure: a mean flow (Q_{mean}) value of < 11.5 ml / min for arterial conduits, a pulsatility index (PI) of > 5.85, and akinetic / dyskinetic wall motion in the CTO territory for SVGs.

Thus, morphological parameters such as graft type, target territory, target vessel caliber, and degree of stenosis, are important factors determining long-term graft patency.

RADIOLOGICAL DIAGNOSTIC METHODS FOR ASSESSING THE CORONARY GRAFT CONDITION. RADIONUCLIDE METHODS AND MAGNETIC RESONANCE IMAGING

According to current guidelines, despite evidence showing graft disease in nearly 1 of 5 patients in early post-CABG, cardiac stress testing and anatomical diagnostic procedures are not recommended to assess graft patency in asymptomatic patients within 5 years after CABG [58]. In addition, the hemodynamic significance of graft stenosis cannot always be accurately determined by coronary angiography [59, 60].

Therefore, various diagnostic strategies (invasive or non-invasive) are important for clinical assessment. They can additionally characterize the hemodynamic consequences of the lesion and identify individuals at risk of death and heart failure following successful CABG. In this situation, radiological methods of non-invasive diagnosis, including nuclear medicine techniques, can play an important role.

RADIONUCLIDE METHODS

It is known that stress myocardial perfusion imaging (MPI) with single-photon emission computed tomography (SPECT) is a well-described non-invasive imaging modality for evaluating patients with suspected or established chronic coronary syndrome (CCS). In addition to evaluating regional myocardial perfusion, ECG gating allows for the assessment of left ventricular (LV) function parameters, i.e. LV ejection fraction (EF) and other parameters [61, 62].

Thus, in the study by F. Ortiz et al. [63], patients underwent SPECT–MPI stress testing 1 year after CABG to determine predictors of adverse cardiac outcomes (combination of death and congestive heart failure). It was shown that three separate stress findings predicted the primary outcome: inability to reach stage 3 of the Bruce protocol (OR 7.3, CI 2.4–22.1, $p < 0.001$), LVEF < 45% (OR 4.0, CI 1.1–15.3,

$p = 0.041$), and a moderate-to-large size of stress-induced perfusion defect (OR 2.31, CI 1.1–1.5, $p = 0.04$). These findings are additional and most conclusive among patients who underwent exercise stress test (hazard ratio (HR) 10.6, CI 3.6–30.6, $p < 0.001$).

Severe stress-induced ischemic LV dysfunction can also be detected on SPECT–MPI by nonperfusion markers, such as transient ischemic dilatation (TID) of the LV [64]. The latter is considered another potent marker of severe CAD and predictor of future cardiac events, even when myocardial perfusion appears to be normal [65].

S.S. Gultekin et al. [66] examined 104 patients who had recurrent CAD symptoms after recent coronary revascularization: 62 patients underwent percutaneous transluminal angioplasty (75 arteries) and 42 patients underwent CABG (104 arteries). Follow-up stress SPECT–MPI and repeat coronary angiography were performed in all patients. Parameters of myocardial perfusion and TID were correlated with the presence of significant obstructive CAD (> 70% CA stenosis) (Figure). SPECT–MPI revealed inducible ischemia in 38 patients (36.5%) and TID > 1.20 in 49 patients (47%). Subsequent coronary angiography (22 ± 7 days after SPECT) showed significant obstructive CAD in 44 patients (42%). The sensitivity of detecting obstructive CAD was 61% for SPECT–MPI alone, but increased significantly to 93% with the addition of TID as a diagnostic criterion ($p < 0.0001$).

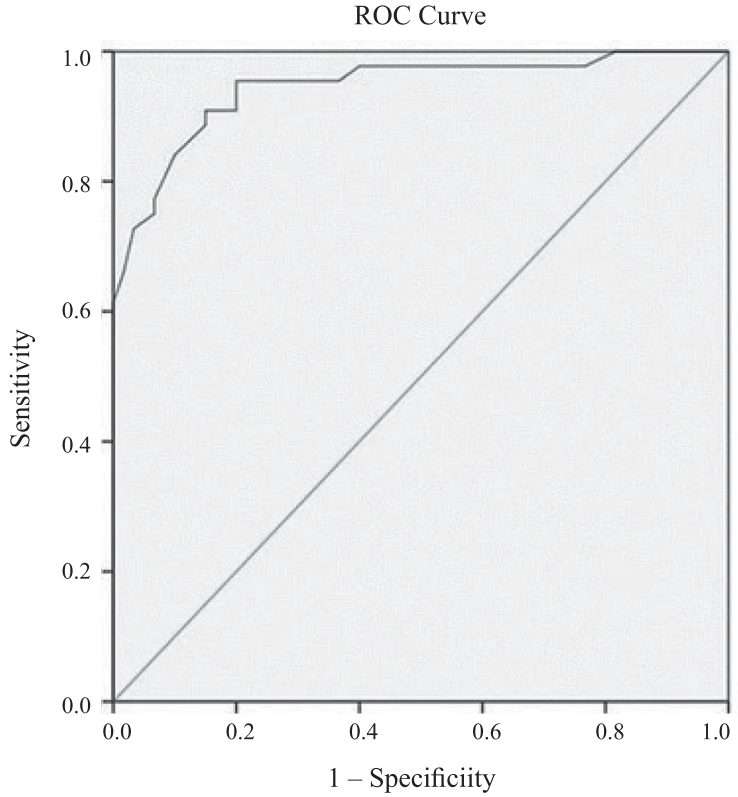

Figure. ROC analysis of LV volume ratio in states of stress and rest in patients with prior coronary revascularization for detection of obstructive (> 70%) CAD [66]

M. Kawasuji et al. [67] compared the flow capacities of ITA and SVG upon exertion by means of radionuclide angiocardiography. 52 patients were divided according to the type of bypass graft performed to the left anterior descending artery: group 1 consisted of 27 patients with the ITA graft, and group 2 included 25 patients with the SVG. SVGs were used to bypass the right and circumflex coronary arteries. Before the surgery, global and regional ejection fractions decreased similarly in both groups with exercise. After the operation, the global ejection fraction measured in groups 1 and 2 increased

significantly from 54 ± 2 to 57 ± 2% and from 54 ± 1 to 60 ± 2%, respectively, ejection fraction in the anteroseptal segment – from 29 ± 1 to 32 ± 2% and from 29 ± 1 to 35 ± 1%, respectively, and ejection fraction in the apical segment – from 75 ± 3 to 82 ± 2% and from 77 ± 2 to 86 ± 2%, respectively.

There were no differences in exercise-induced increases in the global and regional ejection fractions between groups 1 and 2. Six patients in group 1 had exercise-induced wall motion abnormalities at the anteroseptal and (or) apical segments. In contrast, patients in group 2 had no exercise-induced wall motion abnormalities at these segments ($p < 0.05$, group 1 vs group 2).

Results of this study show that ITA grafts respond to the increased demand for blood flow during exercise in essentially the same way as SVGs. However, there seems to be a slightly greater potential in patients with the ITA graft when there is inadequate flow, as evidenced by the small group of patients with exercise-induced wall motion abnormalities.

Thus, methods of radionuclide imaging can identify patients at risk of serious cardiovascular complications, including heart failure, for subsequent identification of coronary bypass graft failure using coronary angiography at various times after CABG.

MAGNETIC RESONANCE IMAGING

Cardiovascular magnetic resonance (CMR) is an accurate diagnostic tool for detecting CAD. It offers both functional studies and tissue characterization for quantification of ischemia and myocardial infarction. Multiple studies have demonstrated high diagnostic accuracy of adenosine myocardial perfusion imaging [68–72] with higher spatial resolution compared with radionuclide imaging. However, only some studies have evaluated stress CMR in patients after CABG; at the same time, they demonstrated good sensitivity and specificity in detecting significant (> 50%) stenoses in grafts and native CA [73–75].

In particular, W.L.F. Bedaux et al. [75] assessed the value of CMR-determined graft flow and flow reserve for differentiating significant graft stenosis from hemodynamically insignificant one. 21 patients scheduled for X-ray angiography due to complaints of recurrent chest pain after CABG were included for evaluation of venous grafts ($n = 40$) by CMR. Three-dimensional contrast-enhanced CMR angiography was performed and followed by flow measurements in grafts at rest and during stress-induced hyperemia. Flow reserve was calculated only if the resting flow exceeded 20 ml / min. The analysis was based on four categories defined by X-ray angiography: occluded grafts ($n = 3$), grafts with > 50% stenosis ($n = 19$), grafts with < 50% stenosis with impaired flow ($n = 8$), and grafts with < 50% stenosis and normal flow ($n = 10$).

The CMR angiography demonstrated occlusion of three grafts out of 40. In 9 of 37 grafts, basal blood flow was < 20 ml / min, and all of them demonstrated significant stenosis at X-ray angiography. In grafts with resting flow of > 20 ml / min ($n = 28$), flow reserve significantly differed between grafts without stenosis and grafts with significant stenosis or with impaired flow (2.5 ± 0.7 vs 1.8 ± 0.9, $p = 0.04$). An algorithm combining basal low of < 20 ml / min and graft flow reserve of < 2 had sensitivity and specificity of 78% and 80%, respectively, for detecting grafts with significant stenosis or impaired flow.

In the study by L.P. Salm et al. [76], a direct comparison between SPECT and CMR in evaluating hemodynamic significance of angiographic findings in bypass grafts was performed. In 25 patients, the function of 57 arterial and venous grafts was assessed by angiography, perfusion SPECT, and coronary flow velocity reserve determination using CMR. Based on the results of angiography and SPECT, 4 groups of conduits could be identified: 1) no significant stenosis (< 50%), normal perfusion; 2) significant stenosis (> 50%), abnormal perfusion; 3) significant stenosis, normal perfusion (no hemodynamic significance); and 4) no significant stenosis, abnormal perfusion (suggesting microvascular disease).

A complete evaluation was obtained for 46 grafts. SPECT and CMR provided similar information in 37 of 46 grafts (80%), illustrating good agreement between the methods (kappa = 0.61, $p < 0,001$). Eight grafts supplied blood to areas of the myocardium with scar tissue. When agreement between SPECT and CMR was restricted to grafts without scar tissue, it improved to 84% (kappa = 0.68). Integration of angiography with SPECT categorized 14 lesions in group 1, 23 – in group 2, 6 – in group 3, and

3 – in group 4. The agreement between SPECT and CMR per group was 86%, 78%, 100%, and 33%, respectively.

Thus, the availability and accessibility of such a non-invasive test as CMR imaging, which allows to exclude significant stenosis of grafts and CA, can be a useful screening tool in the follow-up of patients after CABG.

DOPPLER ULTRASOUND

As noted above, higher patency rates of arterial conduits compared with venous grafts have been explained by histologic characteristics, differences in vascular responsiveness to endogenous agonists, and a greater capacity of arterial endothelial cells to secrete endogenous dilators [11, 77, 78]. At the same time, there is little data describing adaptation of these grafts to an increase in myocardial blood flow demand, for example during exercise. Preserved endothelial function in arterial grafts should contribute to good hemodynamic performance of the graft, allowing it to increase its dimensions when the flow increases [77, 79]. It is well known that arterial grafts *in situ* have a smaller diameter and lower initial flow capacity than venous grafts [80], which can limit any increase in blood flow. However, only a few studies are devoted to the comparative hemodynamic assessment of two types of conduits [67, 81, 82].

Quantitative angiography combined with intravascular Doppler velocity analysis, as proposed by J.W. Doucette et al. [83], allows for accurate measurement of the absolute blood flow and can be used to study adaptation of the vessels to an increase in myocardial oxygen demand. Significantly higher peak flow velocity in the distal ITA graft as opposed to the distal SFG has also been reported in studies using pulsed Doppler echocardiography [82]. Thus, flow dynamics apparently differ in these two types of bypass grafts, but no report has examined these differences in detail. There is a number of other non-invasive Doppler echocardiographic assessments of CA bypass grafts [84, 85], but they are limited to examination of the flow at either the proximal [84] or the distal site [85] of the ITA graft.

A recently developed Doppler guide wire (DGW) for phase velocity analysis in grafts can pass through stenotic CA lesions and be used to measure flow velocities distal to the stenosis [86, 87]. With this method, selective cannulation of the bypass graft during cardiac catheterization allows for phasic flow velocities to be recorded within both the graft and the native CA distal to the site of graft insertion.

O. Gurné et al. [88] conducted a study to evaluate *in vivo* the mechanisms by which different coronary bypass grafts react during an increase in flow demand induced by rapid atrial pacing. The authors compared pediculated and free arterial grafts (LITA and inferior epigastric artery (iEGA)) and venous grafts early and later after bypass surgery. Forty three grafts (13 EGA, 15 ITA, 15 SVG) evaluated early (9 ± 3 days) after bypass surgery were compared with 41 other grafts (15 EGA, 11 ITA, 15 SVG) evaluated later after surgery (mean – 23 months, range – from 6 to 168 months) using quantitative angiography and intravascular Doppler velocity analysis during atrial pacing. Controls included 17 normal CAs.

Baseline graft flow tended to be lower late after surgery compared with the early period (41 ± 16 vs 45 ± 21 ml / min, not significant (NS)). Blood flow increased during pacing by 30 ± 16% early after surgery, less than later after surgery (+46 ± 18%, $p < 0.001$) and less than in normal CAs (+54 ± 27%, $p < 0.001$ vs early grafts; NS vs late grafts). There was no difference between venous and arterial grafts. No significant vasodilatation was observed during pacing early after surgery in arterial and venous grafts. Later after surgery, significant vasodilatation was observed only in arterial grafts (ITA and epigastric grafts), from 2.41 ± 0.37 to 2.53 ± 0.41 mm (+5.1% vs baseline $p < 0.001$). Early after surgery and in venous grafts later after surgery, the increase in flow was entirely due to an increase in velocity. In later arterial grafts, the relative contribution of the increase in velocity to the increase in flow during pacing was lower in arterial grafts (70 ± 22%) than in venous grafts (102 ± 11%, $p < 0.001$) and similar to that in normal CAs (68 ± 28%).

The authors concluded that early and later after surgery, arterial grafts and venous grafts both increase their flow similarly during pacing. Early arterial and venous grafts increase their flow only through a rise in velocity. Later after surgery, arterial grafts act as physiological conduits and increase

their flow in the same way as normal CAs, through an increase in velocity and caliber determined by the endothelium.

As mentioned above, the ITA and GEA graft diameter has been shown to decrease when the native CA stenosis is less than critical [52, 53]. This phenomenon is due to competitive flow in mildly stenosed CAs. In the study by T. Shimizu et al. [54], the shear stress as a significant factor affecting graft patency was compared between the arterial conduit and SVG after CABG. In 101 patients, 40 ITAs, 27 GEAs, and 34 SVGs were examined using DGW during postoperative angiography. The graft flow volume and shear stress were calculated from velocity and diameter data. The study grafts were classified according to the grade of native CA stenosis: group L had 50 to 75% stenosis, and group H had more than 75% stenosis. Group H consisted of 25 ITAs, 17 GEAs, and 21 SVGs, while group L consisted of 15 ITAs, 10 GEAs, and 13 SVGs.

In group H, graft flow volume did not significantly differ among the ITA (34 ± 11 ml / min), GEA (36 ± 16 ml / min), and SVG (41 ± 15 ml / min), while graft shear stress significantly (ITA vs GEA, $p = 0{,}0001$; GEA vs SVG, $p = 0.01$) differed among the ITA (16.0 ± 4.8 dyn / cm^2), GEA (9.1 ± 3.2 dyn / cm^2), and SVG (4.8 ± 1.6 dyn / cm^2). In group L, flow volume was lower ($p < 0.001$) in the ITA (18 ± 6 ml / min) and the GEA (13 ± 8 ml / min) than in the SVG (35 ± 16 ml / min), and shear stress was significantly ($p < 0.001$) greater in the ITA (13.7 ± ± 4.9 dyn / cm^2) than in the GEA (5.6 ± 2.0 dyn / cm^2) or the SVG (4.6 ± 2.0 dyn / cm^2). According to the authors, these data suggest that the superior shear stress of the ITA is maintained despite the reduction of flow volume due to flow competition. Lower shear stress of the GEA in intermediate stenosis may be associated with the development of conduit failure.

There are reports that the postoperative capacity of ITA – LAD grafts is limited compared with that of SVG [67]. Therefore, the aim of study by T. Akasaka et al. [89] was to assess flow dynamics and flow capacities of these conduits to the left anterior descending coronary artery using DGW.

Phasic flow velocity recordings were obtained in the midportion of the bypass graft and within the native LAD artery using a 0.018-in. (0.046-cm) 12-MHz DGW in 53 patients: 27 patients with an ITA graft (16 with a new graft assessed at 1 month postoperatively and 11 patients with an old graft assessed at 1 year) and 26 patients with a SVG (13 patients with a new graft assessed at 1 month postoperatively and 13 patients with an old graft assessed at 1 year). All patients were studied at baseline rest and during hyperemia induced by intravenous infusion of dipyridamole (0.56 mg / kg of body weight) for 4 min.

In the left anterior descending artery itself, systolic and diastolic peak velocities, the time average of the instantaneous spectral peak velocity (time-averaged peak velocity), vessel diameter, and the calculated flow volume did not differ significantly among the four graft groups. The time-averaged peak velocity was significantly greater for new than for old arterial grafts or for new or old venous grafts (27 ± 9 vs 19 ± 6, 11 ± 5, and 12 ± 6 cm / s, respectively, $p < 0.01$). However, since the diameter of new arterial grafts was significantly smaller than that of the other three grafts (2.4 ± 0.1 vs 2.9 ± 0.2 [$p < 0.05$], 3.6 ± 0.6 [$p < 0.01$], and 3.4 ± 0.5 mm [$p < 0.01$], respectively), there was no difference in the calculated flow volumes at rest (62 ± 17 vs 58 ± 15, 61 ± 18, and 58 ± 19 ml / min, respectively, NS) between new arterial grafts and the other grafts.

Although the maximum time-averaged peak velocity during hyperemia was significantly greater in new than in old arterial grafts or new or old venous grafts (47 ± 17 vs 40 ± 7, 31 ± 8, and 34 ± 12 cm / s, respectively, $p < 0.01$), the flow reserve of new arterial grafts was significantly smaller than that of the other three groups (1.8 ± 0.3 vs 2.6 ± 0.3, 2.8 ± 0.5, and 3.0 ± 0.6, respectively, $p < 0.01$), since the baseline time-averaged peak velocity of these new grafts was far greater than that of the other groups.

Thus, ITA graft flow early after the surgery is characterized by higher rest velocity compared with venous graft flow. This high velocity maintains flow volume at baseline values in compensation for the smaller diameter. Although flow reserve does not differ significantly between new and old venous grafts, the reserve for ITA grafts is significantly reduced soon after bypass surgery. This restricted flow capacity improves in the late postoperative period because of an increase in the diameter and a decrease in flow velocity compared with baseline levels.

CONCLUSION

X-ray angiography is considered to be a standard procedure for assessing patency of coronary grafts, because it provides excellent visualization of the graft and quantitative information about the size of the lumen and the presence of obstruction and, thus, provides indications for revascularization. However, the hemodynamic significance of stenosis cannot be accurately determined by coronary angiogram. Therefore, the availability and high information value of other diagnostic radiology methods can be a useful screening tool at any stage of patient monitoring after CABG.

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Current strategies for targeted therapy of liver fibrosis

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ABSTRACT

Liver fibrosis (LF) is an unfavorable event in the natural course of chronic liver diseases (CLD), therefore, early implementation and widespread use of antifibrotic therapy methods is a pressing issue in hepatology. The aim of the review was to describe current approaches to targeted therapy of LF.

PubMed database, Google Scholar search engine, Cochrane Database of Systematic Reviews, eLIBRARY.RU scientific electronic library, as well as reference lists of articles were used to search for scientific articles. The publications that corresponded to the aim of the study were selected for the period from 1998 to 2021 by the terms "liver fibrosis", "pathogenesis", and "treatment". Inclusion criteria were restricted to targeted therapy of LF.

Despite the growing evidence for reversibility of LF, there are currently no effective or clinically approved regimens for its specific therapy. However, taking into account the relevance of the issue, scientific research in this area is necessary. Multiple drugs with a good safety profile have been studied, which, though intended for other purposes, can have a positive effect on LF. In addition, a number of innovative approaches that differ from pharmacotherapy inspire optimism about finding a solution to this problem. It is obvious that studies focused on well-characterized groups of patients with confirmed histologic, elastography, clinical, and radiological parameters are required. This is a challenging task, since the key point will be stratification of risk based on ethnicity, etiology, and clinical status, and very large samples will be required for a reliable assessment. Nevertheless, the solution will increase efficiency of treatment for patients with CLD, improve their prognosis and quality of life, and significantly reduce the need for liver transplantation, a demand for which remains extremely high worldwide.

Keywords: overview, liver fibrosis, pathogenesis, treatment

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Современные стратегии таргетной терапии фиброза печени

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РЕЗЮМЕ

Учитывая, что фиброз печени (ФП) является неблагоприятным событием естественного течения хронических заболеваний печени (ХЗП), скорейшее внедрение и широкое применение методов антифибротической

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терапии являются актуальной проблемой гепатологии. Цель обзора – описать современные подходы к таргетной терапии ФП.

Для поиска научных статей применялись база данных PubMed, поисковая система Google Scholar, Кокрановские систематические обзоры, научная электронная библиотека eLIBRARY.RU, а также пристатейные списки литературы. Соответствующие цели обзора публикации отбирались за период с 1998 по 2021 г. по терминам «фиброз печени», «патогенез», «лечение». Критерии включения ограничивались таргетной терапией ФП.

Несмотря на растущее число доказательств обратимости ФП, в настоящее время пока не существует каких-либо эффективных или одобренных для клинического применения схем его специфической терапии. Однако, принимая во внимание актуальность вопроса, научные поиски в этом направлении необходимы. Были изучены многочисленные лекарственные средства с хорошим профилем безопасности, которые хотя и предлагались для других целей, способны оказывать позитивное влияние на ФП. Кроме того, ряд отличных от фармакотерапии новаторских подходов вселяют оптимизм относительно успешности решения данной проблемы. Очевидно, что необходимы исследования, сосредоточенные на хорошо охарактеризованных группах пациентов с подтвержденными гистологическими, эластографическими, клиническими и радиологическими показателями. Это достаточно сложная задача, поскольку ключевым моментом будет стратификация риска на основе этнической принадлежности, этиологии и клинического статуса и для достоверной оценки потребуются очень большие размеры выборок. Тем не менее ее решение позволит повысить эффективность лечения пациентов с ХЗП, улучшит прогноз и качество их жизни, а также существенно уменьшит необходимость в трансплантации печени, потребность в которой во всем мире остается чрезвычайно высокой.

Ключевые слова: обзор, фиброз печени, патогенез, лечение

Конфликт интересов. Автор декларирует отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

Chronic liver diseases (CLD) cause more than two million deaths worldwide annually, which, taking into account the heavy burden of CLD-related disability and seeking medical care, makes the problem extremely urgent. The most common causes of CLD are HBV and HCV, alcohol abuse, and metabolic disorders. The key step in their natural course is liver fibrosis (LF), which is considered as an abnormal wound-healing response to chronic liver injury. Diffuse excessive deposition and abnormal distribution of the extracellular matrix (ECM) further leads to the development of liver cirrhosis (LC). The severity of clinical manifestations of LC is primarily associated with liver failure and portal hypertension, as well as the occurrence of complications characteristic of them, accompanied by high mortality [1].

The growing evidence of the reversibility of LF, the identification of the main causes and mediators of its progression, as well as advances in non-invasive assessment of this pathological process contributed to treatment development. In general, there is no effective or clinically approved specific therapy of LF yet. Nevertheless, taking into account the relevance of the problem, there is a need for scientific research devoted to it. Of course, these studies should comply with the recommendations adopted in 2014 with the support of the American Association for the Study of Liver Diseases (AASLD) at the conference focused on clinical trials on antifibrotic therapy for CLD aimed at identifying potential unpredictable consequences and / or adverse outcomes associated with antifibrotic therapy in CLD patients [2].

Given that, numerous drugs with a good safety profile were studied, which were offered for other purposes, but had a positive effect on LF. In addition, a number of innovative approaches other than pharmacotherapy are promising when it comes to resolving the issue discussed.

Currently, it has been established that treatment of diseases, the natural course of which is accompa-

nied by LF, is an effective way not only to prevent LF, but also to reverse histologic diseases and restore the structure and function of the liver [3]. In addition, the following strategies of targeted therapy for LF can be distinguished:

– to affect hepatic fibrogenesis and decrease ECM synthesis:

– reduction of inflammatory and immune response;

to affect ligand binding to the receptor and intracellular signaling;

– induction of apoptosis, deactivation and immune clearance of activated hepatic stellate cells (HSCs);

– direct inhibition of collagen synthesis;

2) stimulation of liver regeneration and initiation of LF regression:

– activation of ECM degradation;

– mesenchymal stem cell (MSC) transplantation [4].

STRATEGIES TO AFFECT HEPATIC FIBROGENESIS AND DECREASE EXTRACELLULAR MATRIX SYNTHESIS

Reduction of inflammatory and immune response

The inflammatory response promotes the transition of HSCs from a quiescent to an activated and proliferative state, which causes ECM deposition and at the same time disrupts the immune function of the liver, which aggravates damage to hepatocytes. Therefore, suppression of the inflammatory and immune response is an important approach in the treatment of LF.

Transforming growth factor (TGF)-β is the main profibrogenic cytokine, with the participation of which quiescent HSCs transdifferentiate into myofibroblasts, directly involved in the ECM formation. Therefore, it seems obvious that the suppression of its overexpression and activity may be a promising goal of antifibrotic therapy [5]. For example, J. George et al. [6] reported that the antagonist of the TGF-β receptor ("soluble receptor"), which consists of the extracellular domain of the TGF-β receptor type II (TßRII) fused with chimeric IgG, is an effective inhibitor of experimental hepatic fibrogenesis in rats.

It was shown that the serine protease inhibitor *camostat mesilate* inhibited the production of TGF-β and blocked the HSC activation *in vitro*. In the *in vivo* model, camostat mesilate (1–2 mg / g of diet) markedly attenuated an increase in hepatic plasmin and TGF-β levels, HSC activation, and LF without apparent systemic or local side effects [7].

The chemopreventive drug against carcinogenesis *oltipraz* in an animal experiment inactivated HSCs and suppressed the expression of TGF-β1 [8]. In a randomized, double-blind, placebo-controlled phase II trial involving 83 patients with LF / LC who received oltipraz 60 mg BID, the area of collagen deposition in the liver and plasma TGF-β1 levels tended to decrease by week 24 [9].

Tenofovir is one of the first-line therapies in decompensated HBV-associated LC, as it demonstrated efficacy in preventing progression and reversing CCl4-induced LF in mice through assembling TGF-β1 / SMAD3 and NF-kB / NLRP3 inflammasome signaling pathways through upregulating the expression of NS5ATP9. Tenofovir also regulates the differentiation, activation, and proliferation of HSCs [10].

Numerous animal studies have demonstrated that the activation of platelets, which are a source of profibrogenic cytokines and growth factors, such as TGF-β1, platelet-derived growth factor (PDGF), and epidermal growth factor (EGF), contributes to the development and progression of LF [11]. At the same time, *antiplatelet agents* demonstrate opposite effects, which was confirmed in a systematic review and meta-analysis of four studies with a total population of 3,141 patients [12].

A soluble β-galactoside-binding protein *galectin-3* performs numerous autocrine and paracrine functions. It activates neutrophils, labrocytes, and T lymphocytes, regulates cell adhesion, and induces apoptosis and angiogenesis. Depending on the cell type and the balance between extracellular and intracellular content, galectin-3 can both inhibit and induce cell growth and differentiation. Galectin-3 is crucial for protecting the body from pathogens. It enhances proinflammatory signals, and its monocyte / macrophage chemotaxis contributes to neutrophil adhesion, the release of proinflammatory factors, namely leukocytes and labrocytes. In addition, galectin-3 induces phagocytosis. In preclinical studies on rats, it was noted that GR-MD-02, a galectin-3 inhibitor, reduces collagen deposition and leads to regression of LF and reversal of LC [13]. The first randomized phase I clinical trial, which involved patients with non-alcoholic steatohepatitis (NASH) confirmed by the histologic examination, and extensive LF, demonstrated its safety and good tolerability [14].

Emricasan (IDN-6556), an orally active, irreversible pan caspase inhibitor, was found to reduce hepatocyte apoptosis, inflammation, and LF in CCl4-induced cirrhosis model in rats. It also decreased high

caspase activity and serum alanine aminotransferase (ALT) levels in patients with chronic hepatitis C and non-alcoholic fatty liver disease (NAFLD). Its effect on liver function was studied in a multicenter randomized controlled trial (RCT) including 86 patients with LC (Child–Pugh class A or B, mean score of 6.9; 38% with alcohol-associated LC, 29% with HCV-associated LC, and 23% with NASH; and the Model for End-stage Liver Disease (MELD) score of 11–18 (mean score of 12.8)).

Patients were randomly divided into groups receiving placebo (n = 42) or emricasan (25 mg, n = 44) BID for 3 months. Then the subjects received emricasan (25 mg) BID for 3 months in the open-label second stage. At the 3-month timepoint, emricasan significantly reduced the mean MELD (p = 0.003) and Child–Pugh (p = 0.003) scores in subjects with high MELD scores (15 or more), compared with placebo, with significant reductions in international normalized ratio (INR) (95% confidence interval (CI) from –0.2882 to –0.0866) and total bilirubin (95% CI, from –1.5069 to –0.0823) as opposed to placebo. There were no significant differences between emricasan and placebo groups in the mean MELD (p = 0.466) or Child–Pugh (p = 0.124) scores at 3 months compared with placebo. Serum levels of full-length cytokeratin (CK)-18 (p = 0.02) and caspase (p < 0.001), but not caspase-cleaved CK-18 (p = 0.092), decreased significantly at 3 months in the emricasan group as opposed to the placebo group. Emricasan was well tolerated, and adverse events were balanced between the groups [15].

Other results were obtained in a double-blind, randomized, placebo-controlled trial in which taking emricasan (5 mg / day for an average of 43 weeks or 25 mg / day for an average of 42 weeks) did not reduce the number of cases of decompensation or did not improve liver function in patients with NASH-related decompensated LC (n = 217) [16].

In vitro and *in vivo* models showed a positive effect on liver function and LF of the Chinese herbal medicine, *Gan Shen Fu Fang* (GSFF) consisting of salvianolic acid B and diammonium glycyrrhizinate. GSFF alleviated inflammatory cell infiltration and reduced synthesis of proinflammatory cytokines (tumor necrosis factor α (TNFα) and interleukin (IL)-1β) and NF-kB, as well as ERK phosphorylation in rats with common bile duct ligation after 3 weeks of treatment. *In vitro*, GSFF inhibited viability of HSC-T6 cells and expression of alpha-smooth muscle actin (α-SMA) in them and decreased synthesis of collagen I [17].

By attenuating the effects of reactive oxygen species (ROS) and preventing damage to hepatocytes, antioxidants can be potential antifibrotic agents provided they are directly delivered to the pathological focus [18].

In particular, *S-adenosyl-L-methionine* has shown the ability to reduce ROS and inhibit the HSC activation both in rats with the LF model [19] and in clinical trials. In a long-term, multicenter, double-blind, randomized, placebo-controlled clinical trial in patients with alcoholic LC, it improved survival and delayed the need for liver transplantation [20]. The suppression of ROS and NADPH oxidase 4, which are induced by TGF-β1, is a possible therapeutic target of *polyene phosphatidylcholine* to attenuate HSC activation. However, its effectiveness in patients with alcohol-related liver disease when taken for two years was insignificant [21].

Although *vitamin E* is a prototype of an antioxidant and can induce a strong anti-NASH effect, in a large RCT by A.J. Sanyal et al. [22], it did not have a positive effect on the severity of LF. *Naringenin* is a flavonoid with antioxidant, antifibrogenic, anti-inflammatory and anticancer properties that is capable of preventing liver damage caused by different agents. The main protective effects of naringenin in liver diseases are inhibition of oxidative stress and TGF-β pathway and prevention of HSC transdifferentiation, leading to decreased collagen synthesis. Other effects include inhibition of the mitogen activated protein kinase (MAPK), toll-like receptor (TLR), and non-canonical TGF-β pathways, which further results in drastic reduction of ECM synthesis and deposition. In addition, naringenin demonstrated a beneficial effect on NAFLD by regulating lipid metabolism and modulating synthesis and oxidation of lipids and cholesterol. It is safe, but has low bioavailability and high intestinal metabolism [23].

The use of anti-inflammatory drugs, in particular, *corticosteroids*, as a part of antifibrotic therapy is justified [24]. However, the suppression of hepatic fibrogenesis caused by them is incomplete, which does not prevent the development of LF [25]. Another pathogenetic strategy is neutralization of proinflammatory cytokines, for example, TNFα, by exposure to compounds directed against it, such as *infliximab*, *etanercept,* and *pentoxifylline* [26].

Effect on ligand binding to the receptor and intracellular signaling

Identification of the membrane and nuclear receptors expressed by HSCs revealed new targets for antifibrotic therapy, which include the renin – angiotensin

system, neurotransmitters, endothelin-1 and their receptors, receptor tyrosine kinases, etc., as well as intracellular signaling involving nuclear receptors, such as PPARs, FXR, PXR, LXR, etc. [27].

It is known that angiotensin II is the key mediator of hepatic fibrosis. Its synthesis by HSCs increases as a result of overexpression of angiotensin-converting enzyme [28]. Oral administration of the angiotensin II inhibitor *losartan* (50 mg / day) for 18 months in patients with chronic hepatitis C was associated with a significant decrease in the expression of NADPH oxidase, several profibrogenic type I collagen genes, matrix metalloproteinase (MMP)-2, and urokinase [29]. In a retrospective study, K.E. Corey et al. [30] also found a beneficial role of angiotensin II blockade in hepatitis C virus-related fibrosis [30].

Resolution of NASH as a result of blockade of important inflammation and LF triggers and chemokine receptors CCR2 / CCR5 by their antagonist *cenicriviroc* was described [31].

In recent years, a number of multitargeted inhibitors of the aberrant activity of receptor and non-receptor tyrosine kinase, including sorafenib, erlotinib, imatinib, sunitinib, nilotinib, brivanib, and vatalanib, were investigated as potential drugs for the treatment of LF. Among them, *sorafenib* is the most studied one, which is the first systemic drug that has demonstrated a positive effect on survival in advanced hepatocellular carcinoma [32]. In animals with different models of LC, it also affected some pathogenetic pathways of liver fibrogenesis and angiogenesis by blocking the receptor tyrosine kinases located on the surface of HSCs, the expression of which, especially VEGFR and PDGFR, was increased. As studies revealed, the positive effects of sorafenib were due to:

– the suppression of activated HSC proliferation and the activation of apoptosis;

– the inhibition of cyclin D1 and cyclin-dependent kinase 4 (Cdk-4) with a simultaneous increase in the expression of Fas, Fas-L, and Caspase-3, and a decrease in the Bax / Bcl-2 ratio;

– an increase in the MMPs / tissue inhibitor of matrix metalloproteinases (TIMPs) ratio, and also a decrease in the synthesis of collagen by HSCs;

– the inhibition of phosphorylation of ERK, Akt, and ribosomal protein kinase S6 with a molecular mass of 70 kDa (p70S6K);

– the disturbance of the molecular triad functioning: Kruppel-like factor 6 – angiopoietin 1 – fibronectin [33].

The number of PPARγ located in the HSCs decreases during their activation and progression of LF, whereas stimulation of PPARγ overexpression leads to reduction of collagen production and an increase in metalloproteinase activity of HSCs. Synthetic ligands of PPARγ thiazolidinediones, including *pioglitazone* and *rosiglitazone*, well studied in patients with NASH, showed a positive effect on steatosis, necroinflammation, hepatocyte ballooning, and LF, although in the long term it may be offset by side effects [34].

Animal experiments showed that the transrepressive activity of PPARα in hepatocytes in NASH prevents the progression of steatosis and LF due to the effect on lipid metabolism-related genes [35]. Although the use of *fenofibrate*, a PPARα agonist, in patients with NAFLD at a dose of 200 mg / day for 48 weeks is safe and improves metabolic syndrome, serum glucose levels, and liver tests, its effects on liver histology are minimal [36].

Preliminary data on PPARα/γ agonists (*glitazars*) revealed their positive effect on the lipid profile, blood pressure, atherosclerosis, and inflammation. Overexpression of PPARβ/δ prevented obesity and reduced lipid stores in cardiac cells, and stimulation of PPARα/δ with *elafibranor* caused the resolution of NASH without LF aggravation [37]. The pan-PPAR agonist (α/γ/δ) *lanifibranor* in preclinical models of decompensated LC contributed to a decrease in the severity of LF and portal hypertension [38].

An interim analysis within a multicenter, randomized, placebo-controlled phase III (REGENERATE) trial showed clinically significant histologic improvement in patients with NASH following inhibition of the HSC activation by the FXR nuclear receptor agonist *obeticholic acid* [39]. Another FXR agonist *tropifexor* in the experiment on mice contributed to the regression of the formed LF and reduced the NAFLD fibrosis score (NAS) parameters, the level of triglycerides in the liver, and the expression of profibrogenic genes [40]. Currently, clinical trials are being conducted to assess the safety, tolerability, and efficacy of various doses of this drug in patients with NASH [41].

A powerful FXR agonist EDP-305 in mice with LF models showed significant histologic improvement; in particular, it reduced the deposition of collagen in the liver [42]. In rats with the NASH model, the combined use of the FXR agonist 1INT747 and the angiotensin II inhibitor losartan suppressed hepatic fibrogenesis, reversing intestinal barrier dysfunction and inhibiting the proliferation of activated HSCs [43].

The role of LXR nuclear receptor, which is the key regulator of lipogenesis and modulator of the immune system, in hepatic fibrogenesis is still being investigated. S.W. Beaven et al. [44] in *in vitro* and *in viv*o experiments demonstrated that LXR ligands suppress HSC activation and the expression of fibrogenic genes. In addition, Lxrαβ (-/-) mice showed hypersecretion of inflammatory mediators and increased susceptibility to LF. Nevertheless, to date, researchers express doubts regarding the expediency of using LXR agonists as potential antifibrotic agents due to hepatotoxicity and induction of *de novo* lipogenesis [45].

Induction of apoptosis, deactivation, and immune clearance of activated HSCs

Apoptosis of activated HSCs and their deactivation or direct reduction of their number by immune clearance are currently considered as an important approach in the treatment of LF. It has been established that drug-induced apoptosis of activated HSCs can be achieved as a result of NF-kB inhibition using *fraxetin* (7,8-dihydroxy-6-methoxycoumarin) [46] and 4-hydroxy-2(3H)-benzoxazolone [47]; modulation of alternative splicing of Bcl-x by antisense oligonucleotides [48] and selective STAT1-dependent induction by the synthetic antiviral drug *rilpivirine*, which can also cause STAT3-dependent proliferation of hepatocytes and liver regeneration [49].

In vivo and *in vitro* models showed that *selonsertib*, a selective ASK1 kinase inhibitor with low molecular weight, suppressed HSC activation and reduced collagen production, induction of inflammatory cytokine pathways, and oxidative stress [50]. At the same time, subsequent phase II and III clinical trials did not reveal convincing data regarding its positive effect on LF in patients with NASH [51, 52].

As an important part of innate immunity, invariant natural killer T (iNKT) cells can kill activated HSCs. However, highly activated peripheral iNKT cells also cause MIHAcell line proliferation and HSC line (LX-2) activation through the expression of IL-4 or IL-13, which contributes to the progression of LF [53]. Thus, understanding the iNKT balance in the regulation of HSCs in patients with CLD can help in the development of new antifibrotic drugs.

Curcumin, the major natural polyphenol isolated from the rhizome of *Curcuma longa,* is a well-known hepatoprotector. In an experiment on rats, it decreased CCL4-induced liver damage, oxidative stress, fibrosis, and restored the activity of MMP-9 and MMP-2. Besides, curcumin restored the levels of NF-kB, IL-1, IL-10, TGF-β, connective tissue growth factor (CTGF), type I collagen, MMP-13, and Smad7. It also reduced JNK and Smad3 phosphorylation and the levels of protein and α-SMA and SMAD3. Curcumin normalized GSH and NF-kB, as well as JNK-Smad3 and TGF-β-Smad3 pathways, leading to reduction of the number of activated HSCs and contributing to the antifibrotic effect [54].

In a randomized, double-blind, placebo-controlled trial, 70 patients with LC were randomly divided into two groups: group 1 received 1,000 mg / day curcumin (n = 35), group 2 received placebo (n = 35) for 3 months. At the final stage, the patients receiving curcumin (n = 29) showed a decrease in the MELD (i) (from 15.55 ± 3.78 to 12.41 ± 3.07; $p < 0.001$), MELD (from 15.31 ± 3.07 to 12.03 ± 2.79; $p < 0.001$), MELD-Na score (from 15.97 ± 4.02 to 13.55 ± 3.51; $p = 0.001$), and Child – Pugh score (from 7.17 ± 1.54 to 6.72 ± 1.31; $p = 0.051$). At the same time, in the placebo group (n = 31), these parameters increased significantly ($p < 0.001$ in all cases) [55].

Direct inhibition of collagen synthesis

Another promising strategy for the treatment of LF is to directly affect collagen synthesis. For example, in a multicenter, open-label PROMETEO study involving 281 patients with CLD of various etiology and advanced LF, the safety and efficacy of 12-month use of *pirfenidone*, an immunosuppressant (600 mg orally, every 12 h), in combination with standard treatment was studied. The results showed that 35% of the patients who received it had a significant decrease in the severity of LF. The Child – Pugh score improved in 29.7% of cases. Serum ALT and aspartate aminotransferase (AST) decreased by 40.6% and 43.3%, respectively. In addition, the patients receiving the immunosuppressant showed lower serum levels of TGF-β1 than those treated only according to the standard scheme [56].

Colchicine is a plant alkaloid that inhibits polymerization of microtubules and, thereby, prevents collagen secretion. In the RCT involving 38 patients, the effect of colchicine on LF of various etiologies was studied. Patients included in group A (n = 21) were prescribed the drug at a dose of 1 mg per day. Those who did not receive it made up group B (n = 17). After 12 months, the average serum albumin levels increased only in group A ($p < 0.05$). After 12 months of the treatment in group A, the average serum values of PIINP, a biomarker of LF, did not change significantly and decreased in 7 patients after 24 months ($p < 0.05$).

No significant histologic changes on the Knodell score were found in both groups after 12 months of follow-up [57].

The efficacy and safety of colchicine was evaluated in the Cochrane meta-analysis combining the results of 14 RCTs and including a total of 1,150 patients with LF of alcoholic, viral, and unidentified etiology. There was no significant effect of colchicine on the all-cause mortality (odds ratio (OR) 0.90, 95% confidence interval (CI): 0.63–1.29), liver-related mortality (OR 1.05, 95% CI: 0.61–1.80), complications (OR 1.01, 95% CI: 0.63–1.62), and hepatic biochemical and histologic parameters (OR 1.02, 95% CI: 0.58–1.79). In addition, its administration was accompanied by an increased risk of adverse events (OR 4.92, 95% CI: 2.66–9.10; $p < 0.001$) [58].

STIMULATION OF LIVER REGENERATION AND INITIATION OF LIVER FIBROSIS REGRESSION

Activation of extracellular matrix degradation

Given that MMPs and TIMPs are crucial for the development of LF, and the balance between them is important for the homeostasis of ECM components, it is expected that they will become new therapeutic targets in the treatment of CLD [59]. An increase in the activity of enzymes that destroy ECM, as well as their introduction by gene therapy, can enhance the degradation of ECM. For example, in experiments on rats with different LC models, the attenuation of LF was associated with increased regulation of MMP-1 and MMP-8 caused by transfection of their genes [60].

An experimental study by O. Ohayon et al. [61] demonstrated that treating fibrotic rat livers with *halofuginone*, a multipotent antifibrogenic drug, and subsequently subjecting them to hydrodynamics-based transfection with human VEGF-165 resulted in elevated expression of heparan sulfate-degrading heparanase. Moreover, these rats demonstrated an improved capacity to regenerate following partial 70 % hepatectomy. *In vitro*, halofuginone stimulated heparanase and vascular endothelial growth factor (VEGF) expression in HSCs.

An experiment on mice showed the ability of *polaprezinc* to attenuate LF at the late stages of NASH by inhibiting the expression of TIMPs [62]. Lysyl oxidase like-2 (LOXL2) mediates type I collagen crosslinking and ECM stabilization during hepatic fibrogenesis, and also independently promotes differentiation of fibrogenic hepatic progenitor cells. *In vitro* and *in vivo* models demonstrated that the blockade of these two convergent profibrotic pathways by therapeutic inhibition of LOXL2 with the monoclonal antibody AB0023 attenuates LF and leads to its regression [63].

An open-label, pilot phase II clinical trial studied the safety and tolerability of *simtuzumab*, a monoclonal antibody directed against LOXL2, in subjects with advanced CLD, caused by HCV, human immunodeficiency virus (HIV), or HCV – HIV coinfection. The drug was administered at a dose of 700 mg intravenously every 2 weeks for 22 weeks. The treatment was well tolerated, but there were no significant changes in the severity of morphological signs of LF, as well as values of the hepatic venous pressure gradient [64].

It was established that an immunosuppressant *pirfenidone* can successfully reduce the expression of HSP47 protein and, by regulating the activity of the TGF-β signaling pathway, inhibit HSC proliferation, reduce the abnormal accumulation of type I and type III collagen, as well as suppress the expression of type II collagen, TIMP-1, and MMP-2. Its efficacy was studied in 28 patients with chronic HCV who received pirfenidone (1,200 mg / day) for 24 months. Six patients dropped out after 12 months of the therapy. At the end of the treatment, necroinflammation grades were reduced by an average of 3.2 points in 82% of patients ($p < 0.05$) and Ishak fibrosis score decreased by an average of 2 points in 67% of patients ($p < 0.05$). Liver steatosis decreased in 61% of patients. Serum IL-6 and TGF-β1 levels decreased significantly in 93% and 67% of patients ($p < 0.05$), respectively, while TNF-α decreased in 47% of patients. Serum ALT and AST levels tended to normalize in 81% of patients. The levels of mRNA of antifibrogenic cannabinoid receptors CB2 in the liver increased in 86% of cases, and the expression of receptors CB1 decreased in 29% of patients. Improvements in the quality of life and the Child – Pugh score were reported in all patients [65]. Despite the promising results, there are still concerns about the potential side effects and adverse events associated with pirfenidone [66].

Mesenchymal stem cell transplantation

Mesenchymal stem cell (MSC) transplantation is an important and promising approach in the treatment of LF. These multipotent fibroblast-like cells are characterized by the expression of CD73, CD90, and CD105 surface antigens, the absence of expression of CD45, CD34, CD14, Cd11b, CD19, CD79a, and HLA-DR, adhesion to plastic, and the ability to differentiate into osteoclasts, chondrocytes, adipocytes,

and hepatocytes. The largest source of MSCs is bone marrow. In addition, they can be obtained from adipose tissue, umbilical cord tissue, amniotic fluid, breast milk, synovial membrane, placental cells, tooth pulp, lungs, and liver (in both adults and fetus).

In addition to the immunomodulatory effect on T cells, B cells, and macrophages, MSCs have antifibrotic effects both through immunomodulation and by direct inhibition of the proliferation of activated HSCs, increasing the activity of MMPs and suppressing the synthesis of ECM [67]. They also contribute to an increase in proliferation and reduction of hepatocyte apoptosis and elevated expression of certain anti-inflammatory and antifibrotic cytokines with potential hepatotropic properties, including hepatocyte growth factor (HGF), VEGF, basic fibroblast growth factor (bFGF), placental growth factor (PlGF), monocyte chemoattractant protein (MCP)-1, stem cell factor (SCF)-1, chemokine SDF-1, CD135 or FMS-like tyrosine kinase 3 (FLT-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), and numerous ILs [68]. In addition, the MSCs-stimulated proliferation of hepatocytes from an increased population of hepatic progenitor cells leads not only to a decrease in the severity of LF, but also to liver regeneration [69].

MSCs are chosen for transplantation due to their availability and low immunogenicity. In addition, researchers using MSCs face fewer ethical problems due to their non-somatic origin, and the procedure is considered safe and was highly evaluated in clinical settings for various diseases, showing promising results [70]. The therapeutic efficacy of MSCs is affected by many factors, namely the cultivation method, strategy, and delivery routes. 3D cell culture is considered the most suitable and physiologically similar microenvironment for cell growth. There are many methods of 3D cell culture for the formation of MSC spheroids, such as hanging drop, magnetic cell levitation, chitosan membranes, microgravity bioreactor, and spinner flask [71].

In CLD, both direct (portal vein, hepatic artery) and indirect delivery routes of MSCs can be used, for example, intrasplenic, intraperitoneal, through peripheral veins, as well as through an extracorporeal liver support system. It is obvious that direct delivery provides a higher retention rate [72]. However, in patients with decompensated LC, it may be associated with an increased risk of bleeding associated with portal hypertension [73].

Preliminary experimental data are promising regarding the prospects of this method in the treatment of LF, while the results of clinical trials still require further evaluation [67]. For example, in a prospective study involving 90 patients with decompensated LC, bone marrow MSCs were transplanted simultaneously into the portal and peripheral veins (ratio 1 : 1) after 2 weeks, which led to a statistically significant improvement in serum levels of albumin, bilirubin, and INR. This effect persisted for 6 months in the patients who underwent one procedure and for 12 months in those who underwent a second procedure four months after the first one. By the end of the study, 36.7% and 66.7% of patients, respectively, had a decrease in ascites. In addition, there was an improvement in the hepatic functional reserve according to the Child – Pugh and MELD scores. The safety of the procedure was indicated by a low complication rate [74].

In a multicenter, randomized, open-label, phase II trial involving 72 patients with biopsy-confirmed alcoholic LC, single or double injections of 5×10^7 bone marrow MSCs into the hepatic artery after 6 months were associated with a 25% and 37% decrease in the proportion of collagen ($p < 0.001$), respectively, and an improvement in liver function parameters according to the Child – Pugh scores ($p < 0.05$) [75].

A RCT including 40 patients with HCV-induced decompensated LC showed that normalization of serum enzymes and improvement of liver protein synthesis were observed in 54 % of cases after infusion of bone marrow MSCs through peripheral veins. Three months after the transplantation, the values of serum PIIICP and PIIINP LF biomarkers decreased from 9.4 ± 4.2 to 8.1 ± 2.6 and from 440 ± 189 to 388 ± 102, respectively ($p = 0.7$) [76].

On the contrary, in a RCT involving 27 patients with decompensated LC, absolute changes in the Child – Pugh and MELD scores, serum levels of albumin, aminotransferases, and INR in patients who underwent infusion of bone marrow MSCs through peripheral veins and received placebo after 12 months of follow-up did not differ significantly [77].

CONCLUSION

Despite the relevance of the problem, it should be noted that before considering antifibrotic therapy as a gold standard of treatment for diseases which are accompanied by LF, studies should be conducted with a focus on well-characterized groups of patients with confirmed histologic, elastography, clinical, and radiological data. The above-mentioned is quite a difficult task, since the key point will be stratification of risk based on ethnicity, etiology, and clinical status, and

very large sample sizes will be required for a reliable evaluation. Nevertheless, the solution to the problem will increase the effectiveness of treatment in patients with CLD, improve their prognosis and quality of life, and significantly reduce the need for liver transplantation, which remains extremely high worldwide.

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Pathogen-specific molecular imaging and molecular testing methods in the prognosis of the complicated course of diabetic foot syndrome, the risk of amputation, and patient survival

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ABSTRACT

The aim of this review was to provide extended information on current trends in the diagnosis of complicated diabetic foot syndrome (DFS), the most frequent and severe complication of diabetes mellitus, including high-tech medical imaging methods and instrumental and laboratory predictors of the complicated course and risk of amputation in DFS.

The article provides an analytical review of modern publications over the past 5 years on diagnosis and therapy. Pilot data on the use of high-tech medical imaging methods, assessment of skin microbiota and ulcers in DFS, molecular testing methods in terms of predicting the amputation risk and survival of patients with DFS, as well as the effectiveness of biosensing systems have been systematized, summarized, and subjected to analytical evaluation.

The review provides an expert assessment of the capabilities of pathogen-specific molecular imaging using modern positron emission tomography (PET), single-photon emission computed tomography (SPECT), and high-energy radionuclides in bacterial infection to understand its pathogenesis, minimize diagnostic problems, improve antimicrobial treatment, and address fundamental and applied aspects of DFS. Literature data on the assessment of foot perfusion in diabetic patients with varying degrees of limb ischemia by hybrid technologies (SPECT / CT and PET / CT) and new modalities of magnetic resonance imaging (MRI) are also systematized, which contributes to new understanding of the response to revascularization, surgical shunting, and stimulation of angiogenesis within ischemic tissue, as well as potentially to healing of foot ulcers.

The review is aimed at substantiating a multidisciplinary approach in DFS, selection, development, and implementation of innovative strategies for diagnostic modalities to identify diabetic foot pathologies, and choice of an adequate method for treating and monitoring the results of therapy in the context of personalized medicine.

Keywords: diabetes mellitus, diabetic foot syndrome, osteomyelitis, angiosome, perfusion, microbiota, molecular imaging

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Патоген-специфическая молекулярная визуализация и методы молекулярного тестирования в прогнозе осложненного течения синдрома диабетической стопы, риска ампутаций и выживаемости пациентов

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РЕЗЮМЕ

Цель настоящего обзора – расширение информации об актуальных направлениях в диагностике осложненного течения синдрома диабетической стопы (СДС), частого и тяжелого осложнения сахарного диабета, включая высокотехнологические методы медицинской визуализации и инструментально-лабораторные предикторы осложненного течения и риска ампутаций при СДС.

Представлен аналитический обзор современных публикаций за последние 5 лет по диагностическим и терапевтическим направлениям; систематизированы и обобщены, а также подвергнуты аналитической оценке пилотные данные, касающиеся использования высокотехнологических методов медицинской визуализации, оценки микробиоты кожи и язвенных дефектов при СДС, методов молекулярного тестирования с точки зрения прогноза риска ампутаций и выживаемости пациентов с СДС и эффективности применения систем биосенсирования.

Дана экспертная оценка возможностей патоген-специфической молекулярной визуализации с использованием современных технологий позитронно-эмиссионной томографии (ПЭТ) и однофотонной эмиссионной компьютерной томографии (ОФЭКТ) и высокоэнергетических радионуклидов при бактериальной инфекции для понимания ее патогенеза, минимизации диагностических проблем, улучшения антимикробного лечения и для решения фундаментальных и прикладных аспектов СДС. Систематизированы литературные данные об оценке перфузии стоп у больных сахарным диабетом с различной степенью ишемии конечностей методами гибридных технологий (ОФЭКТ/КТ и ПЭТ/КТ) и новых модальностей магнитно-резонансной томографии, что способствует новому пониманию ответной реакции на реваскуляризацию, хирургическое шунтирование и стимулирование ангиогенеза в пределах ишемизированной ткани, а также потенциально – и для заживления язвы стопы.

Статья направлена на обоснование мультидисциплинарного подхода при СДС, а также выбор, развитие и внедрение инновационных стратегий диагностических модальностей в установлении патологических процессов при СДС, выбор адекватного метода лечения и мониторирования результатов терапии в рамках развития персонифицированной медицины.

Ключевые слова: сахарный диабет, синдром диабетической стопы, остеомиелит, ангиосома, перфузия, микробиота, молекулярная визуализация

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

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INTRODUCTION

The problem of diabetic foot syndrome (DFS), which is the most common and severe complication of diabetes mellitus (DM) due to high percentage of post-amputation deaths, remains critical and requires a multidisciplinary approach to monitor and predict the therapeutic response. The aim of this review was to provide extended information on current trends in the diagnosis of complicated DFS, including high-tech medical imaging methods and instrumental and laboratory predictors of the complicated course and risk of amputation in DFS.

The main manifestation of DFS is trophic ulcers or other purulent and destructive processes starting from the integumentary system. However, the cause and pathogenetic mechanisms of phenotypically similar DFS manifestations can be completely different. It complicates the choice of adequate therapeutic strategies (conservative, surgical, as well as their combinations) and the prognosis of this DM complication, increasing the incidence of poor outcomes and the number of amputations of lower extremities and post-amputation deaths.

The main pathogenetic mechanisms of DFS are the development of diabetic macroangiopathy, microangiopathy, neuropathy, in complicated forms – bacterial infection in the soft tissues and bones of the feet. The underlying pathology in a number of patients, which could contribute to the main mechanisms of DFS formation (joint damage, chronic venous insufficiency, radicular syndrome, etc.), complicates the choice of the correct treatment strategy. Under these conditions, it is extremely relevant to identify the leading pathogenetic mechanisms of the DFS formation, evaluate the contribution of the underlying pathology, and develop and validate new pathogenetically grounded radiological methods and methods of molecular diagnosis and biosensing.

MODERN DIAGNOSTIC RADIOLOGY METHODS IN DFS

Magnetic resonance imaging in diabetic foot syndrome

Most authors consider magnetic resonance imaging (MRI) as the gold standard in diagnostic radiology of complicated DFS [1–3]. Technical capabilities of MRI in recent years make it possible to use this method in DFS both to visualize structural changes in the foot tissues and to assess the vascular bed using contrast-enhanced and non-contrast-enhanced MR angiography. An important advantage of MRI, according to a recent review by C. A. Ruiz-Bedoya et al. (2019), is recognition of bone marrow edema with precise anatomical localization at an early stage – 3–5 days after the onset of the infectious process [4].

Recent literature sources describe MR-semiotics of foot lesions in diabetes mellitus (DM) in detail. According to D. H. Massel et al. (2020), MRI has high sensitivity in the diagnosis of osteomyelitis (OM) of the foot due to typical changes in signaling characteristics and allows to accurately localize purulent complications and assess their prevalence and clinical risk of complications [5]. Recent publications indicate the possibility of using certain MR signs as predictors of purulent and destructive complications in DFS. Thus, bone marrow edema and an adjacent at this level subcutaneous adipose tissue ulcer with the depth of more than 50% are unambiguous predictors of the development of OM of the foot [6, 7].

Moreover, Y. H. Jang et al. (2020), based on the results of a multivariate analysis of MRI data, indicated a new pattern of DFS complication development: confluent pattern of decreased T1 marrow throughout the entire bone fragment, as well as “blotches” of unchanged bone marrow against the background of a “thin / reticular mesh” [7].

H. M. Kotecha et al. (2020) currently consider the possibility of using native MRI in emergency departments in patients with DFS as an abbreviated MRI protocol (only coronal T1-weighted and sagittal T2-weighted FMPIR images) lasting an average of 8 minutes [8]. A number of authors have substantiated MRI in patients with DFS for planning the scope of surgical interventions. The data obtained by M. Jbara et al. (2016) indicate a significant association of preoperative MRI with a decrease in postoperative mortality [9, 10]. The relevance of repeat MRI for assessing treatment outcomes in patients of reproductive age with DFS is reported by C. Lauri et al. (2018) [11].

However, D. Duryea et al. (2017) note that, despite high sensitivity and positive and negative predictive power of MRI in the diagnosis of purulent complications, the specificity of the method is not

so high due to the difficulties in differential diagnosis of aseptic and inflammatory bone marrow edema [5, 7]. Therefore, differential diagnosis of pathological changes in the feet remains a frequent clinical problem in DM patients [12].

Contrast-enhanced and non-contrast-enhanced MR angiography

Contrast-enhanced MR angiography (CE-MRA) is of great importance in assessing the anatomy and pathology of the foot vessels. The advantages of CE-MRA over digital subtraction angiography (DSA) in patients with DFS are visualization of a larger number of stenotic vessels and simultaneous clear visualization of inflammatory complications, such as OM, soft tissue abscesses, and fistulas [13]. However, CE-MRA is inferior to SA in assessing distally located vessels, as well as in critical ischemia cases. A number of publications indicate the priority of this technique before revascularization procedures in patients with ischemic or mixed forms of DFS and refusal to use X-ray techniques in foreign medical centers [12].

Preoperative CE-MRA assesses arterial patency, the presence of diffuse calcification in distal vessels and microaneurysms, the state of collateral blood flow, and targeted vessels for surgical bypass [14]. CE-MRA is useful for identifying active-phase Charcot foot, monitoring a response to treatment, and predicting the healing time of wound-related defects [1, 3]. The diagnostic efficiency parameters of CE-MRA exceed those of CT angiography (CTA) and duplex ultrasound scanning of the foot vessels in DFS [13].

MR-semiotics of complicated DFS has been sufficiently studied to date. Thus, M.A. Zamyshevskaya et al. (2016) presented MRI data on blood supply to the foot in case of intraosseous purulent inflammation, acute diabetic osteoarthropathy, and arteriovenous shunting. The authors described the distribution of the contrast agent with an increase in vascular permeability in inflammation sites [15].

The quantitative assessment of dynamic contrast-enhanced MRI is described in sufficient detail [13, 16]. D. Liao et al. (2018), using the extended linear Tofts model, evaluated three quantitative parameters of CE-MRA: the transfer constant – Ktrans, the contrast rate index – Kep, and the volume fraction of the extravascular extracellular space – Ve [16]. These parameters demonstrated statistical significance in the differential diagnosis of Charcot foot and OM in DFS, however, the limited number of patients and the peculiarities of post-processing do not yet confirm the possibility of wide use of the quantitative assessment of MR angiography data in clinical practice.

Technological progress made it possible to conduct not only contrast-enhanced, but also non-contrast-enhanced studies of the peripheral vessels in the lower extremities, which are based on either an increase or decrease in the blood flow signal. N. Zhang et al. (2016) in their publication concluded that non-contrast-enhanced MRA can be used as a safe and reliable screening tool for assessing the state of the foot arteries in patients with DM [17].

Quiescent-interval single-shot MR angiography (QISS MRA) is a two-dimensional, balanced, steady-state, free precession synchronized with ECG, which has several advantages and demonstrates high accuracy compared with CTA and cMRA in the diagnosis of critical lower limb ischemia in DFS. A variant of this modality, QISS MRA with arterial spin labeling (ASL), is a related technique that has the potential to visualize the foot vessels due to theoretically improved background suppression.

A. Lam et al. (2020) described in detail the use of QISS MRA and QISS ASL-MRA in patients with critical ischemia due to DFS, which was not done in earlier studies [18]. The authors describe these non-contrast-enhanced MRI techniques as fast, relatively simple, and at the same time highly effective imaging methods that are insensitive to patient movements, pulse wave, and blood flow characteristics, as well as applicable in the presence of severe diabetic nephropathy with a decrease in the estimated glomerular filtration rate (eGFR). At the same time, QISS MRA is considered as a method of targeted visualization for performing shunting and revascularization of the foot vessels. The use of more powerful MRI machines (3T) and improvement of a number of technical aspects can improve this non-contrast-enhanced MRI technique and contribute to its introduction in clinical practice.

Diffusion-weighted MRI in DFS

Foreign publications on the use of diffusion-weighted MRI to detect the inflammatory process in DFS appeared in 2017–2020 [12, 19, 20]. The publication by A.A. K. Abdel Razek and S. Samir (2017) indicates that one of the advantages of this technique is the ability to quantify the signal from altered foot tissues by calculating the diffusion coefficient (DC) [19]. The DC values in the affected bone are significantly higher in acute diabetic neuroarthropathy compared with those in OM. Thus, diabetic osteoarthropathy is associated with bone marrow edema with a relatively smaller inflammatory cell number and higher DC values, while OM is associated with the presence of microorganisms, as well as inflammatory and dead cells, hence, with subsequent limited diffusion and lower DC values. However, diffusion-weighted MRI does not completely exclude the combination of osteoarthropathy and OM, especially at the early stage of inflammation and with the subacute course of neuroarthropathy, which reduces the diagnostic efficiency of the technique [19].

The use of 3.0T MRI systems with new technical characteristics aimed at suppressing perfusion effects can improve the accuracy of DC calculations, contributing to the improvement of the qualitative and quantitative characteristics of diffusion-weighted images in DFS [12]. At the same time, it is too early to raise the issue of using diffusion-weighted MRI in clinical practice due to a lack of standardization for DC calculation, subjectivity of interpretation of the obtained values, and technical capabilities of MRI machines, which requires a larger study or meta-analysis of the use of diffusion-weighted MRI in DFS.

Radionuclide diagnosis in DFS

Scintigraphy with 99mTc-HMPAO- and In111-oxime-labeled leukocytes is regarded as the gold standard in radionuclide diagnosis of OM in DFS [1, 21]. In 2018, European Association of Nuclear Medicine (EANM) in order to standardize the procedure for labeling leukocytes developed research protocols and criteria for interpreting the method results. These criteria include comparative characteristics of various degrees of radiopharmaceutical hyperfixation at control points (after 1, 3, and 20 hours) depending on sterile and non-sterile inflammation [22].

Concomitant Charcot osteoarthropathy, leading to radiopharmaceutical hyperfixation due to increased hematopoietic activity in the bone marrow, secondary to chronic inflammation, contributes to lower specificity of the method. To overcome this limitation, it is proposed to perform additional bone marrow scintigraphy using nanocolloids [11]. In case of using a dual technique, two diagnostic criteria for OM in Charcot arthropathy are described: capture of labeled leukocytes without corresponding activity on bone marrow scintigraphy images and incongruent spatial distribution of two radiopharmaceuticals [11, 23].

The main reasons for the decrease in the specificity of this technique are low anatomical spatial resolution, hyperfixation of labeled leukocytes at the site of sterile inflammation in the Charcot foot, and leukopenia [24]. Combined use of labeled leukocyte scintigraphy with a modality that is highly informative in terms of imaging anatomical structures can increase the specificity of this method.

The effect of long-term antibiotic treatment on the sensitivity of labeled leukocyte scintigraphy is still a matter of debate. Judging by preliminary data, the use of radioactively labeled leukocytes is allowed 2 weeks after the end of therapy, even in the presence of false negative results [1, 22].

As an alternative to labeled leukocytes, monoclonal antibodies (MoAbs) or antibody fragments (Fabs) against specific antigens expressed by activated granulocytes can be proposed. However, the role of MoAbs and Fabs in assessing complicated DFS has not been widely studied, there are no standardized protocols for collecting and interpreting research results [11].

There are some recent studies developing highly specific biomolecules and new agents, since molecular imaging of bacterial infections may provide a unique opportunity to monitor treatment of patients with DFS complicated by infections. Currently, in this category of patients, according to A.O. Ankrah et al. (2018), the results of using a number of isotope indicators, such as 18F-FDS, 99mTc-UBI 29-41, and 68Ga-NOTA-UBI, are evaluated. Using these isotope indicators does not require manipulations with blood and is able to differentiate between inflammatory and infectious processes with high specificity [25]. However, the sensitivity of these agents requires further evaluation and confirmation

in larger clinical trials, especially in cases of chronic infections with low bacterial load [4].

Scintigraphy with labeled antibiotics, such as 99mTc-labeled ciprofloxacin, can identify the infectious process and differentiate between sterile and non-sterile inflammation, but this radiopharmaceutical has lower diagnostic efficiency than radiopharmaceuticals with labeled leukocytes, which is probably due to a non-specific accumulation mechanism. In 2019, a group of researchers led by N. Ahmed published the results of 99mTc-labeling of ceftizoxime, a third-generation cephalosporin with a broad spectrum of antibiotic activity compared with ciprofloxacin [24].

J. Vouillarmet et al. (2017) report the use of single-photon emission computed tomography (SPECT) / CT with labeled leukocytes to predict remission after a 6- or 12-week course of antibiotic therapy in the case of non-surgical treatment of foot OM in DM [26]. However, W. J. Jeffcoate, basing on a number of publications (2016–2017) on comparing the effectiveness of clinical and instrumental monitoring of patients with complicated DFS, concludes that the use of this hybrid technique can be overestimated, and the method itself does not have a great impact on daily clinical practice [27].

According to R. Ahluwalia et al. (2020), SPECT / CT is a useful method of functional and structural imaging for both foot OM and Charcot foot [28]. At the same time, a broader and more prospective approach to the study of SPECT / CT as a method for identifying predictors of Charcot foot formation is required, taking into account the advantages of this method over MRI in assessing structural changes, as well as a method used in patients for whom MRI is contraindicated [1].

Positron emission tomography in DFS

18F-fluorodeoxyglucose (18F-FDG) positron emission tomography (PET) provides non-invasive 3D imaging with higher spatiotemporal resolution and sensitivity compared with SPECT and MRI, and moderate hyperglycemia does not affect the diagnostic performance of 18F-FDG [2, 29]. The disadvantage of the method is low specificity. In addition, glucose uptake may remain impaired for 3–4 months after surgery or injury [4].

C. Ruiz-Bedoya et al. (2019) note that molecular imaging of bacteria at the site of infection facilitates selection of appropriate empiric antimicrobial therapy [4]. A drug like 18F-fluorodeoxysorbitol (18F-FDS), selectively targeting the Enterobacteriaceae family of bacteria, can be used in combination with a broad-spectrum imaging marker (e.g., 11C-para-aminobenzoic acid) for PET indication of infection and differentiation between Gram-positive and Gram-negative bacteria.

Taking into account the advantages of PET over SPECT imaging, it is recommended to study and develop PET equivalents of indicators that are potential for visualizing infectious agents. The use of radiometals, such as 68Ga, offers more labeling possibilities, especially for peptides. Radionuclides with longer half-lives, such as 64Cu and 89Zr, can also be a good alternative for labeling peptides currently labeled with 68Ga. These relatively longer-acting radionuclides will provide delayed imaging, which is a requirement for infection indication, including those in DFS. Radioimmunotherapy with antibiotics using nanoparticles labeled with new radionuclides for drug delivery can shorten the duration of antibiotic therapy and affect resistant microorganisms [25].

According to C. Lauri et al. (2020), 18F-FDG-PET / CT is an alternative to scintigraphy, however, to date, there is no clear definition of criteria and standardization of this hybrid technique [1]. Thus, some authors note lower sensitivity of the hybrid technology in diagnosing complicated DFS compared with MRI, while the specificity and accuracy of the PET / CT method are significantly higher than those of MRI [12].

A semi-quantitative analysis of the maximum standardized absorbance value (SUVmax) was developed when performing 18F-FDG PET / CT. C. Lauri et al. (2020) report significantly higher SUVmax values in cases of OM in DFS compared with the same parameter in Charcot foot and uncomplicated DFS, which indicates the significance of the SUVmax parameter for the differential diagnosis of these pathological processes [23]. Although A.I.G. Diez et al. (2020) also concluded that the SUVmax parameter can be useful for differentiating pathological processes in the foot in DM, some authors did not find statistically significant differences when comparing this parameter in patients with Charcot foot and OM [12].

Publications on the possibilities of PET / MRI in assessing the state of the bone marrow and monitoring the course of the disease in DFS patients with the subsequent prospect of differentiating damage to soft tissue and bone structures [12] reflect the development of hybrid techniques. A.W.J.M. Glaudemans et al. (2019) believe that PET / MRI, as opposed to PET / CT, will increase the accuracy of diagnosing foot infections by improving the differentiation of OM itself and soft tissue infections [30]. The potential possibility of obtaining hybrid SPECT / MRI images using workstation software is studied [31].

A few foreign articles describe various approaches to the quantitative / semi-quantitative assessment of visual data in patients with DFS and the creation of clinical diagnostic scales. A variant of the MR scoring system for patients with Charcot foot was presented by L. Meacock et al. in 2017. The presented semi-quantitative scale was based on such MRI symptoms as bone marrow edema and the presence of the affected bone fracture [32].

V.D. Udodov et al. patented and described in the dissertation thesis (2018) a scoring system for assessing combined SPECT / MRI in patients with DFS and suspected OM. This system is currently one of a kind for diagnostic practice. This scoring system includes a number of MR symptoms of inflammatory processes in DFS and two scintigraphic symptoms based on the results of labeled leukocyte scintigraphy. Based on the results of the ROC analysis, the threshold value for OM in DFS was obtained with a total score of existing visual symptoms of more than 12. This quantitative criterion is highly specific and sensitive and may become promising in the clinical diagnostic evaluation of the foot damage in DM patients.

ASSESSMENT OF TISSUE PERFUSION IN DFS

A significant problem in DM patients is impaired tissue perfusion and, as a result, endothelial dysfunction, capillary microangiopathy, and critical ischemia of the lower extremities [4, 33]. Targeted treatment of ischemic non-healing foot ulcers requires angiosome-directed revascularization, which could lead to a significantly higher rate of wound healing and reduce a risk of large limb amputations in patients with peripheral arterial disease (PAD) [34].

J.L.Alvelo et al. in 2018 described the use of 99mTc-tetrofosmin for perfusion imaging by SPECT / CT. The method demonstrated the possibility of a qualitative and quantitative assessment of foot microcirculation at rest with the ability to detect perfusion defects in areas containing non-healing foot ulcers in patients with critical lower limb ischemia and PAD [33]. It should be noted that 99mTc-sestamibi, used in cardiology, also showed high informative value and certain advantages at the preclinical and clinical stages in the diagnosis of PAD and was considered as an alternative to 99mTc-tetrofosmin in future investigations [33].

Regardless of previous scientists, a research group led by T. Chou in 2020 published their own data on the use of SPECT / CT for the quantitative assessment of angiosome perfusion as an additional method in relation to X-ray angiography and measurement of ankle – brachial index (ABI) and toe – brachial index in patients with DM and critical limb ischemia with planned lower limb revascularization [35].

Non-contrast-enhanced MRA is a highly effective method for the absolute assessment of foot perfusion in patients with DM. The results presented by H. Chen in 2018 suggest that the parameters of microvascular arterial injury in MRI will allow for a better understanding of the pathophysiology of ongoing changes in the tissues [36].

J. Zheng et al. (2016) published data on the possibilities of constructing non-contrast MRI perfusion angiosome foot maps in healthy individuals and patients with DM [37]. Foot perfusion measurements were taken during exercise. As a result, according to non-contrast MRA data, regional differences in the perfusion of the foot muscles in the isolated angiosomes were clearly visualized. In DM patients, perfusion during exercise was statistically significantly lower than in healthy volunteers.

In 2019, the same group of researchers led by M. Edalati published an extended version of the foot MR perfusion assessment using arterial spin labeling (ASL) [38]. The authors found that the MR perfusion reserve of the foot in DM patients was significantly lower than in patients without DM. The second important point was a statistically significant decrease in MR perfusion of the muscles in the periulcer anatomical region both at rest (reserve) and during exercise compared with areas of the foot

located at a distance from the diabetic ulcer. Thus, the authors confirm and describe the angiosome theory of blood supply to the feet and its impact on the DFS course, however, due to the small sample size, additional experimental and clinical studies are required to confirm the results obtained. The main focus of this study, except for the angiosome theory, is the fact that the perfusion reserve of muscles around healed ulcers in DFS was significantly higher than that in long-term non-healing foot ulcers. This fact justifies the prospect of further study of MR perfusion reserves of skeletal muscles as predictors of foot wound healing in DFS [38].

Despite the initial success of angiosome-directed revascularization, up to 54% of foot ulcers cannot be unambiguously attributed to a specific angiosome due to dual blood supply. Recent studies of foot tissue in the near-infrared spectrum have made it possible to consider the image of oxygen saturation of the foot tissue (StO2) as a modified angiosome theory. This new "angiosome" turned out to be better than the traditional angiosome model for detecting ischemic skin lesions associated with foot ulcers. Studies of tissue oxygenation in DM patients have continued in the evaluation of skeletal muscle oximetry [37].

An MRI-based assessment of microcirculation was carried out to measure the skeletal muscle oxygen extraction fraction (SMOEF) of the foot in DFS as well as to compare two angiosome models – classical and oximetric ones. According to the results, an absolute mean value of SMOEF at rest in DFS patients was higher than in healthy people. This difference may be due to reduced tissue perfusion in DFS patients, and, therefore, tissue oxygen demand must be met by increasing oxygen extraction even at rest. A clinically important point is the increase in SMOEF in healthy volunteers during the transition from rest to physical activity, rather than in patients with DFS when assessing similar angiosomes. It is worth noting that the oximetric angiosome model of the foot allows to directly determine the areas with low or high oxygen content delivered through one or more foot arteries. This distribution of angiosomes may be more appropriate for DM patients, since local microcirculation disruption is a frequent and clinically important aspect for patients with DM, even without peripheral arterial occlusive disease, reflected by the classical angiosome theory [38].

Blood oxygenation level-dependent non-invasive functional MRI (BOLD-MRI) is a method for assessing dynamic changes in oxygenation of skeletal muscles, which reflects changes in the volumetric blood flow, especially in the microvasculature. The ability of the method to detect vascular anomalies of the foot may be especially valuable in patients with macro- and microvascular diseases [39].

Despite the widespread development of contrast-enhanced and the introduction of non-contrast-enhanced techniques for blood flow MR examination in DFS, most scientific publications are still limited to determining vascular patency. Other aspects of the blood flow are studied to a limited extent, and the data obtained are not enough to formulate reliable conclusions. Therefore, further exploring MRA possibilities, particularly, MR perfusion, in DFS is needed.

OTHER NON-INVASIVE DIAGNOSTIC METHODS AND PREDICTORS OF LOWER LIMB AMPUTATION IN PATIENTS WITH DFS

The relative simplicity and availability of radiation-free non-invasive methods for diagnosing DFS and predicting its course can provide the possibility of their use by a doctor directly during a patient consultation, as well as by DM patients themselves for daily monitoring and control of the foot state. The monitoring of blood pressure in the lower extremities, plantar temperature and pressure, and gait changes are among these methods. Complex biosensor systems designed to assess and / or monitor the presence of various markers of a poor disease prognosis in DFS, in particular, metalloproteinases and their tissue inhibitors, should also be mentioned. In addition to the above, a promising area of study is the assessment of the wound microbiome and microflora in DFS in order to diagnose and select a patient management strategy.

Monitoring of blood pressure and blood flow in the lower extremities

Evaluation of such physiological parameters as blood pressure and blood flow in the lower extremities showed high clinical significance in assessing the condition of patients with DFS. In a systematic review, R.O. Forsythe et al. (2020) noted the effec-

tiveness of 6 non-invasive clinical tests in predicting wound healing or a risk of amputation in DFS. The researchers identified 4 signs that showed the greatest accuracy in assessing the degree of a decrease in lower extremity perfusion, which in turn is an important indicator of a high risk of amputation: ankle pressure < 50 mm Hg, ABI < 0.5, blood pressure in the toes < 30 mm Hg, as well as transcutaneous oxygen tension < 25 mm Hg.

On the other hand, in the same study, favorable signs indicating a higher likelihood of wound healing in DFS were also identified. Good outcome sings included skin perfusion pressure ≥ 40 mmHg, toe blood pressure ≥ 30 mmHg, and transcutaneous oxygen tension ≥ 25 mmHg. It is promising to use the results of these non-invasive tests in combination with other clinical predictors to select a strategy for further patient management – a variant of conservative treatment, a detailed assessment of perfusion disorders with possible subsequent revascularization or surgical treatment [40].

Monitoring of plantar foot temperatures

Monitoring of plantar foot temperature has shown itself to be a promising method for indirectly assessing the state of the blood flow in the lower extremities in order to early identify focal lesions of the feet. It was established that an increase in plantar temperature occurs as a result of repeated loading on the foot and inflammatory changes, in particular, enzymatic autolysis [41].

J. Golledge et al. in their research (2020) showed that monitoring the plantar foot temperature with a portable infrared thermometer on a daily basis is effective in preventing new or recurrent ulcers [42]. The parameter of temperature differences between symmetrical areas of opposite feet (2.2°C) turned out to be the most effective, as opposed to absolute temperature values and temperature comparison between areas of the same foot. However, despite the proven effectiveness, this method did not receive wide application value. The reasons for this are not entirely clear, but are likely to be explained by the difficulties for daily use and, as a result, low adherence of patients to this remote-control method. To overcome this barrier, innovative and ergonomic models are offered: floor mats for portable thermography, socks and insoles with built-in infrared sensors, portable infrared cameras for mobile phones with the ability to continuously monitor changes in the parameter and send data to the attending physician [42,43].

In terms of compliance issues, the focus of researchers is increasingly shifting to complex multifunctional devices. In particular, temperature sensors that can be built into orthopedic shoes for diabetic feet are being developed. These sensors would make it possible to measure both the quality of foot unloading and the regularity of its wearing [44].

Thus, the greatest attention is paid to the development of complex sensors with the possibility of remote monitoring of parameters. This is due to increased attention both to telemedicine issues (taking into account the current epidemiological situation) and to issues of patient adherence to modern methods of diagnosis and treatment.

Plantar pressure measurement

Pressure, pressure gradients, shear stress, and peak plantar pressure are a group of mechanical loading parameters that contribute significantly to the formation of ulcers. The key point in the management of patients with DFS is the use of unloading devices and specialized shoes, made individually in accordance with the foot characteristics of each patient. In particular, one of the most important targets is reduction of peak plantar pressure to less than 25%, which makes a significant contribution to wound prevention in DFS [43, 44].

Plantar pressure monitoring is performed using pressure plates or insoles with built-in sensors in medical centers. However, some of the existing devices, such as Pedar® *(Novel, Munich, Germany)*, F-Scan ™ (Tekscan Inc., USA), and SurroSense Rx (Orpyx Medical Technologies, Canada), track the effectiveness of specialized orthopedic footwear in reducing peak plantar pressure in the outpatient setting [45].

A number of prototypes are able to warn the user about a prolonged increase in pressure (more than 35–50 mm Hg for more than 15 minutes) in a certain area of the foot using an audio signal, as well as to assess the degree of subsequent unloading of the foot [46]. Today, these systems are used primarily for research purposes, but are not integrated into clinical practice. Long-term monitoring of plantar pressure with the ability to provide feedback when “alarm

levels" occur is a promising avenue and certainly requires further study.

Monitoring gait changes

It is known that patients with diabetic polyneuropathy are characterized by impaired gait, which also contributes to changes in plantar pressure and to an increased risk of ulcers [47]. The greatest problem is the objectification of gait changes, since the patient himself can sometimes subjectively miss even pronounced disturbances in motor activity. In this regard, removable devices for remote monitoring of the step pattern have already been developed and tested [48]. At the next stage, these data can be used to develop training programs using artificial intelligence systems to correct gait disorders. For example, Samsung has created Gait-Enhancing Mechatronic System (GEMS) to redistribute foot pressure and reduce the risk of ulcers [49].

Biosensor systems

Wound bed assessment today relies mostly on subjective interpretation without the use of objective instruments. The use of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) as biomarkers in DFS is increasingly discussed by researchers.

To date, there is no suitable device that uses an objective quantitative index *in situ* to determine the severity of chronic ulcers, as well as a need for surgical treatment (in the outpatient setting, delayed surgical treatment often leads to aggravation of wounds). MMP-2 and -9 are considered as markers of active inflammation. Their expression increases during wound process exacerbation.

S. Kang et al. (2019) describe a hydrogel biosensor device for detecting the expression of MMPs in wound exudate, which allows to assess the severity of the ulcer and timely provide a surgical intervention. The mechanism of this biosensor model operation is as follows: upon immobilization of a fluorescent peptide cleaved by a specific metalloproteinase, its amount can be measured by an increase in the fluorescence of the hydrogel substrate [50].

A group of scientists from Bangkok in 2018 described changes in MMP-1, MMP-9, and TIMP-1 levels by ELISA during healing of diabetic ulcers. The authors concluded that the level of MMP-9, MMP-1, and TIMP-1 could be used as a potential screening test during the patient's first visit. The researchers propose a scoring system which allows to divide patients into 2 prognostic groups at the clinical stage: 'good healer' and 'poor healer'. This scoring can help to correctly determine a further treatment strategy. Moreover, it is expected that highly selective dressings with components that reduce the expression of the above inflammatory markers will be effective for diabetic ulcers that are difficult to treat with standard treatment methods [51].

Another promising technology is the multidimensional MS / MS protein identification system in tissue samples. Using this technology, the protein composition of foot exudates in DFS patients was analyzed. The study showed the overexpression of both metalloproteinases (MMP-1, MMP-2, and MMP-8) and proteins with antiangiogenic properties, such as collagens CO15A1 and CO18A1 [52].

J. Jones et al. in 2019 noted excessive activation of MMP-8 and MMP-9 in diabetic wounds in mice and humans, with MMP-9 being associated with longer healing of diabetic wounds, while MMP-8 expression was, on the contrary, a favorable prognostic factor for the disease [53]. Speaking of other biomolecules that play a key role in the healing of chronic ulcers, one cannot but mention growth factors (GF), such as EGF, bFGF, VEGF, PDGF, and IGF. It is known that the level of the above GFs in the wound is significantly reduced in DFS, which therapeutically justifies their use as topical drugs. The limiting factor in this case is low stability of the biomolecules *in vivo* due to degradation under the influence of tissue proteases in the wound bed. A promising direction is the development of sustained release drug delivery systems: solid lipid nanoparticles, nanostructured lipid carriers, polymeric microspheres and nanospheres, hydrogels, and nanofibrous scaffolds. Many studies in recent decades have confirmed their high therapeutic efficacy [54].

Microbiome

The microbiome is a collection of bacteria, viruses, unicellular eukaryotes, and other microorganisms coexisting with the host organism. Their presence creates a complex system of physiologically and metabolically interconnected processes

that affect a human life. The study of ulcer microbiome in DFS is of great importance. Identification of various bacterial strains in DFS plays an essential role in the prognosis of the disease. Ulcers can be colonized by various aerobic and anaerobic bacteria depending on various factors.

In superficial ulcers, the microbiome mainly consists of Gram-positive cocci, including *Staphylococcus aureus, Streptococcus pyogenes*, beta-hemolytic Streptococci, or coagulase-negative Staphylococci. Patients with deeper ulcers may be infected with Enterococci, Pseudomonas, or anaerobic bacteria [55].

One of the modern methods for studying the bacterial diversity of tissue samples is the method of culture analysis. This method involves the use of large-scale cultivation conditions for bacterial strains, followed by detailed identification of colonies using matrix-assisted laser desorption / ionization (MALDI) or 16S ribosomal RNA gene PCR. The method, according to the authors, makes it possible to identify all bacterial strains in the sample, including the most minimal ones, as a result, the entire microbiome of skin lesions in DFS was defined and described in detail. The authors found extremely high bacterial diversity, namely 53 species of various bacteria in the diabetic foot wound. A detailed analysis of bacterial diversity in each specific clinical case will allow for a more thorough approach to the selection of antibiotic therapy, however, this area requires further study.

Many studies demonstrate that the predominant bacterial strain in diabetic foot wound tissue is *Staphylococcus aureus*. It has also been found to be associated with a worse prognosis in diabetic foot infections, while the presence of *E. faecalis* in the wound significantly correlates with better wound healing. A number of factors have been found to influence the dominant bacterial strain establishment, such as demographic characteristics, personal hygiene, severity of the lesion, glycemic control, and current or previous antibiotic treatment. In addition, the laboratory research method used to identify bacteria also has a high impact [56].

M. Malone et al. in 2017 conducted a study using DNA sequencing to analyze the microbiota of the wound tissue in DFS. The study included groups of patients who received targeted antimicrobial therapy according to the results of a standard culture method. According to the results, no significant differences were found in the composition of the microbiome in patients with successful therapy and those who had no effect from the treatment, which calls into question the clinical significance of the standard culture method for determining bacterial wound strains in DFS [57].

In another research, J.U. Park et al. in 2019 used a DNA sequencing method to compare the microbiota of normal skin and ulcer in DFS. An important finding was the fact that the overall diversity of the bacterial flora was significantly poorer in diabetic foot wound tissue. It is assumed that the dominant development of opportunistic flora and local inflammation are mutually potentiating factors that lead to unfavorable clinical outcome. Moreover, it is noted that chronic inflammation in ulcers is a background for anaerobic flora, especially in the deep layers of the wound, which in turn causes an unfavorable clinical outcome. The predominant types of bacteria in the intact skin were Actinobacteria, Staphylococci, Corynebacteria, and Propionibacteria, while in ulcers, Anaerobes, Bacteroids, Enterococci, and Pseudomonads were found [58]. Summary data on the composition of the bacterial flora in normal skin tissue and tissue of diabetic foot ulcers are given in the Table [58].

Table

The microbiome of diabetic ulcers			
Parameter	Gram-positive bacteria	Gram-nagative bacteria	Anaerobes
Predominant strains in diabetic foot ulcers	*Staphylococcus aureus* (MSSA and MRSA). *Streptococcus pyogenes* (beta hemolytic)	*Pseudomonas aeruginosa. Streptococcus pyogenes* (beta hemolytic). *Proteus* (different species)	*Peptostreptococcus, Bacteroides, Prevotella, Clostridium*
Localization of bacteria in the ulcer	Superficial layers of the wound	Superficial layers of the wound	Deep layers of the wound
Frequency of detection in DM	Non-predominant type of bacteria	Predominant type of bacteria	Present

Note: MSSA – methicillin-sensitive Staphylococcus aureus; MRSA – methicillin-resistant Staphylococcus aureus.

CONCLUSION

DFS and its complications are a common clinical problem. Delaying an accurate diagnosis can lead to an increase in patient complications, including amputation. The key questions regarding the diagnosis of foot infection, its location and spread, the type of pathogen, and the response to treatment are still not fully resolved, as accurate identification and differentiation of different types of DFS continues to be a challenge for clinical practitioners. Systematized in this article, special methods for providing valuable information about DFS and its complications will contribute to a better understanding of this disease. Multimodal imaging and a multidisciplinary clinical diagnostic approach, according to the authors, are mandatory in order to plan the most appropriate therapeutic strategy for an individual patient with DFS.

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Authors contribution

Zorkaltsev M.A – conception and design. Zavadovskaya V.D., Saprina T.V. – editing of the manuscript and expert assessment. Zamyshevskaya M.A. – drafting and editing of the sections “Magnetic resonance diagnosis in DFS” and “Methods for assessing tissue perfusion in DFS”. Udodov V.D. – drafting and editing of the section “Radionuclide diagnosis in DFS”. Shestakov A.V. – drafting and editing of the section “Other non-invasive diagnostic methods and predictors of lower limb amputation in patients with DFS”. Mikhailova A.A. – drafting and editing of the section “Other non-invasive diagnostic methods and predictors of lower limb amputation in patients with DFS”. Loyko Yu.N. – drafting of the section “Methods for assessing tissue perfusion in DFS”. Musina N.N. – primary screening of the data, analysis and interpretation of the data. All authors approved the final version of the article before publication, agreed to be responsible for all aspects of the work, implying the proper study and resolution of issues related to the accuracy or integrity of any part of the work.

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Promising directions in the treatment of chronic heart failure: improving old or developing new ones?

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ABSTRACT

Unprecedented advances of recent decades in clinical pharmacology, cardiac surgery, arrhythmology, and cardiac pacing have significantly improved the prognosis in patients with chronic heart failure (CHF). However, unfortunately, heart failure continues to be associated with high mortality. The solution to this problem consists in simultaneous comprehensive use in clinical practice of all relevant capabilities of continuously improving methods of heart failure treatment proven to be effective in randomized controlled trials (especially when confirmed by the results of studies in real clinical practice), on the one hand, and in development and implementation of innovative approaches to CHF treatment, on the other hand. This is especially relevant for CHF patients with mildly reduced and preserved left ventricular ejection fraction, as poor evidence base for the possibility of improving the prognosis in such patients cannot justify inaction and leaving them without hope of a clinical improvement in their condition. The lecture consistently covers the general principles of CHF treatment and a set of measures aimed at inotropic stimulation and unloading (neurohormonal, volumetric, hemodynamic, and immune) of the heart and outlines some promising areas of disease-modifying therapy.

Keywords: chronic heart failure, treatment, neurohormonal modulators, sacubitril / valsartan, pecavaptan, fineron, vericiguat, sodium – glucose cotransporter type 2 inhibitors, omecamtiv mecarbil, gene therapy, cardiac resynchronization therapy, cardiac contractility modulation, heart transplantation, implantation of a circulatory assist device

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Перспективные направления лечения хронической сердечной недостаточности: совершенствование старых или разработка новых?

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РЕЗЮМЕ

Беспрецедентные достижения последних десятилетий в области клинической фармакологии, кардиохирургии и имплантационной аритмологии значительно улучшили прогноз у пациентов с хронической сердечной недостаточностью (ХСН), однако, к сожалению, сердечная недостаточность продолжает ассоциироваться с высокой смертностью. Решение этой проблемы видится одновременно в максимально полном применении в клинической практике всех актуальных возможностей непрерывно совершенствующихся методов лечения сердечной недостаточности, доказавших свою эффективность в рандомизированных контролируемых исследованиях (особенно при подтверждении результатами исследований реальной клинической практики), с одной стороны, а также в разработке и оперативном внедрении инновационных подходов к терапии ХСН – с другой. Больше всего в этом нуждаются пациенты с ХСН с умеренно сниженной и сохранной фракцией выброса левого желудочка, бедная доказательная база возможности улучшения прогноза у которых не может обосновывать бездействие и оставление их без надежды хотя бы на клиническое улучшение состояния. В лекции последовательно рассмотрены общие принципы лечения ХСН, комплекс мероприятий, направленный на инотропную стимуляцию и разгрузку (нейрогормональную, объемную, гемодинамическую и иммунную) сердца, а также обозначены некоторые перспективные направления болезнь-модифицирующей терапии.

Ключевые слова: хроническая сердечная недостаточность, лечение, нейрогормональные модуляторы, сакубитрил/валсартан, пекаваптан, финерон, верицигуат, ингибиторы натрий-глюкозного котранспортера 2-го типа, омекамтив мекарбил, генная терапия, сердечная ресинхронизирующая терапия, модуляция сердечной сократимости, трансплантация сердца, имплантация аппарата вспомогательного кровообращения

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

Due to the development of clinical pharmacology, cardiac surgery, and implantation arrhythmology, we have seen significant advances in the treatment of patients with chronic heart failure (CHF) in the last decades. However, the long-term results of the so-called optimal medical therapy are often still disappointing [1–4]. The prognosis in patients with CHF is one of the worst, though this fact is often poorly understood by practitioners [5–10].

On the one hand, it becomes clear that there is a high need for fundamental and applied research aimed at improving existing disease-modifying approaches to the treatment of patients with CHF. On the other hand, this research should be also aimed at finding new breakthrough directions for the pharmacological and non-pharmacological treatment of heart dysfunction.

The aim of this paper was to discuss promising treatment options for patients with CHF.

GENERAL PRINCIPLES OF TREATMENT FOR HEART FAILURE

Therapeutic approaches to treating CHF are numerous and include general interventions, pharmacotherapy, electrophysiology therapies, surgery, and mechanical circulatory support. Naturally, in each specific case, these methods are combined differently [11, 12]. Achieving sustainable treatment effect is hindered by unreasonably ignoring any of these treatments (for example, non-pharmacological options) [12].

Etiotropic and pathogen-specific therapy can improve the quality of life and life expectancy of patients with CHF. A personalized approach to treatment primarily makes it necessary to take into account the etiological heterogeneity of the group of patients with CHF [13–15]. Since the conditions that are complicated by the development of HF differ in their pathogenesis, it is difficult to create universal therapy algorithms [11, 14]. Nevertheless, it is obvious that properly selected treatment of the disease underlying CHF in many cases can significantly reduce the severity of manifestations of cardiac decompensation and sometimes allows to eliminate them completely (for example, after successful surgical correction of heart disease) [1, 16]. First of all, we refer to treatment of ischemia and acute myocardial infarction, prevention of recurrent heart attacks, careful identification and active treatment of people with arterial hypertension, diabetes mellitus, obesity and dyslipidemia, elimination of the causes of specific myocardial damage, and timely correction of valvular pathology and heart defects [1, 11].

In a clinical situation, when it is temporarily impossible to eliminate the cause of the disease (for example, if due to severe circulatory failure, radical treatment of the underlying disease is not feasible), pathogen-specific and symptomatic therapy should be aimed at reducing the clinical manifestations of CHF and creating conditions that would allow physicians to reconsider etiotropic treatment [16].

All modern methods of treating CHF aimed at improving the prognosis can be divided into several main groups with a specific target [4, 17]: 1) blockade of cardiomyocyte death (necrosis and apoptosis) and loss of cell organelles (autophagy); 2) improvement of lusitropic and inotropic functions of the heart (increased cardiac output, cardiac resynchronization, and cardiac contractility modulation); 3) decreased severity of pathological cardiac remodeling (chamber dilatation and spherification, increase in myocardial mass); 4) preservation of and increase in the number of actively contracting cardiomyocytes (when cardiomyocytes are no longer in the hibernation state; myocardial stunning or generation of new cardiomyocytes). The discreteness of these targets is rather conditional, since in many cases the use of specific modern methods for treating CHF (for example, angiotensin-converting enzyme (ACE) inhibitors) provides a complex of sanogenic effects that go beyond one goal.

Since heart failure is typically an aging-associated disease, it is often associated with other diseases and syndromes, such as diabetes mellitus, obesity, anemia, kidney failure, chronic obstructive pulmonary disease, sleep disorders, depression, and hyperkalemia, which increase the likelihood of a negative outcome and should be scrupulously recorded in the diagnostic report [18–21]. Adaptation of existing and new regimens for CHF treatment aimed at solving these problems simultaneously can theoretically improve the survival rate of patients with comorbid pathology, especially those with preserved left ventricular ejection fraction (LVEF) [1, 22–24].

NEUROHORMONAL MODULATORS

The modern concept of medical treatment of patients with CHF can be narrowed down to two main principles: inotropic stimulation and unloading

(volumetric, hemodynamic, neurohormonal, and immune) of the heart [16, 25]. As inotropic stimulants did not prove to be very effective, at the end of the last century, the dominant role of contractile myocardial insufficiency in HF mechanisms (especially at an early stage) was revised as part of the evolution of the HF pathogenesis paradigm [26–29]. In the early 2000s, undoubtedly, the greatest increase in the survival rate of patients with CHF was provided by neurohumoral unloading of the cardiovascular system, and the use of ACE inhibitors (or angiotensin II receptor antagonists), beta-blockers, and mineralocorticoid receptor antagonists was considered reasonable [30, 31]. However, even with the combined use (the so-called triple neurohormonal blockade), the drugs of these groups did not become a "panacea" in the treatment of CHF, and known possibilities of neurohormonal modulators (risk of death is reduced by 23–35%) made researchers search for fundamentally new targets for drug exposure to affect the functional state of neuroendocrine systems activated in this syndrome at the circulatory and, more importantly, at the tissue level [17, 22, 30, 32, 33].

Unfortunately, testing the hypothesis that the addition of new selective blockers of the neurohormonal system may still bring additional benefits has resulted in obtaining unsatisfying results when studying the effectiveness of endopeptidase inhibitors (omapatrilat), renin inhibitors (aliskiren) or endothelin receptor antagonists (bosentan, darusentan) [30]. Well-known cardiologists did not see much sense in creating new effective neurohormonal inhibitors for the treatment of CHF due to the fact that in reality it was quite difficult to achieve a complete neurohormonal blockade (largely due to the effect of neurohormonal escape), which is not physiological, since endocrine, paracrine, and autocrine regulatory effects of hormones in the HF development should not be considered as purely pathological [17, 34].

When pharmacology seemed to have stopped creating new effective neurohormonal modulators for the prevention and treatment of HF with reduced LVEF, in the search for preferred directions for the development of pharmaceutical substances, the researchers began to focus on balanced modulation with simultaneous stimulation of the activity of "beneficial" hormonal regulatory axes rather than on isolated fight against the so-called "bad" neuroendocrine responses [22, 34, 35]. This concept was proven to be successful in clinical studies, in which the neprilysin inhibitor sacubitril as part of a single crystalline supramolecular complex, containing six molecules of sacubitril and six molecules of valsartan [36], was more efficient than the "pure" renin – angiotensin – aldosterone system (enalapril) in terms of improving the prognosis and quality of life in patients with CHF [17, 37–40]. Restoration of the normal balance of neurohormones with different directions of action was achieved by inhibiting the degradation of vasoactive peptides (natriuretic peptide, bradykinin, substance P, and calcitonin gene-related peptide), which provided sanogenic effects (vasodilation, increased level of diuresis / natriuresis, and slower pathological remodeling of cardiomyocytes and cardiac extracellular matrix).

The latest European Society of Cardiology Guidelines for the diagnosis and treatment of acute and chronic HF emphasized the need for timely triple therapy, including a beta-blocker, a mineralocorticoid receptor antagonist, and a neurohumoral modulator sacubitril / valsartan, which prolongs life in patients with CHF with reduced LVEF [1]. However, J. Lindenfeld and M. Jessup were right to quote C.E. Koop saying that "drugs do not work in patients who do not take them" [41], which also contributes to the common practice when doctors do not actively prescribe such therapy, which altogether increases the patient's risk of death by 2–3 times [42, 43]. Unsatisfactory adherence of internists to modern principles of CHF therapy, the effectiveness of which was proven in large-scale clinical trials, requires great educational efforts [11]. Physicians should understand that despite the known limited possibilities of neurohormonal modulators, the best practical way to increase the effectiveness (increase life expectancy) of the treatment for both decompensated and stable patients with CHF and reduced LVEF is to prescribe this therapy to a larger number of patients [44–47]. In other words, while waiting for a miracle cure, practicing physicians should not expose CHF patients to an unacceptably high risk of death by not taking the needed measures. The use of all relevant possibilities of continuously improving neurohumoral modulation should become a fundamental rule for them.

Moreover, the results of a sub-analysis of the PARAGON-HF study and a meta-analysis of the PARAGON-HF and PARADIGM-HF studies, which demonstrated the potential for disease-modi-

fying activity of sacubitril / valsartan and a decrease in the number of hospitalizations due to HF decompensation, which could not be attributed to CHF with reduced LVEF, make it possible to discuss the advisability of using a combination of these drugs, regardless of the value for this LV contractile function parameter [1, 48, 49]. In this regard, there is a reason to expect the evolution of the evidence base [50–52].

The discussed direction of pathogen-specific CHF therapy is still to be developed further. In particular, the known role of the hypothalamic peptide hormone vasopressin (antidiuretic hormone) accumulating in the posterior lobe of the pituitary gland (neurohypophysis) in the mechanisms of fluid retention and the role of cardiac and vascular remodeling and dysfunction in patients with CHF allow to consider the latter as a target for pharmacological intervention [53, 54]. The so-called aquaretics (vaptans) are successfully used in patients with CHF associated with severe hypervolemic hyponatremia [1]. Since therapy with tolvaptan, which is the most studied selective, competitive V2 receptor antagonist, does not affect the prognosis of a patient with CHF, the current focus is placed on the use of dual vasopressin receptor antagonists, which have a potential advantage due to simultaneous blockade of vasopressin V1a receptors [55, 56]. In particular, several experimental and clinical studies are currently underway focused on the analysis of the efficacy and safety of the non-selective V1a / V2 receptor antagonist pecavaptan [57, 58].

Sixty years after H. Selye's publications, which emphasized the importance of aldosterone in the mechanisms of heart and kidney fibrosis, several pharmaceutical companies, inspired by the success of using its steroid antagonists (spironolactone, eplerenone) for the treatment of CHF, began to search for new non-steroidal mineralocorticoid receptor antagonists with certain pharmacokinetic and pharmacodynamic properties that can make them more beneficial than first- and second-generation drugs [59]. Several substances of third-generation mineralocorticoid receptor antagonists are under development, but fineron seems to meet the search criteria to the greatest extent. The first results of its clinical use indicated that its advantages over classical first- and second-generation drugs are not only theoretical, but also practical [60–64].

In patients with acute decompensated heart failure whose systemic blood pressure is normal or elevated, hormonal vasodilators can be added to overcome refractoriness to diuretics, of which the use of serelaxin (a recombinant analogue of human relaxin-2) and low doses of nesiritide (recombinant human brain natriuretic peptide) is the most promising [65, 66].

Finally, it is possible to modulate in the required direction (increase or decrease) the biological effects of a number of hormones and neurotransmitters by influencing their second messengers. For example, sanogenic effects of the stimulator of the soluble guanylate cyclase vericiguat receptor (vasodilation and a decrease in the severity of coronary microvascular dysfunction, slowdown in the development of fibrosis and myocardial hypertrophy, an increase in the speed and degree of cardiomyocyte relaxation in diastole, an improvement in the ventricular – arterial coupling, and an increase in cardiac reserve) improve the prognosis (reduced risk of death caused by cardiovascular diseases) and reduce the need for hospitalization due to decompensation in patients with HF with reduced LVEF if used on a long-term basis [25, 67, 68].

The latest European Society of Cardiology Guidelines for the diagnosis and treatment of acute and chronic HF indicate that vericiguat may be considered in CHF patients with functional class II–IV reduced LVEF with aggravating heart failure despite combination therapy with an ACE inhibitor, beta-blocker, and mineralocorticoid receptor antagonist [1]. Another soluble guanylate cyclase stimulator, riociguat, is currently recommended (also in combination with endothelin receptor antagonists or prostanoids) for the correction of pulmonary arterial hypertension and portopulmonary hypertension [69].

SODIUM-GLUCOSE COTRANSPORTER TYPE 2 INHIBITORS

As for the search for new directions in the treatment of CHF, the most successful option involved the use of hypoglycemic drugs from the group of the sodium-glucose cotransporter type 2 (SGLT2) inhibitors. Convincing evidence of the effectiveness of the so-called gliflozins allowed the experts of the European Society of Cardiology to designate in 2021 two selective SGLT2 inhibitors (dapagliflozin and empagliflozin) as the 4th component of optimal

first-line CHF therapy, including the neurohormonal modulators discussed above [1, 70]. At the same time, the diuretic (osmotic diuresis) and slight natriuretic effects of the considered SGLT2 are not associated with blood glucose level, and their administration to patients with functional class II–IV CHF and reduced LVEF decreases cardiovascular mortality and the need for hospitalization due to decompensated heart failure, regardless of the presence and severity of carbohydrate metabolism disorders [70, 71].

The positive additive effect of these drugs on the survival of CHF patients with reduced LVEF already receiving optimal therapy based on a triple neurohormonal blockade (sacubitril / valsartan or an ACE inhibitor, a beta-blocker, a mineralocorticoid receptor antagonist) should encourage practitioners to transfer the achievements of clinical research into practice more quickly [47]. At the same time, it would appear that accumulating data on the high efficacy of the discussed gliflozins in a wide range of LVEF values will make it possible in the near future to justify the addition of indications for their prescription in cases of CHF with mildly reduced and preserved LVEF [52, 72–74].

It is supposed that SGLT2 has a so-called class effect, and we can expect addition to the line of drugs in this group of drugs which are intended for the treatment of CHF due to other selective cotransporter type 2 inhibitors (for example, canagliflozin and ertugliflozin) and non-selective cotransporter type 1 and 2 inhibitors (for example, sotagliflozin) [75–79].

Perhaps, other hypoglycemic agents can be as effective as SGLT2. Long-acting analogues of glucagon-like peptide-1 were the most likely to prove their efficacy in treating heart failure [80–82].

INOTROPIC DRUGS

Until the end of the 20th century, cardiac glycosides were mostly used for the treatment of CHF, currently they are only used as adjuvants (digoxin and, possibly, digitoxin) that do not affect the disease prognosis, but improve symptoms in certain clinical situations. In practice, there are only two such clinical situations. The first one is overt heart failure associated with atrial fibrillation and a high heart rate, when other therapeutic approaches (e.g., pulmonary vein isolation or effective doses of beta-blockers for arterial hypotension) cannot be applied [1]. We should also keep in mind that in sinus rhythm with a resting heart rate of 70 bpm or more, when, despite combination therapy with optimal doses of first-line drugs, tachysystole persists, it is recommended to add the selective sinus node If channel inhibitor ivabradine for patients with symptomatic heart failure and the LVEF value of 35% or less, in order to reduce the risk of hospitalization and death from cardiovascular causes [1, 83]. The second case includes symptomatic CHF with reduced LVEF in patients with sinus rhythm, when symptoms persist despite treatment with an ACE inhibitor (or sacubitril / valsartan), a beta-blocker, and a mineralocorticoid receptor antagonist [1, 55].

It should be noted that the main mechanism of digoxin at low doses recommended for clinical practice is associated not with the inotropic effect of the drug, but with its neuromodulatory activity. We are talking about weakening of sympathetic nervous activity and a decrease in renin secretion, associated with inhibition of K^+-Na^+-dependent ATPase in the afferent fibers of the vagus nerve and renal tubules, respectively [12, 55].

Short-term use of non-glycoside inotropic stimulating agents is limited by the clinical situation with reduced cardiac output and hemodynamic instability in progressive and acute heart failure [1, 4, 55]. Vasoconstrictors (e.g., norepinephrine, midodrine, and vasopressin), inotropes with vasoconstrictor properties (e.g., dopamine, epinephrine, and droxidopa), cardiotonic agents (e.g., dobutamine, milrinone), and inodilators, among which, according to some experts, levosimendan is the most promising one and can be used in the absence of a pronounced decrease in systolic blood pressure – > 85 mm Hg [1, 84, 85]. However, despite a short-term improvement in hemodynamics and clinical status in patients with decompensated heart failure, long-term therapy with non-glycoside inotropic drugs may be associated with an increased risk of death [12, 86].

The use of a representative of a new class of myotropic compounds, omecamtiv mecarbil, which is a selective activator of cardiac myosin, may become a treatment option for patients with severe heart failure associated with low cardiac output and hemodynamic instability, as modern pharmacotherapy options are limited for such patients [87, 88]. Post hoc analysis of the results of the randomized clinical

trial GALACTIC-HF, which took into account an episode of CHF decompensation or cardiovascular death as the primary endpoint, demonstrated a positive effect of omecamtiv mecarbil on the prognosis of patients with severe functional class III and IV heart failure and reduced LVEF (< 30%) [88].

Another relatively new direction of inotropic support is the modulation of the sarcoplasmic reticulum Ca2+-ATPase 2a (SERCA2a), the expression and activity of which is reduced in CHF, which leads to disruption of intracellular calcium movement between the cytosol and the lumen of the sarcoplasmic reticulum and negatively affects the mechanics of systole and diastole. Modulation of SERCA2a in patients with CHF can be achieved through gene therapy (intracoronary or endomyocardial administration of viral and plasmid vectors encoding SERCA2a or other proteins of the Ca^{2+}-modulating protein family) [89, 90].

Since the excitation – contraction coupling in myocardium in CHF is impaired at various levels (receptors, ion channels, and transporters, phosphorylation of proteins that modify the function of the cardiomyocyte, etc.), a wide variety of targets can be chosen for the targeted correction of maladaptation shifts: from enzymes to structural proteins and cytoprotective factors. These approaches are especially important when treating patients suffering from hereditary diseases with heart damage. Despite the controversial results that followed attempts to transfer the promising results of laboratory studies on the modification of genetic programs and additional translational mechanisms into practice, this direction of research is still of interest, but much remains to be done to prove the perfection of delivery and intracellular transfer systems, as well as the effectiveness and safety of the discussed approaches to therapy for CHF before gene therapy and postgenomic medicine are included in standard treatment protocols [90–94].

ELECTROPHYSIOLOGY THERAPIES

In addition to optimal medical therapy in selective groups of patients with CHF with reduced LVEF, for more than 20 years electrophysiology treatment has been successfully used, namely implantation of conventional pacemakers (relevant for patients with sick sinus syndrome and high-grade atrioventricular block), cardiac resynchronization therapy (effectiveness of triple chamber pacing has been proven in patients with severe systolic dysfunction and wide QRS) and implantation of a cardioverter – defibrillator (used for primary and secondary prevention of life-threatening cardiac arrhythmias). The last two methods that can be combined (a pacemaker with a defibrillator function) in one patient are recommended in all modern guidelines for treatment of CHF with detailed indications, which depend on the duration of the QRS, the reduction of the LVEF value, the CHF functional class, the main heart rhythm (sinus or atrial fibrillation), the risk of fatal arrhythmia, the etiology of heart failure, the presence and severity of comorbidity, age, and life expectancy [1, 12, 95, 96].

Electrophysiology therapies are continuously being improved, and currently the effectiveness and safety of cardiac contractility modulation devices implanted in patients with symptomatic CHF with reduced LVEF, who cannot undergo cardiac resynchronization therapy (with narrow QRS) or who have not received sufficient clinical effect from this therapy, are still actively studied. Cardiac contractility modulation is based on two-electrode stimulation of the interventricular septum with a biphasic high-voltage signal in the absolute refractory period, and its use provides an increase in heart contractility due to positive changes in intracellular calcium homeostasis (increased expression of SERCA2a or other proteins of the Ca2+-modulating protein family) without increasing myocardial oxygen consumption and also improves the functional state and quality of life and, possibly, prevents hospitalization of these patients [1, 97].

So far, there are still insufficient data regarding the evaluation of the efficacy and safety of other implantable electrotherapy devices in patients with CHF, in particular, of those aimed at modifying the autonomic nervous system activity (for example, baroreflex activation therapy), in order to decide on the possibility of their use in clinical practice [1, 98, 99].

TREATMENT OF ADVANCED HEART FAILURE

Patients with symptoms of CHF corresponding to functional classes III and IV that persist despite optimal medical therapy and cardiac resynchronization therapy (if indicated) and are associated with objective signs of severe cardiac dysfunction, such

as severe systolic and (or) diastolic LV dysfunction, elevated ventricular filling pressure, and increased plasma natriuretic peptide levels, require timely referral to a specialized center, where advanced methods of treating heart failure are used, which are not available in a clinic [1, 100–102].

If other methods of dehydration are ineffective in these patients, extracorporeal ultrafiltration (sparing modes are preferable with a minimum volume of extracorporeal blood and an ultrafiltration rate of no more than 250 ml / h), and peritoneal dialysis can be used [12, 102–104].

Considering that a well-founded conclusion about advanced heart failure leaves little hope for the success of pharmacotherapy, the only options for patients include surgical treatment, heart transplantation, or implantation of a circulatory assist device [102, 105, 106].

Conventional surgical treatment is aimed at correcting the etiological factors, as well as the main mechanisms underlying CHF. For instance, this treatment would include revascularization of ischemic but viable myocardium in patients with LVEF value not exceeding 35%, aortic valve replacement (transcatheter implantation is preferable when perioperative risk is high) in severe symptomatic aortic valve stenosis with an average pressure gradient above 40 mm Hg or in severe aortic regurgitation in all symptomatic and asymptomatic patients with LVEF of less than or equal to 50%, as well as surgery to correct mitral regurgitation (endovascular mitral valve clip placement theoretically seems more reasonable in a situation of high perioperative risk), also in secondary (due to LV dilatation) severe mitral regurgitation (especially in patients with LVEF less than 30%), which cannot be corrected with pharmacotherapy and electrophysiology treatment [102, 105, 107, 108].

Despite the lack of well-designed controlled studies, it is widely believed in the cardiology community that heart transplantation in end-stage CHF significantly improves survival (one-year survival rate of about 90%, median survival rate of 12.2 years), physical performance, and quality of life compared with conventional treatment, provided that appropriate selection criteria are carefully observed (the mainstay for the treatment of refractory CHF) [102, 109].

Long-term mechanical circulatory support is increasingly being considered as an alternative to heart transplantation in patients with end-stage CHF, in whom transplantation is not feasible for objective or subjective reasons [1, 102, 110, 111].

OTHER PROMISING DIRECTIONS OF TREATMENT FOR PATIENTS WITH CHF

A list of some promising approaches to the treatment of patients with CHF which are currently being developed or have already proven their effectiveness but need wider application in clinical practice is presented in the table.

CONCLUSION

Unprecedented advances in secondary prevention significantly improved the prognosis in patients with CHF, but, unfortunately, heart failure is still associated with high mortality. Sustainable progress in solving this problem is seen in the fullest possible application of all relevant continuously improving methods of treating heart failure in clinical practice, which have proven their effectiveness in randomized controlled trials (especially when confirmed by the results of studying real clinical practice), as well as in development and rapid implementation of innovative approaches to the treatment of CHF. CHF patients with mildly reduced and preserved LVEF need this most of all, the poor evidence base for the possibility of improving their prognosis cannot justify a lack of measures taken by physicians and leaving patients without a hope for even a clinical improvement [174].

Table

Some promising approaches to the treatment of patients with CHF

Treatment approaches	Note
Correction of iron deficiency (serum ferritin concentration of less than 100 mg / l or in the range from 100 to 300 mg / l in combination with the iron transferrin saturation coefficient of less than 20%), which is detected in approximately every second patient with CHF [112]	Anemia, which is iron-deficiency in 75% of patients with CHF, is an independent factor of poor prognosis for any etiology of heart failure and any value of LVEF [12, 113, 114]. Iron deficiency should be considered as an independent clinically significant concomitant condition and, therefore, in order to reduce the need for hospitalization, reduce the clinical severity of CHF, as well as improve the functional capabilities and quality of life of patients, it is advisable to correct even latent iron deficiency [1, 113].

Table (continued)

	The results of the Cochrane Review confirm that the effectiveness of iron salt preparations (mainly divalent) and Fe^{3+} preparations based on hydroxide polymaltose complex in the treatment of iron-deficiency anemia is the same in the general population, with a better tolerability profile in the latter [115, 116]. Nevertheless, there is a widespread opinion among cardiologists that oral iron preparations are ineffective in the treatment of patients with CHF, and in most modern guidelines for iron deficiency, only intravenous administration of iron carboxymaltose is recommended [1, 113, 117]. It should be noted that the ferinject annotation states that it should be used in hospital departments with the necessary equipment to provide emergency medical care in case of anaphylactic reactions. Erythropoietin should not be used in the treatment of patients with CHF even with a reduced hemoglobin level [1, 113].
Stimulation of the potential for cardiac muscle regeneration	In advanced scientific centers, material is being accumulated in four main areas [118–128]: – Some researchers are investigating the efficacy and safety of various methods of transplantation (endovascular, transthoracic or during heart surgery) of own or autologous stem cells and myoblasts isolated from skeletal muscle, as well as other cells (including genetically modified ones). Researchers encounter pending problems related to the need to prepare sufficient material for transplantation, develop methods for cell preconditioning, their targeted administration, survival / rejection of transplanted cells, and their commitment towards cardiomyogenesis. – Other researchers are developing a technique for stimulating the production and release of own stem cells into the bloodstream from the bone marrow (for example, using granulocyte and granulocyte – macrophage colony-stimulating factors), which does not require surgery or complex invasive intervention and can be a good alternative to cell transplantation. – Still others are exploring the possibility of direct reprogramming of cardiac fibroblasts, allowing the transformation of terminally differentiated cells into cardiomyocytes. At the same time, the search is underway for optimal cell reprogramming factors (transcription factors, such as GATA4, MEF2c, and TBX5, which are usually combined in different ratios, cytokines, microRNAs, and other epigenetic modifiers), and delivery systems are being improved. – Finally, there are researchers convinced that heart regeneration can be achieved by re-activating the proliferation of own cardiomyocytes (no more than 5% of cells express proliferative activity marker Ki-67) and try to stimulate the potential of cardiac muscle regeneration using, for example, acellular biomaterials. The results of these experimental and clinical studies have not yet allowed to revise clinical guidelines for the treatment of CHF [129, 130].
Myocardial cytoprotection	Despite a rather impressive list of drugs that can be attributed to the so-called myocardial cytoprotectors, from the standpoint of evidence-based medical practice, only the use of long-acting trimetazidine is justified for the treatment of patients with ischemic CHF, in whom it demonstrates high antianginal and anti-ischemic efficacy and provides increased tolerance to physical activity, positive dynamics of indicators characterizing LV remodeling and its functional state, as well as a decrease in the risk of death and repeated hospitalizations [1, 12, 131, 132]. The development and clinical trials on myocardial cytoprotectors continue; myocardial cytoprotectors under study are potentially effective in CHF and are aimed at inhibiting fatty acid oxidation, stimulating glucose oxidation, activating the cytochrome chain, optimizing the transport of an energy substrate into mitochondria, and increasing the antioxidant potential of cardiomyocytes [4, 133–143].
Correction of hyperkalemia classified as mild (5.0–5.4 mmol / l), moderate (5.5–6.0 mmol / l), and severe (> 6.0 mmol / l).	Associated with an increased risk of adverse (including fatal) outcomes, hyperkalemia is becoming more common in patients with CHF, partly due to an increase in the incidence of comorbidities, but, apparently, to a greater extent due to the widespread use of combination therapy with neurohumoral modulators [144]. The prescription of potassium sequestrants (for example, patiromer or sodium zirconium cyclosilicate) increases the safety of such therapy and the likelihood of achieving target doses of renin – angiotensin – aldosterone system inhibitors [1, 96, 145–147]. Whether the correction of hyperkalemia with these drugs will improve clinical outcomes in patients with CHF is still to be determined [96].
Interventions aimed at slowing down the cardiac extracellular matrix remodeling	All neurohormonal modulators, which have become the core of CHF therapy, have antifibrotic activity to a greater or lesser extent. In recent years, the possibility of potentiating this effect by targeting the key mechanisms of pathological collagen accumulation and changing its composition in the interstitium has been studied; the search is underway for possible therapeutic targets (galectin-3, matrix metalloproteinases, metallopeptidase inhibitor 1, growth differentiation factor-15, osteopontin, etc.) [148–157].
Correction of proinflammatory status	It is a well-proven fact that the pathology of the immune system is essentially important in the mechanisms of CHF [158–161]. Toll-like receptors, inflammasomes (including NOD-like receptors), cytokines, and apoptotic and pyroptotic effector mechanisms are most often considered as promising targets of therapy aimed at reducing the severity of proinflammatory shifts at the systemic and local levels, as well as the readiness of cardiomyocytes to implement apoptosis, pyroptosis, and autophagy programs [162–172]. The use of nonsteroidal anti-inflammatory drugs can reduce the effectiveness of the main drugs used to treat CHF, provoke the development of acute decompensation, and increase the risk of thrombotic events (especially selective cyclooxygenase blockers) [1, 173].

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The role of cathepsin S in the pathophysiology of bronchial asthma

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ABSTRACT

To date, the study of the role of proteases in the pathogenesis of various diseases remains relevant. The variety of cathepsin functions is associated with the peculiarities of their localization, expression, and regulation, due to which cathepsins are involved in development of many pathologies. Dysregulation of proteases, their inhibitors, and substrates can lead to the development of multiple organ dysfunction.

The review presents data on the characteristics of the entire family of cathepsins and cathepsin S, in particular. The pathophysiological role of cathepsin S in the formation of bronchopulmonary pathologies, as well as in bronchial asthma is described, and intra- and extracellular implementation mechanisms are considered. The authors believe it is this enzyme that could be targeted in targeted asthma therapy to prevent airway wall remodeling at the earliest stages of the disease. The literature search was carried out in the search engines Medline, eLibrary, Scopus, the Cochrane Library, and RSCI.

Keywords: cathepsin S, bronchial asthma, pathophysiology, proteases, airway remodeling

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Роль катепсина S в патофизиологии бронхиальной астмы

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РЕЗЮМЕ

До настоящего времени сохраняет свою актуальность изучение роли ферментов – протеаз в патогенезе различных заболеваний. Многообразие функций катепсинов обусловлено особенностями их локализации,

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экспрессии и регуляции, благодаря чему они принимают участие в развитии многих патологических процессов. Дисрегуляция активности протеаз, их ингибиторов и субстратов может привести к развитию полиорганных заболеваний.

В обзорной статье представлены данные о характеристике всего семейства катепсинов и катепсина S в частности; описаны его патофизиологические роли при формировании бронхолегочных патологий, а также при бронхиальной астме; освещены внутри- и внеклеточные механизмы реализации. Авторы считают, именно этот фермент может стать мишенью для таргетной терапии астмы с целью предотвращения ремоделирования бронхиальной стенки на самых ранних этапах заболевания. Поиск литературы осуществлялся в поисковых системах Medline, eLibrary, Scopus, The Cochrane Library, РИНЦ.

Ключевые слова: катепсин S, бронхиальная астма, патофизиология, протеазы, ремоделирование дыхательных путей

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

Cathepsins are found in lysosomes of different cell types, including endothelial cells, vascular smooth muscle cells, and macrophages [1]. These enzymes are secreted as inactive forms (zymogenes) maturating due to a cascade of pathological chemical reactions. Their proteolytic activity partially depends on the balance between proteases and the endogenous inhibitor cystatin C [2]. Over the past two decades, scientists have revealed that it is cathepsins K, L, and S that are highly potent elastases in the cathepsin family [3]. Cathepsin S is able to destroy different components of the basement membrane. Apart from this, it has been demonstrated that it is cathepsin S that is involved in atherosclerosis, angiogenesis, inflammation, rheumatoid arthritis, chronic obstructive pulmonary disease, and bronchial asthma [4]. It is important to note that activity of proteases requires strict regulation, since disturbances in the close interaction between proteases, substrates, and inhibitors may promote progression of different pathologies: both multiple organ dysfunctions and disorders impairing a specific organ [5, 6].

GENERAL CHARACTERISTIC OF CATHEPSINS

International research performed over the past 60 years has demonstrated that proteases make a decisive contribution to the pathophysiology of pulmonary diseases. Initially, these molecules were known as enzymes cleaving the protein with a limited range of substrates [7]. However, modern data have shown that the variety of protease substrates and biological effects induced by their processing is enormous [8, 9].

The cathepsin molecules are a group of lysosomal enzymes, the proteolytic activity of which may manifest both in the intra- and extracellular space. All cathepsins fall into three protease families: serine proteases (A, G), aspartic proteases (D, E), and cysteine proteases (B, C, F, H, K, L, O, S, V, X, and W), amounting to 31%, 25%, and 4% from the total number of cathepsins, respectively [10]. The gene of cathepsin S was found in human chromosome 1q21, and, as all lysosomal cathepsins, it is translated into a prezymogen before transition to the mature and active state [11, 12]. These enzymes are participants of such physiological processes as food digestion, blood clotting, and bone resorption. They are also directly associated with pathogenesis of diseases of almost all organs and systems in the body [8, 12]. The diversity of functions and properties of cathepsins is explained by the specifics of their localization, expression, and regulation.

The ability to irreversibly cleave peptide bonds requires strict regulation of activity of these enzymes. All cathepsins manifest the highest activity in an acid environment particularly characteristic of lysosomes [8, 10]. Moreover, it is known that in-

flammation as a non-specific physiological process is accompanied by development of acidosis, which may lead to an increase in protease activity in the extracellular space. Apart from that, the ability of certain cathepsins to retain proteolytic activity in a neutral environment broadens the spectrum of their activity. Thus, it is known that cathepsins K and H retain activity at pH = 7.4, and the optimal pH for cathepsin S is 6.5 [10, 12]. The regulation of cathepsin synthesis may be carried out at the transcriptional, translational, posttranslational, and epigenetic levels [10]. In particular, methylation of CpG islands, as an example of epigenetic regulation, is typical of cysteine cathepsins [10].

It is important to note that the activity of proteases is strictly regulated by the tissue cytokine profile [5]. The release of active molecules of cathepsin S takes place under the influence of many regulating factors, including such proinflammatory molecules as interleukin (IL)-1β, IL-4, IL-13, and tumor necrosis factor (TNF) α [12]. Disturbances in the close interaction of the studied enzymes with their substrates and inhibitors may promote activation of pathological cascades and progression of different pulmonary diseases, including such mucosal inflammatory diseases as cystic fibrosis, chronic obstructive pulmonary disease (COPD), and idiopathic pulmonary fibrosis (IPF), as well as secondary bacterial infections [13].

It is an important observation that some proteases have limited expression in the body, which defines the specificity of their functions. For example, cathepsin K is specifically localized in osteoclasts, while cathepsins E and S are localized in immune cells [14].

In order to understand the disease pathogenesis, it is also important to know which protein is the substrate in the specific situation. Depending on the cathepsin localization, it is possible to suggest the presence of one substrate or another. Cathepsins are known to possess high collagenolytic and elastolytic activity that plays a special role in tissue remodeling [15].

SPECIFICS OF CATHEPSIN S, ITS ROLE IN BRONCHOPULMONARY PATHOLOGY

In the human body, a large volume of cathepsin S is localized in smooth muscle cells, macrophages, and dendritic cells, which allows for local degradation of the basement membrane and elastic layer in the bronchial and vascular wall. The launch of a cascade of lysosomal pathophysiological reactions activates wall remodeling in small bronchi and progression of atherosclerotic changes in the intima. Activation of the vascular and bronchial endothelium results in deterioration of the comorbid patient's condition, which makes it relatively difficult to determine the primary pathology leading to clinical exacerbation [16].

Studies register a higher level of cathepsin S and its activity in the bronchoalveolar lavage fluid of COPD patients compared with healthy individuals [17]. Apart from that, cathepsin S is a potent elastin-destructing proteinase, which participates in adaptive immune responses. Analysis of COPD pathogenesis in mouse models showed that cathepsin S contributes to damage to pulmonary interstitium and development of pulmonary hyperinflation through destruction of elastic fibers in the lung tissue [18, 19].

Cathepsin S is especially relevant in the context of pulmonary pathology, since its ability to potentiate elastase activity and inactivate protective proteins in the airways induces extracellular matrix remodeling and impairs mucus secretion in a wide pH range. Respiratory acidosis or alkalosis, characterised by alteration of partial pressure of CO_2 in arterial blood due to the change in alveolar ventilation, and, as a result, insufficient removal of CO_2 from the blood are often revealed in such diseases as pneumonia, bronchial asthma, COPD, and adult respiratory distress syndrome [20].

In diseases with high neutrophil counts, registered in patients with bronchopulmonary pathology, a frequent imbalance between proteases and their inhibitors (neutrophil elastase, α_1-antitrypsin, secretory leucoprotease inhibitor, and elafin) is found [6, 21, 22]. The reactions emerging during the antiprotease overload lead to chronic inflammation in the airways determined by mucociliary clearance dysfunction, extracellular matrix remodeling activation, and a decrease in the susceptibility threshold to secondary bacterial infection [23].

INTRACELLULAR FEATURES IN MECHANISMS OF CATHEPSIN S ACTION

Cathepsin S plays an important role in various intracellular processes, including proteolysis and

formation of the immune response mediated by the major histocompatibility complex class II (MHC II) [24]. Biochemically, cathepsin S also differs from many members of the cathepsin family in its ability to retain activity in neutral pH [25].

Upon delivery of the antigen to the endolysosomal pathway, the invariant chain (Ii) of the MHC II is cleaved with formation of a fragment of class II-associated invariant chain peptide (CLIP), which allows for subsequent binding of the exogenous antigen. The proteolytic cleavage of Ii is catalyzed by active cathepsin S and other proteases. The CLIP fragment is then cleaved, moving to the plasma membrane of the antigen-presenting cell to activate CD4+T lymphocytes. Cathepsin S-mediated cleavage of Ii is of key significance not only for the presentation but also for the activation of mobility of dendritic cells [26].

CATHEPSIN S IN THE CONTEXT OF BRONCHIAL ASTHMA PATHOGENESIS

Extracellular cathepsins directly participate in activation of extracellular matrix remodeling through degradation of structural components of the latter: collagen and elastin [27]. The representative of the protease family under study is also noted to possess elastolytic and collagenolytic properties, which makes it possible to consider its elevated expression as a predictor of pulmonary dysfunction development [28, 29].

Studies have shown that patients with bronchial asthma (BA) undergoing therapy with systemic glucocorticoids (GCs) have lower serum level of cathepsin S [30]. Polymorphisms in the molecular structure of the enzyme may define susceptibility of patients to BA development and severity of its progression [31].

The results obtained using animal models have also shown the association between the expression of the protein and allergic BA pathogenesis, as well as atopy in general. High levels of cathepsin S are registered in modeling of eosinophilic inflammation in the airways. The knockout of cathepsin S or preventive introduction of its inhibitor leads to a decrease in the bronchial wall inflammation and limitation of eosinophilia in the bronchoalveolar lavage fluid [32].

Elevation of cathepsin S level leads to skin itching and atopic dermatitis in mice due to binding of protease-activated receptors, such as PAR-2 and PAR-4. Activation of PAR-2-induced maturation of dendritic cells and subsequent differentiation of CD4+T-cells lead to an increase in skin inflammation and chronic scratching of the defects. It is by no means unimportant that the volumes of expired air in the studied mice with allergic BA were significantly lower than those of the control group. This facilitates proteolytic activity of cathepsins, including cathepsin S. Therefore, this enzyme may be associated with inflammation, atopy, and susceptibility to BA and dermatitis [33].

Due to its key role in the antigen presentation pathway, cathepsin S may potentially promote progression of asthma [34]. This thesis is confirmed by a number of preclinical models: the profiles of antigen expression in BALB/c and C57BL/6J mice infected with ovalbumin (OVA), a classical mouse model of allergic pulmonary inflammation, have shown that the expression of the cathepsin S gene were elevated by 4.0 and 3.2 times, respectively [35]. A study researching levels of the protein revealed an increase in cathepsin S in the bronchoalveolar lavage fluid after infecting mice with OVA [36]. Apart from that, treatment of wild-type mice with a reversible inhibitor of cathepsin S reduced inflammation in the OVA-infected mice compared with the levels in the cathepsin S knockout model, which highlights the pharmaceutical ability of protease in this disease [37].

CLINICAL APPLICATION

Considering the whole variety of processes emerging at the molecular level, cathepsin S performs extracellular and intracellular functions that may impact on many physiological changes in the lung tissue and, most importantly, define the trend of pathological and chemical processes in disease progression [38]. A number of recent theoretical studies as well as studies based on mouse models indicate the potential of the enzyme as a predictor of lung tissue deformation and irreversible airway remodeling [39].

Therefore, these features underline the fact that cathepsin S is an ideal target for disease treatment: its strict therapeutic inhibition must minimize the potential adverse effects [40]. Moreover, its higher stability in neutral pH compared with the other members of the cathepsin family highlights its

increased potential for participation in extracellular proteolytic activity [36].

It has been shown that preventive dosage of the irreversible inhibitor of cathepsin S decreases pulmonary eosinophilia in mice, which confirms the hypothesis that inhibition of the enzyme before inflammation in the airways is beneficial in lung tissue diseases. Additionally, foreign studies have shown that the studied molecule participates in later manifestations of the allergic reaction [41].

Our improved understanding of the structure and activity of cathepsin S will lead to explanation of the immunological role of the protein and determination of the therapeutic strategy – extracellular inhibition of cathepsin S aimed at preventing wall remodeling in small bronchi at the earliest stages.

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Kraposhina A.Yu. – conception and design, analysis and interpretation of the data. Sobko E.A. – substantiation of the manuscript, critical revision of the manuscript for important intellectual content. Demko I.V. – final approval of the manuscript for publication. Kazmerchuk O.V. – conception and design. Kacer A.B. – analysis and interpretation of the data. Abramov Yu.I. – analysis and interpretation of the data.

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Phenotype variation of hypertrophic cardiomyopathy in carriers of the p.Arg870His pathogenic variant in the *MYH7* gene

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ABSTRACT

The review analyzes variability of clinical manifestations of p.Arg870His in the *MYH7* gene, which is repeatedly registered in patients with hypertrophic cardiomyopathy (HCM). The analysis involves the data from scientific publications obtained as a search result in the PubMed, ClinVar, and eLibrary.ru databases, as well as authors' own results. A wide range of phenotypic manifestations have been revealed in carriers of p.Arg870His, from the asymptomatic to severe course, rapid progression, and early death. The review considers possible factors that modify the effect of the pathogenic variant (i.e. dosage of the pathogenic variant, the presence of other unfavorable genetic variants, etc.). The importance of accumulating information on the clinical features of HCM in the carriers of specific gene variants is emphasized in order to clarify their pathogenicity and to identify factors modifying the clinical outcome, which is important for the choice of the treatment strategy for HCM.

Keywords: hypertrophic cardiomyopathy (HCM), myosin heavy chain 7 (*MYH7*) gene

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Фенотипическая вариабельность гипертрофической кардиомиопатии у носителей патогенного варианта p.Arg870His гена *MYH7*

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РЕЗЮМЕ

Обзор посвящен анализу вариабельности клинических проявлений неоднократно зарегистрированного у пациентов с гипертрофической кардиомиопатией (ГКМП) патогенного варианта p.Arg870His гена *MYH7*. К анализу привлечены данные научных публикаций, полученных в результате поиска в базах данных PubMed, ClinVar, eLibrary.ru, а также собственные результаты. Выявлен широкий спектр фенотипических проявлений у носителей патогенного варианта p.Arg870His: от бессимптомного носительства до тяжелого течения, быстрого прогрессирования и ранней смерти. Обсуждаются возможные факторы, модифицирующие эффект патогенного варианта (доза патогенного варианта, наличие других неблагоприятных генетических вариантов и др.). Подчеркивается важность накопления информации о клинических особенностях течения ГКМП у носителей конкретных вариантов генов с целью уточнения их патогенности, выявления модифицирующих клиническую картину факторов, что имеет значение для определения тактики ведения пациентов с ГКМП, уточнения прогноза, определения стратегии обследования членов их семей.

Ключевые слова: гипертрофическая кардиомиопатия (ГКМП), ген тяжелой цепи миозина (*MYH7*)

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

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INTRODUCTION

Hypertrophic cardiomyopathy (HCM) is a myocardial disease with hypertrophy of the left and / or right ventricle. The development of the disease cannot be explained by extra load, arterial hypertension, the presence of another heart pathology, systemic disease, or other conditions associated with left ventricular hypertrophy. Asymmetric thickening of the interventricular septum is the most common form of the disease [1]. Symptoms include dyspnea, heart pain, faints, dizziness, tachycardia, and sudden cardiac death. HCM is diagnosed with a frequency of 1: 500 [2], which may be underestimated due to the asymptomatic course of the disease in some patients. Estimated disease prevalence may vary in different populations. Thus, HCM prevalence was estimated as 0.19% in Spain [3], and as 0.031% in Korea [4].

According to the modern concepts, HCM is a hereditary disease characterized by high genetic heterogeneity and clinical polymorphism [5–7]. In the curated database ClinGen [8], HCM genes are classified according to the strength of the association with the disease: proven, or strong (*MYBPC3*, *MYH7*, *TNNT2*, *TNNI3*, *TPM1*, *ACTC1*, *MYL2,* and *MYL3* genes), moderate (*CSRP3*, *TNNC1*, *JPH2* genes), and weak (16 genes). Genes with a proven, strong and moderate association with the disease encode proteins of thick and thin filaments of the sarcomere, Z-disc, and sarcoplasmic reticulum complex [9].

The online resource ClinVar lists 40 genes [10], whose rare variants are considered to cause the development of this disease. Among them, there are 427 pathogenic variants in 29 genes and 408 likely pathogenic variants in 31 genes. The most significant contribution to the genetic causes of HCM is made by pathogenic (P) / likely pathogenic (LP) variants localized in two genes of sarcomeres – *MYBPC3* and *MYH7* [10–14]. In addition, about

6,000 variants with uncertain significance (VUS) in 110 genes have been described, but the HCM-related data are contradictory [10]. At the same time, pathogenicity estimates for particular genetic variants are regularly reviewed as new clinical data on the variant carriers and their family members are accumulated. In particular, such a revision in relation to *MYH7* gene variants led to a decrease in the number of VUS from 42 to 30%. [15]. In some cases, variants were initially categorized as "benign", i.e. non-pathogenic, but later were detected in patients with severe HCM [16, 17].

A number of studies have assessed correlations between genotype and phenotype in HCM [11–13, 18–27]. For example, they focused on HCM patients with pathogenic mutations in the *MYH7* and *MYBPC3* genes. The study revealed more surgical interventions, a higher risk of sudden cardiac death, and a shorter life expectancy in patients with pathogenic variants in the *MYH7* gene, compared with carriers of the *MYBPC3* gene variants. During 6 years of follow-up, 26% of patients with pathogenic variants in the *MYH7* gene had clinical manifestations of HCM, while those with the *MYBPC3* gene variants remained asymptomatic [28]. Another study showed that patients with pathogenic variants in the *MYH7* gene, compared with the *MYBPC3* gene, had a larger left atrium, a high risk of atrial fibrillation, and a worse disease prognosis [24]. In addition, myectomy or percutaneous alcohol septal ablation was more often performed in individuals with pathogenic variants in the *MYH7* gene [29]. The meta-analysis (which included 51 studies with 7675 HCM patients) showed that *MYH7* pathogenic variants, on average, led to earlier HCM development and a more severe course, compared with patients with HCM associated with other genes. Besides, the incidence of impaired cardiac conduction, ventricular arrhythmias, and heart transplantation is higher in patients with pathogenic variants in the *MYH7* gene than in those with variants in the *MYBPC3* gene [13]. In case of pathogenic variants in the sarcomeric genes (*MYBPC3*, *MYH7*, *TNNT2*, *MYL2*, *MYL3*, *TNNI3*, *ACTC1*, *TNNC1*), the disease manifests earlier than in patients who do not have mutations in these genes [26].

However, given the high genetic heterogeneity of HCM and, therefore, a small number of patients with the same mutation, some questions still remain unresolved. In this regard, the role of some pathogenic variants in HCM development is questionable, and the factors contributing to the development of pathogenic phenotypes and the disease course remain unclear. The problem of HCM phenotypic description and variability, as well as pathogenicity estimates of genetic variants is actively discussed in the scientific and clinical community [30].

HCM is an autosomal dominant disease, and the vast majority of patients have a single pathogenic variant. At the same time, cases of compound heterozygotes and even homozygotes for particular mutations have been described. Such patients usually have an earlier onset and a more severe course of the disease.

One of the mutations repeatedly identified in HCM patients is the p.Arg870His amino acid replacement in the beta-myosin heavy chain protein encoded by the *MYH7* gene. Cases of both the disease and asymptomatic carriage of this variant are known. One study describes a pedigree where two members, who are descendants of closely related marriages, were found to be homozygous for the pathogenic variant [18]. In our practice of HCM genetic diagnosis, this mutation was also registered and showed incomplete penetrance [31]. Thus, this variant is of interest for a detailed analysis of the clinical characteristics in patients from different families.

The aim of the study was to systematically review publications describing the phenotypic features of HCM in carriers of the pathogenic p.Arg870His variant in the *MYH7* gene, including the use of our own findings.

MATERIALS AND METHODS

Articles were found in the PubMed, ClinVar, and eLibrary databases using the following keywords and their combinations: hypertrophic cardiomyopathy (HCM), myosin heavy chain 7 (*MYH7*) "p.Arg870His", without any restrictions by the study design and native language of authors. Titles and abstracts were checked in order to assess whether studies corresponded with the topic of the review. All the clinical data from the articles were included in the table: diagnosis, gender, age, family history, description of the main symptoms, NYHA classification of heart failure, data of instrumental heart

examinations (echocardiography (Echo-CG), interventricular septum (IVS), left ventricular posterior wall (LVPW), ejection fraction (EF), peak gradient in the left ventricular outflow tract (LVOT), and electrocardiography (ECG)).

RESULTS

In total, we found 66 publications mentioning the pathogenic genetic variant p.Arg870His of the *MYH7* gene in HCM patients. Only 6 studies [18–20, 32–34] described clinical characteristics of the disease in patients. Except for the case described by us [31], there were no Russian publications that provided information on the clinical characteristics of patients with this mutation (based on the information given in the scientific electronic library eLibrary.ru). Several studies lacked some instrumental methods for assessing the heart state (Echo-CG and ECG).

CLINICAL CHARACTERISTICS OF HYPERTROPHIC CARDIOMYOPATHY IN CARRIERS OF THE PATHOGENIC P.ARG870HIS VARIANT IN THE *MYH7* GENE

A single-nucleotide variant 2609:G>A in exon 22 of the *MYH7* gene results in an arginine to histidine substitution at codon 870 (p. Arg870His, R870H, rs36211715) in the alpha-helical S-2 domain of the beta-myosin heavy chain protein. The p. Arg870His substitution is one of the most frequently reported pathogenic variants detected in patients with HCM. Even though the amino acids arginine and histidine are both positively charged, they differ in several properties (hydrophilicity, size, donor – acceptor properties) [35], which can determine the structural and functional features of the protein. Amino acid substitutions in this region can affect myofilament assembly, protein stability, tensile strength, and stiffness [34, 36]. The p.Arg870His variant causes a drastic decrease (more than ten-fold) in the binding affinity of the C1–C2 domains of *MYBPC3* (MyBP-C) [37]. The variant reduces the relative sliding velocity between actin and myosin filaments [38]. The mutation destabilizes the interactions (as well as the structure) between *MYH7* and *MYL3* and between *MYH7* and *MYL2* [34].

The p. Arg870His substitution is very rarely registered in populations (with frequency corresponding to the rate of mutational events, – 4×10^{-6}–1.6×10^{-5}) [39], but it is detected in HCM patients of various nationalities (both in sporadic and familial cases) [18–21, 32, 33, 40–44]. Although most researchers consider this pathogenic variant relatively benign, carriers of p. Arg870His show a wide range of phenotypic manifestations. These are cases of asymptomatic carriage [20, 32], severe clinical manifestations of HCM [33], as well as cases of sudden cardiac death in families with this pathogenic variant [19, 21] (Table).

Clinical symptoms in carriers of this variant vary both between members of the same family and between members of different families. Extensive information was obtained in the study of a large Indian family with HCM, with the p.Arg870His substitution as the cause of the disease [18, 32] (Table). The authors described high clinical heterogeneity of HCM in the carriers (from asymptomatic carriage, usually in younger people, to early death). The age of onset also varies widely. However, as some authors note [32], the onset age is accurately established only for the probands, since the health status of other carriers is assessed during subsequent family screening (i.e., the age of onset must be earlier). Among members of the family with HCM from India, 75% of men and 44% of women with the p.Arg870His variant had clinical symptoms of HCM. The average penetrance of the variant was 59% [32].

In general, asymptomatic carriage of this variant is typical of young people. In the elderly, clinical symptoms of this pathology were recorded even in the absence of HCM echocardiographic signs. The age of diagnosis varied from 16 to 47 years in men and from 20 to 69 years in women (Table). Some female carriers of the pathogenic variant of different age (19, 48, and 55 years) with normal heart ultrasound results had HCM symptoms (dyspnea, palpitations). Furthermore, one woman aged 25 years was diagnosed with HCM according to echocardiographic data but had no disease symptoms.ECG changes in carriers of the pathogenic p.Arg870His variant are typical of HCM (Q waves, depression of the ST segment, T wave inversions (Table)) [45]. Sometimes ECG changes precede clinical manifestations of the disease [19, 20, 46–48]. At the same time, ECG changes arising in already diagnosed HCM patients indicate a high risk of ventricular tachyarrhythmia and sudden cardiac death [49].

Table

Phenotype variation of HCM in carriers of the p.Arg870His pathogenic variant in the *MYH7* gene

Population	Pathology (type)	Case	Sex	Age at the examination	Genotype	Clinical symptoms	NYHA	Echo-CG				ECG	Source
								IVS, mm	LVPW, mm	LVOT, mm Hg	EF,%		
Russia	HCM (O)	P-mother	f	45	het	Syncope, dyspnea, weakness	III	20	9	105	63	ST depression in leads I, II, aVL, aVF, V5–V6	Own data
				53		Deterioration of the condition	–	24	16	70.8	91	–	
				53[a]		Surgical treatment	–	15	11	12	55	–	
				54		–	–	19	13	8.43	62	–	
				57		–	–	18	12	8.8	65	–	
	Normal	P-daughter	f	27	het	No	–	7.8	7.6	–	72	–	
India	HCM	P1	m	69	het	Palpitations, syncope	II	15	10	–	69	Enlarged LA	[32]
	Normal	P1	f	55	het	Palpitations, angina, dyspnea	II	10	9	–	73	WNL	
	Normal	P1	f	48	het	Palpitations, dyspnea	II	12	10	–	65	WNL	
	HCM	P1	f	58	het	Dyspnea	II	17	11	–	65	Abnormal Q wave	
	HCM (O)	P1	m	52	het	Dyspnea, syncope	III	21	11	40	75	–	
	HCM	P1	m	47	het	Dyspnea, palpitations, angina	II	16	10	–	65	T wave inversion	
	HCM (NO), ASH	P1[b]	m!	37 (30)	homo	Palpitations, syncope	III	20	11	–	55	T wave inversion, abnormal Q wave	
	HCM	P1	f	34	het	Palpitations	II	13	8	–	73	T wave inversion	
	HCM (O)	P1	m!	32 (31)	het	Palpitations, dyspnea	III	23	13	64	76	Enlarged LA, left axis deviation	
	HCM	P1	f	25	het	No	I	14	11	–	78	Short PR interval	
	HCM (NO), ASH	P1	m!	23 (19)	homo	Dyspnea, syncope	III	19	10	–	76	T wave inversion, abnormal Q wave, lower ischemia	
	Normal	P1	f	20	het	No	I	11	9	–	80	WNL	
	Normal	P1	m	16	het	No	I	12	10	–	82	WNL	
	HCM	P1	f	20	het	Dyspnea	I	13	10	–	78	WNL	
	Normal	P1	m	17	het	No	I	8	7	–	81	WNL	
	Normal	P1	f	19	het	Dyspnea	I	10	9	–	72	WNL	
	Normal	P1	f	16	het	No	I	7	7	–	75	WNL	
	HCM (O)	S[c]	m!	36 (34)	het	No	II	32	21	–	70	–	

Table (continued)

Population	Pathology (type)	Case	Sex	Age at the examination	Genotype	Clinical symptoms	NYHA	Echo-CG				ECG	Source
								IVS, mm	LVPW, mm	LVOT, mm Hg	EF,%		
Japan	HCM, ASH	P2/father	m	40	het	–	–	20	11	–	78	Complete right bundle branch block	[20]
	HCM, ASH	P2/son	m	16	het R870H+ Arg54 Ter	–	–	20	11	–	83	Abnormal Q waves in leads II, III, aVF, V5–V6, T wave inversion in leads I and aVL	
				19				25	12	–	82	Abnormal Q waves in leads II, III, and aVF disappeared, ST depression with T wave flattening in V5 and V6 emerged	
Spain	HCM (NO), ASH	P7/sister	f	65 (59)	het	–	II	–	20	–	–	–	[19]
	HCM, ASH	P7/brother[d]	m	50 (38)	–	–	–	–	22	–	–	–	
Czech Republic	Severe HCM	S	–	36	het	Chronic atrial fibrillation, chest pain, fatigue, dyspnea	–	–	28	60	–	–	[33]
Italy	Arrhythmogenic cardiomyopathy	P8/father	m	43 (2005)	het (*de novo*)	Syncope, sustained ventricular tachycardia	–	–	–	–	–	Tachycardia, wide QRS complex	[34]
				45 (2007)		Syncope, implantable cardioverter – defibrillator	–	–	–	–	–	Monomorphic ventricular tachycardia after syncope	
				59 (2021)		Ventricular arrhythmias were not registered for 13 years	II	–	–	–	60	Epsilon wave in V3–V4, T wave inversion in V3–V6, and Q wave in V5–V6	
	Normal	P8/daughter	f	18	het	Syncope, palpitations	I	WNL (not given)				Episodes of sinus tachycardia during palpitations and syncopal episodes	

Note: P – familial case; S – sporadic case; (O) – obstructive form of HCM; (NO) – non-obstructive form of HCM; ASH – asymmetric septal hypertrophy; het – heterozygous carrier of the pathogenic variant; homo – homozygous carrier of the pathogenic variant; WNL – within normal limits; IVS – interventricular septum thickness; LVPW – left ventricular posterior wall thickness; LVOT – left ventricular outflow tract gradient; EF – ejection fraction; NYHA – class of heart failure; a – medical examination data after septal myectomy with mitral valve repair; b – the patient died due to heart failure a few months after the pacemaker was implanted; c – the patient served in the armed forces for more than 10 years (prolonged physical activity); d – after 12 years of follow-up, the patient developed severe systolic dysfunction resulting in sudden cardiac death; ! – proband.

In general, only 6% of individuals with apparent echocardiographic evidence of HCM at the time of diagnosis did not have ECG changes. Patients with abnormal ECG had more severe symptoms, higher peak pressure gradients in the LVOT, and a greater degree of ISW thickness. They were more likely to have severe syncopal symptoms requiring surgical myectomy and / or implantation of a cardioverter – defibrillator [45]. In addition, ECG results may change during the follow-up of patients with HCM (Table).

Interestingly, in a recent study [34], the pathogenic p.Arg870His variant of the *MYH7* gene was considered to be the cause of arrhythmogenic cardiomyopathy (Table). A 43-year-old man had a syncopal episode while taking amiodarone, and ECG showed a wide QRS complex and complex tachycardia. Two years later, he was implanted with a cardioverter- – defibrillator due to the monomorphic ventricular tachycardia following syncope (registered by ECG). The pathogenic p.Arg870His variant of the *MYH7* gene in this man arose *de novo* and was inherited by his daughter. At the age of 18, she showed no abnormalities, according to echocardiography and Holter ECG monitoring, as well as heart computed tomography. However, dyspnea, palpitations, and episodes of sinus tachycardia with palpitations and syncope were observed [34].

Despite the fact that most studies describe a relatively mild clinical course of the disease for p.Arg870His in the *MYH7* gene, some HCM families with this mutation had early and (or) sudden deaths, including deaths of homozygous patients [18, 19, 44].

It should also be noted that the HCM course may differ depending on an amino acid that was replaced in the protein structure, even if it occurred in the same codon. Thus, when cysteine replaces arginine at codon 870 (p.Arg870Cys), it leads to a severe course of HCM with an early onset and a high risk of sudden cardiac death [44].

Data on the follow-up of carriers with HCM-inducing pathogenic genetic variants are also interesting. Such studies are scarce, and the results of dynamic observation of groups with individual pathogenic mutations are rarely published [20, 50]. At the same time, pharmacotherapy and (or) surgery can change the clinical presentation of HCM (Table).

Thus, the spectrum of phenotypic manifestations of the p.Arg870His mutation shows that carriers of the same pathogenic variant may have a wide range of HCM clinical symptoms as well as other forms of cardiomyopathies. In this regard, it is important to establish the factors that can modify clinical symptoms in the pathogenic variant carriers.

FACTORS MODIFYING THE CLINICAL PRESENTATION OF HCM

Causes of clinical variability in the manifestation of pathological signs in carriers of pathogenic mutations (including p.Arg870His in the *MYH7* gene) may include both genetic and epigenetic factors, as well as lifestyle.

Genetic factors influencing the clinical course include gene dosage (homozygous and (or) heterozygous genotype for the pathogenic variant), the presence of other P- and LP-variants in the same or another HCM gene [12, 17, 19, 20, 51–54], as well as a set of genetic variants in other genes [55, 56]. A rare case was described for the p.Arg870His mutation: two patients homozygous for this mutation were born in two different inbred marriages in the same pedigree [32, 18]. One of them died suddenly at the age of 36, a few months after the implantation of the pacemaker due to the developed heart failure. Another patient was diagnosed with HCM (asymmetric septal hypertrophy without obstruction) at the age of 19 and had abnormal T and Q waves on the ECG (Table).

One of the first published cases combining two mutations in the *MYH7* gene, i.e., a compound heterozygous genotype, was also associated with the p.Arg870His mutation. In addition to that variant, the patient had a nonsense mutation in exon 3, which formed a stop codon at codon 54 (p.Arg54Ter). This variant combination led to the early development of HCM (at the age of 16) and rapid progression of the disease (worse ECG and Echo-CG results) [20]. The pathogenic p.Arg870His variant was inherited from the father (who developed the disease at the age of 40). The second variant, p.Arg54Ter, was inherited from the maternal grandmother (both the grandmother and mother were healthy). Thus, the combination of the two mutations inherited from parents led to HCM development at an early age and its more severe course. In addition, the authors supposed that heterozygous nonsense mutations in

the *MYH7* gene did not lead to the disease manifestation. That study identified the p.Arg870His variant in three pedigrees, and 9 out of 10 variant carriers had myocardial hypertrophy at the time of the medical examination [20].

As we are accumulating more and more data on HCM gene sequencing, it turns out that patients with more than one pathogenic variant are not so rare [12, 53, 57]. For example, the combination of pathogenic variants p.Arg787His and p.Ile736Thr in the *MYH7* gene led to severe HCM [19]. It is interesting that the probands with compound heterozygous variants in the *MYH7* gene demonstrated a higher left ventricular myocardium mass and higher QRS, SV1, and RV5 + SV1 amplitudes on ECG than those with double mutations on the same chromosome [58].

Based on the study of mutations in the *MYH7*, *MYBPC3*, *TNNT2*, and *TNNI3* genes, Y. Zou et al. [12] concluded that neither a specific gene nor a specific mutation were correlated with the clinical phenotype of HCM, while the number of mutations was associated with the maximum thickness of the left ventricular wall. Multiple pathogenic variants (either in the same or in different sarcomeric genes) were registered in 9.5% of HCM patients. Another study of 2,912 HCM probands showed that 8% of probands had more than one pathogenic (P) or likely pathogenic (LP) variant or variant of uncertain significance (VUS); 0.6% of probands had 2 or more P / LP variants (including homozygous ones, and 1 proband had 3 P / LP variants, the age of such patients was 10 years younger than that of patients with single variants). 5% of probands had 1 P / LP and at least 1 VUS, and 2.4% had 2 or more VUS [53].

In addition to the combined effect of P and LP variants, the phenotype may be influenced by rare polymorphic variants in sarcomeric genes. For example, in 60 HCM patients without pathogenic variants in the genes of sarcomeric proteins, functionally significant variants in the intron and 3'UTR of the *MYH7* gene were identified. They were localized in the promoter region at the binding sites of transcription factors [59]. So, the clinical presentation of HCM is determined not only by individual mutations in the sarcomeric genes, but also by a combination of mutations and (or) variants in several genes (not only sarcomeric). Therefore, HCM is more and more often referred to as not a monogenic but an oligogenic disease [7]. A detailed examination and genotyping of patients with severe HCM (as well as other cardiomyopathies) for the presence of other P / LP genetic variants is essential for predicting the course of the disease both in probands and in their relatives who inherited these variants and (or) their combinations.

The potential significance of the general genetic background for the clinical presentation of the disease may be evidenced by the data of genome-wide association studies (GWAS) for HCM [55, 56]. Thus, a study by A.R.Harper et al. [55] identified 12 loci associated with HCM. Moreover, single nucleotide polymorphisms affect the HCM severity in carriers of mutations in the genes of sarcomeric proteins [55, 60].

The spectrum of genes modifying the clinical presentation of HCM is constantly expanding [61, 62]. Thus, the presence of mutations in the genes of ion channels (*KCNQ1*, *KCNH2*, *CACNA1C*, *SCN5A*, and *ANK2*) in HCM patients increases the risk of life-threatening arrhythmias and sudden cardiac death and affects their prognosis and treatment [63]. At the same time, the genes modifying the clinical course of HCM may differ in men and women, as has been shown for the development of cardiac fibrosis in this disease [61].

The influence of environmental and epigenetic factors on the penetrance of pathogenic variants and the HCM course is described by data from twin studies. Monitoring the HCM progression in 11 pairs of monozygotic twins (with 9 pairs having pathogenic variants in the sarcomeric genes) for 5–14 years revealed inconsistency of morphological changes (thickness of the left ventricular wall, left atrial diameter, and left ventricular ejection fraction). It led the authors to the conclusion that epigenetics and environmental factors play an essential role in the progression of this disease [64]. Behavioral features (such as doing sports, physical activity, etc.) can also act as factors modifying the clinical presentation of HCM [32].

Unfortunately, modifying factors (multiple pathogenic variants, effects of regulatory elements and polymorphic variants of different genes, whose products ensure functioning of the cardiovascular system, general genetic background, epigenetic

modifications, etc.) have not been sufficiently studied despite the clinical significance of possible results of such studies.

CONCLUSION

The complexity of describing the genetic component of HCM and assessing the pathogenetic significance of individual variants is due to the fact that this disease is characterized by incomplete age-dependent penetrance and variability of the clinical course even in carriers of the same pathological variant [14, 27, 32, 64–66]. For instance, it was demonstrated by the pathogenic p.Arg870His variant in the *MYH7* gene. Sometimes HCM develops without a clear clinical presentation, and sudden cardiac death may be its first manifestation. According to epidemiological data, sudden cardiac death in individuals with even minor signs of HCM often occurs in case of a sedentary lifestyle or light activity (66%), often in bed or during sleep (32%), less often during physical activity (22%), including participation in competitions [67].

Accurate pathogenicity classification of variants in HCM genes is of great clinical importance. Thus, it is known that patients with P / LP variants in HCM genes had a lower survival rate compared with patients with no such variants in these genes [28]. At the same time, genetic testing in HCM allows not only to confirm the clinical diagnosis, but also to identify family members with pathogenic variants who are at risk of developing the disease, which opens up the possibility of prevention [68, 69]. Moreover, examining relatives of HCM patients with a genetically determined cause sometimes helps to identify mutation carriers already having HCM manifestations [70]. Therefore, if pathogenic variants in cardiomyopathy genes are detected in an individual (even in the absence of complaints), it is recommended to examine relatives and start cascade genetic screening, if pathological phenotypes are detected [69].

In the future, accumulation and analysis of data on the phenotypic variability in carriers of specific pathogenic variants will help to determine the conditions of mutation penetrance and predict features of the disease course more accurately. In this regard, the ClinVar expert group proposed to expand and unify the criteria used for the phenotypic description of patients in order to clarify the pathogenicity of variants [30], which will contribute to improving the care provided to patients with this pathology.

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Authors contribution

Kucher A.N. – conception and design; drafting of the manuscript; substantiation of the manuscript or critical revision of the manuscript for important intellectual content; final approval of the manuscript for publication. Valiakhmetov N.R., Salakhov R.R., Golubenko M.V., Pavlyukova E.N. – analysis and interpretation of the data; drafting of the manuscript; substantiation of the manuscript or critical revision for important intellectual content. Nazarenko M.S. – conception and design; substantiation of the manuscript or critical revision of the manuscript for important intellectual content; final approval of the manuscript for publication.

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Collagen synthesis in the skin: genetic and epigenetic aspects

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ABSTRACT

One of the most important functions of the skin, mechanical, is provided by collagen fibers and their interaction with other elements of the extracellular matrix. Synthesis of collagen fibers is a complex multistep process. At each stage, disturbances may occur, leading, as a result, to a decrease in the mechanical properties of the connective tissue. In clinical practice, disorders of collagen synthesis are manifested through increased skin laxity and looseness and premature aging. In addition to the clinical presentation, it is important for the cosmetologist and dermatologist to understand the etiology and pathogenesis of collagenopathies. The present review summarizes and systematizes available information about the role of genetic and epigenetic factors in the synthesis of collagen fibers in the skin. Understanding the etiology of collagen synthesis disorders can allow doctors to prescribe pathogenetically grounded treatment with the most effective results and minimize adverse reactions.

Keywords: skin collagen, collagen synthesis, collagenopathy, gene polymorphism

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Синтез коллагена в коже: генетические и эпигенетические аспекты

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РЕЗЮМЕ

Одна из важных функций кожи, механическая, обеспечивается коллагеновыми волокнами и их взаимодействием с другими элементами внеклеточного матрикса. Синтез коллагеновых волокон – это сложный многоэтапный процесс. На каждом этапе может возникнуть нарушение, приводящее в итоге к снижению механических свойств соединительной ткани. Клинически нарушения коллагенообразования проявляются в виде повышенной дряблости, рыхлости кожи, раннего проявления признаков старения лица. Кроме клинической картины, врачу косметологу и дерматологу важно понимать этиологию и патогенез коллагенопатий. В нашем обзоре мы обобщили и систематизировали имеющуюся информацию о роли генетических и эпигенетических факторов в процессе синтеза коллагеновых волокон кожи. Понимание патогенеза нарушения коллагенообразования может позволить врачам назначать патогенетически обоснованное лечение с достижением наиболее эффективных результатов и минимизацией нежелательных реакций.

Ключевые слова: коллаген кожи, синтез коллагена, коллагенопатия, полиморфизм генов

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 order to prescribe pathogenetically grounded therapy for aesthetic skin imperfections, it is important to understand the physiological and pathological processes in the skin and, based on this, prescribe a set of measures aimed at restoring its physiological properties [1]. To do this, it is necessary to perform in-depth study on synthesis of collagen fibers, including the genetic aspects of collagen synthesis. Uniting fragmented data about genes encoding key proteins, including enzymes, at all stages of collagen synthesis in the skin can help to develop new predictive strategies in medical cosmetology (aesthetic medicine).

Collagen accounts for up to 25% (in dry weight) of all proteins in the human body, providing structural support for connective tissue, including the skin [2]. A large number of modern methods of aesthetic medicine are aimed at improving and stimulating collagen synthesis in the skin [3]. At the same time, some companies have undertaken clinical trials, and histologic studies and have suggested that particular techniques bring significant results. However,

in clinical practice, we are far from achieving consistent clinical effects in all patients. In the context of diverse results in our patients, we most often talk about the "individual characteristics" of a particular person. So, what underlies these individual characteristics?

There are two groups of factors that can influence collagen synthesis in the skin: external and internal ones [4]. External factors include nutrition (the completeness of intake of nutrients necessary for collagen synthesis) and the impact of environmental factors. Internal factors include the hormonal status, the inherent genetic code for the structural elements of the skin, and epigenetic regulation of the activity of genes encoding key proteins and enzymes of collagen synthesis [5]. The genetic aspects of collagen fiber turnover (synthesis, function, degradation), as well as their role under normal conditions and in pathology are being actively studied. The largest number of studies are devoted to the collagen of bone tissue and internal organs. The number of studies concerning genetic predictors of collagen synthesis in the skin has been increasing in recent years, but there is a need to systematize the existing data.

THE STRUCTURE OF THE COLLAGEN MOLECULE

n the extracellular matrix, two main classes of macromolecules are distinguished: glycoproteins (fibronectin, proteoglycans, and laminin) and fibrous proteins (collagen and elastin). The extracellular matrix proteins are called "matrisomes" [6]. A collagen molecule is a fibrillar glycoprotein characterized by versartility in the construction of various tissues. The natural form of the collagen fiber provides necessary mobility during skin stretching, but in scar tissue, fibers are straighter and thinner, and, consequently, the tensile strength of the collagen fiber decreases [7]. Depending on the type of collagen, its supramolecular structure can be fibrillar and non-fibrillar. Among the 28 types of collagens in the skin, type I, III, and V fibrillar collagens are of the greatest importance for the skin, while non-fibrillar collagens (type IV collagen located in the basement membrane and type VI, VII, XIV, and XVII collagens) are less important.

All collagens, at least partially, are supercoils twisted in a left-handed fashion consisting of three polypeptide chains [8]. These polypeptide chains can have the same sequence of amino acid residues (in this case, the collagen molecule is known as homomeric) or a different sequence (heteromeric collagen molecule) [9]. So, the dominant form of type I collagen is a heterotrimer. The homotrimeric can be found in fetal tissues, tumors, and some fibrous lesions in various tissues; it is more resistant to the action of collagenases [10]. In contrast, the dominant form of type III collagen is a homotrimer. Its fiber diameter is smaller than that of type I collagen. However, when type I and III collagen appear together, the latter regulates the diameter of the collagen fiber [11]. The collagen molecule consists of repeating triads of (X-Y-Gly)n, where Gly is the amino acid glycine. The X and Y positions may be attributed to any other amino acids, but quite often they are filled by proline or hydroxyproline [12]. Glycine is the smallest of the amino acids, and its lateral hydrogen is always in the center of the helix. This amino acid contributes to the coiling of the three helices and provides tight packing of collagen into the helix [13, 14]. Mutations in genes that lead to replacement of glycine with another amino acid, lead to a change in the structure of the helix and thus a disruption in the protein function. For example, more than 650 mutations in the *COL3A1* gene encoding the pro-alpha 1 chain of type III collagen have been identified, among which missense mutations replacing glycine with a bulkier amino acid are the most common. Most glycine substitutions lead to the formation of a more thermolabile protein with greater susceptibility to proteinases [15]. Most patients with such mutations are heterozygous and can produce both normal and abnormal α-chains of type III procollagen, so they can have both normal and mutant homotrimers and triple chains containing one or two abnormal chains [16].

COLLAGEN SYNTHESIS

The main producers of extracellular matrix components, including collagen, are fibroblasts. Collagen fiber synthesis is a complex multi-stage process that begins with transcription of the gene encoding collagen in the cell nucleus and ends with the assembly of the collagen fiber in the extracellular space [17]. At each stage, it is possible to identify genes that contribute to the fiber formation. At the initial stage, these are genes that contain the structure code for the polypeptide chain. Also, at this stage, the role of epigenetic regulation can be noted. At the next

stages of the assembly (post-translational changes), the role of genes responsible for spatial arrangement of collagen fibers affecting the functionality of the fiber is important [18].

The assembly of the collagen polypeptide chain occurs in ribosomes, where information is read from the messenger ribonucleic acid (mRNA), and the polypeptide chain is assembled (translated) from amino acids with the participation of transfer RNA (tRNA). The primary collagen polypeptide chain consists of three domains: N-propeptide, triple-helical (makes up 95% of the molecule), and C-propeptide. These domains are transported to the endoplasmic reticulum, where they undergo subsequent post-translational modification [19]. A key step in the collagen formation is formation of a triple supercoil, or trimerization, which begins at the C-terminal end at the site of disulfide bonds and proceeds at a lightning speed to the N-terminal end of the molecule. Each individual polypeptide chain is folded into a left-handed helix. Then all three chains are folded together into a right-handed helix. Before the assembly of the supercoil, post-translational changes occur in each of the chains, such as hydroxylation, glycosylation, and oxidative deamination. All these changes occur inside the cell [20]. For subsequent thermal stability of collagen, prolyl residues in the triple-helical domain are hydroxylated to 4-hydroxyproline by prolyl-4-hydroxylase encoded by the genes *P4HA1, P4HA2, P4HB,* and *P4HA3* (Table) [21]. For subsequent collagen reticulation, some of the lysine residues are hydroxylated by procollagen-lysine, 2-oxoglutarate-5-dioxygenase encoded by the *PLOD* gene and then glycosylated [22, 23]. As seen from the Table, under physiological conditions, *PLOD1* and *PLOD3* are highly expressed in the skin. Hydroxylation requires the presence of oxygen, vitamin C (for reduction of iron ions in the composition of enzymes), and α-ketoglutarate [24]. Ascorbic acid (vitamin C) is a cofactor of prolyl hydroxylases and lysyl hydroxylases, which are involved in collagen biosynthesis [25].

Table

Genes encoding enzymes involved in post-translational changes in the collagen fiber [26]

Gene, encoded protein / enzyme	Localization on the chromosome	Clinical manifestations of mutation / polymorphism	Expression in the skin (RPKM)
P4HA1 (α-subunit of the prolyl 4-hydroxylase)	10q22.1, 17 exons	Poor prognosis in malignant neoplasms	7.129 ± 2.121
P4HA2 (α-subunit of the prolyl 4-hydroxylase)	5q31.1, 17 exons	Poor prognosis in malignant neoplasms, risk of myopia	4.6 ± 0.816
P4HB (β-subunit of the prolyl 4-hydroxylase)	17q25.3, 10 exons	Poor prognosis in malignant neoplasms	89.377 ± 8.824
PLOD1 (LH1) (lysyl hydroxylase (procollagen lysine, 2-oxoglutarate 5-dioxygenase 1))	1p36.22, 20 exons	Ehlers – Danlos syndrome type VI	11.061 ± 2.249
PLOD2 (LH2) (lysyl hydroxylase (procollagen lysine, 2-oxoglutarate 5-dioxygenase 2))	3q24, 23 exons	Ehlers – Danlos syndrome type VIB, Brook's syndrome	0.988 ± 0.202
PLOD3 (LH3) (lysyl hydroxylase (procollagen lysine, 2-oxoglutarate 5-dioxygenase 3))	7q22.1, 19 exons	Ehlers – Danlos syndrome type VIB, Stickler-like syndrome	7.062 ± 2.361
LOX (lysyl oxidase)	5q23.1, 8 exons	Aortic aneurysms, vascular disorders	4.234 ± 1.207
ADAMTS1 (disintegrin and metalloprotease with thrombospondin motif 1)	21q21.3, 9 exons	Impaired growth, fertility, and organ morphology	2.796 ± 0.682
ADAMTS2 (disintegrin and metalloprotease with thrombospondin motif 2)	5q35.3, 23 exons	Ehlers – Danlos syndrome type VIIC	1.395 ± 0.248
ADAMTS10 (disintegrin and metalloprotease with thrombospondin motif 10)	5q35.3, 23 exons	Disruption of growth and development of the skin, lens, and heart, Weil – Marchesani syndrome	1.485 ± 0.952
BMP1 (bone morphogenetic protein 1)	8p21.3, 21 exons	Osteogenesis imperfecta, disruption of morphogenesis and tissue regeneration	5.382 ± 1.39
BMP2 (bone morphogenetic protein 2)	20p12.3, 3 exons	Impaired development of bone and cartilage tissue	2.551 ± 0.444
BMP4 (bone morphogenetic protein 4)	14q22.2, 6 exons	Dental system pathology, orofacial cleft, microphthalmia, cardiovascular pathology	2.207 ± 0.446
BMP7 (bone morphogenetic protein 7)	20q13.31, 7 exons	Pathology of the skeletal system, kidneys, and brown adipose tissue	7.722 ± 0.536

Other lysine and hydroxylysine residues undergo oxidative deamination using lysyl oxidase (LOX), thus forming reactive aldehydes that are capable of forming covalent intramolecular and intermolecular cross-links [27]. Trimerization occurs in the endoplasmic reticulum and is facilitated by chaperone proteins. The folding of the procollagen molecule begins only after the translation of the entire protein molecule is completed, with the autonomous folding of the C-propeptide domain on each monomer strand. After the folding, cysteine-rich C-propeptide is stabilized by disulfide bonds. After the folding, C-propeptide domains "recognize" each other and assemble together; in fibrillar proteins this process is mediated by Ca^{2+} and intermolecular disulfide bonds [28]. The assembled C-propeptide trimer then initiates almost instantaneous folding of the triple-helical domain, which is rich in proline and glycine, with preliminary isomerization of proline peptide bonds into a trans configuration [10]. In the resulting triple helix, further hydroxylation of procollagen is weakened and preparation for protein secretion begins (in a non-canonical way). After the formation of the supercoil, large globular domains are removed from both sides of the molecule to produce tropocollagen. Then, collagen reticulation occurs – cross-links are formed between some lysine and hydroxylysine residues [29].

Regulation of the displacement and orientation of various collagen chains occurs by additional globular non-collagen domains. After the formation of the triple helix, N-terminal propeptides are removed by zinc-dependent proteases belonging to the ADAMTS group (A disintegrin and metalloproteinase with thrombospondin motifs). The C-terminal propeptides of collagen are cleaved off by a group of metalloproteases belonging to BMP-1 (bone morphogenetic protein 1) (Table) [30]. As seen from the Table, the expression of the *ADAMTS1, ADAMTS2,* and *ADAMTS10* genes in the skin under physiological conditions is approximately the same, whereas the remaining enzymes of the ADAMTS group have only low-level gene expression in the skin. However, the key enzyme involved in the cleavage of the N-terminal propeptide is the N-protease encoded by the *ADAMTS2* gene. In the group of *BMP* genes, *BMP7* and *BMP1* have the greatest expression in the skin under physiological conditions, but the key role in the cleavage of the C-terminal propeptide in the skin belongs to BMP-1 [31].

The assembly of a collagen molecule is spatially arranged depending on the type of collagen and is enzymatically supported by additional molecular organizers, such as fibronectin, integrins, and minor collagens [32]. First, supramolecular structures of 4–5 protofibrils are assembled from a tropocollagen molecule; then, microfibrils are formed, from which a fibril (with a diameter from 10 to 300 nm) is produced with the participation of proteoglycans [33]. Proteoglycans on the surface of fibrils form a kind of a shell. Then, during the autogenesis, fibrils form a collagen fiber, which also includes glycosaminoglycans, glycoproteins, and non-collagen proteins. Fibrillogenesis is a spontaneous process (self-assembly), which is evidenced by spontaneous formation of fibrils by collagen fibers *in vitro*. However, *in vivo*, fibrillogenesis of type I collagen is controlled by cellular mechanisms – it occurs only in the presence of type V collagen, fibronectin, and integrins (fibronectin-binding and collagen-binding) [34]. At the same time, it is believed that type V collagen is important for the nucleation of type I collagen fibrils, while fibronectin and integrins are important during its assembly.

The tissue specificity of the collagen fiber is determined by the final composition of various collagens in heterotypic fibrils, and this composition is influenced by various signaling molecules involved in the fibrillogenesis [35]. In the collagen molecule, there are intrahelical and interhelical bonds. Reticulation is carried out by two mechanisms: specific (enzymatically controlled) and non-specific (spontaneous). According to the first mechanism, lysine is oxidized by lysyl oxidase with subsequent formation of aldimines. Then the reaction with histidine occurs to form chemically stable histidinohydroxylysino-norleucine [36]. Lysyl oxidase, which hydroxylates the lysyl residues of type I and II collagens, is encoded by the *LOX* gene [37]. The second mechanism may include multiple non-specific reactions with glucose and its oxidation products, resulting in the formation of advanced glycation end-products. This mechanism is especially important in aging and in diseases, such as diabetes mellitus. Carbohydrates and oxidized carbohydrates react with arginine, lysine, and hydroxylysine to form a glycated protein. Reticulated collagen is resistant to enzymatic and chemical degradation.

REGULATION OF COLLAGEN SYNTHESIS

The synthesis and assembly of the collagen fiber are influenced by many signaling molecules and proteins. Some of the most important of these are N-propeptides of type I collagen; fibronectin; lysyl oxidase; tenascin-X; thrombospondin; matrillins; perlecan; decorin; biglycan; fibromodulin; and lumican. Thus, a mutation of the gene encoding tenascin-X leads to the development of Ehlers – Danlos syndrome. In this syndrome, collagen fibrils of the usual size and shape are detected, but with a lower packing density. As a result, the total collagen content in the skin is reduced by 30% [38]. In addition, collagen and N-propeptides inhibit further synthesis of procollagen through negative feedback regulation. One of the most common glycoproteins of the extracellular matrix is fibronectin, which plays an important role in the development, cell growth, differentiation, adhesion, and cell migration through integrin-mediated signaling [39].

The formation of type I collagen fibrils requires the presence of type V collagen, as type V collagen acts as a central nucleus in the formation of type I collagen fibers. Transforming growth factor β1 (TGFß1), Wnt / β-catenin, and p38 mitogen-activated protein kinase (MAPK) also play a role in regulating the expression of collagen genes [40]. TGFß also binds to the extracellular matrix through binding to latent TGFß-binding protein 1, which is associated with fibronectin 1 and fibrillin microfibrils [41]. TGFß1 stimulates the differentiation of myofibroblasts, resulting in pathological fibrosis (scarring) during tissue regeneration. An additional factor in the differentiation of myofibroblasts is mechanical tension (stiffness) of the tissue that supports profibrotic activation [42]. Various cytokines, including interferon-gamma (IFNy), interleukin (IL)-1, and basic fibroblast growth factors (bFGF, FGF-2), may participate in the suppression of TGFß1 activity. As a result of their action, collagen deposition decreases, and apoptosis is induced [43]. Hypoxia can lead to a decrease in the level of mRNA and type III collagen in chondrocytes and, on the contrary, to their increase in the lungs, resulting in alveolar fibrosis. In the skin, adenosine and purine, which are formed from ATP and ADP, are released in response to hypoxia, trauma or metabolic stress. In fibroblasts, adenosine, acting through its receptors, activates the expression of the *COL3A1* gene [44]. Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) also enhance the expression of *COL3A1* mRNA and protein in human skin fibroblasts through MAPK signaling [45].

EPIGENETIC REGULATION

Epigenetics studies inherited changes in protein synthesis that are not determined by changes in the nucleotide sequence. Typically, such changes are caused by the action of protein synthesis regulators (de-/methylation of DNA, de-/acetylation of histones, de-/phosphorylation of transcription factors, the action of regulatory microRNA (miRNA)) and other intracellular mechanisms. Modification of DNA and histones (involved in DNA packaging in the cell nucleus) alters the histone – histone and histone – DNA interactions, regulating the availability of transcription factors and influencing gene transcription [46]. Among other factors, modification of epigenetic mechanisms underlies the mechanisms of aging of the skin and collagen fibers. The role of DNA and histone methylation, as well as histone acetylation, is the most studied [47]. In particular, DNA methylation results in transcription repression and long-term maintenance of genome stability. However, in some sporadic cases, DNA methylation leads to gene activation in several types of cells [48]. Demethylation of DNA is facilitated by the influence of some external and internal factors. Maintenance of methylated DNA is important for the preservation of progenitor cells and self-renewal of the skin [49].

With aging of the skin, the so-called epigenetic drift accumulates, as a result of which both hypomethylated and hypermethylated DNA regions accumulate. At the same time, ultraviolet (UV) radiation makes a great contribution to DNA hypomethylation, and the degree of hypomethylation is correlated with clinical parameters of skin photoaging [50]. An example of epigenetic changes is a decrease in the regulation of the gene encoding LOX in old fibroblasts, resulting in a decrease in the mechanical properties of the skin [51]. Methylation of histones, depending on the modified site, can lead to activation or suppression of transcription. Acetylation (deacetylation) of histone tails has the opposite effects of methylation (demethylation): acetylation leads to chromatin relaxation and transcription activation; deacetylation, on the contrary, leads to tighter chromatin coiling and transcription inhibition.

Specific NAD^+-dependent enzymes (sirtuins (SIRT)), due to their participation in histone acetylation, play a key role in epigenetic regulation and facilitate transcription. Moreover, they participate in the control over energy metabolism and oxidative stress, cell survival, response to UV damage, DNA repair, tissue regeneration, and inflammation [52]. In the dermis, SIRT can inhibit collagen degradation, regulate DNA repair, and increase the activity of type I collagen synthesis by fibroblasts. The activity of SIRT decreases with age and under conditions of oxidative stress [53].

CONCLUSION

A large number of genetic and epigenetic factors affect the functioning of collagen fibers and, consequently, the mechanical properties of the skin. Gene mutations leading to various collagenopathies may be associated with one of the genes encoding collagen proteins, enzymes involved in post-translational collagen modifications, MMP or glycosaminoglycans [10]. In Russian medicine, the terms differentiated and undifferentiated hereditary connective tissue dysplasias were previously proposed. The introduction of modern methods of molecular genetic diagnosis indicates that the most common hereditary ("differentiated") collagenopathies include osteogenesis imperfecta, Ehlers – Danlos syndrome, Caffey disease, and Marfan syndrome [25], which should be taken into account by doctors of aesthetic medicine. These are monogenic syndromes of Mendelian inheritance caused by causal (pathogenic) gene mutations, in which the contribution of the environment is minimal or absent. For example, the genes involved in the development of Ehlers – Danlos syndrome include *COL5A1, COL5A2, COL3A1, PLOD1, COL1A1, COL1A2, ADAMTS2, TNXB, FMNA, CHST14, SLC39A13, B4GALT7,* and *FKBP14* [54].

On the other hand, the number of associative genetic studies on multifactorial collagenopathies is increasing. In these diseases, both the carriage of polymorphisms in candidate collagen genes and the influence of external environmental factors are important. This is due to higher incidence of multifactorial collagenopathies in the population compared with monogenic collagenopathies, many of which are rare (orphan). The study of the contribution of single nucleotide polymorphisms (SNPs) to the development of multifactorial connective tissue diseases, in general, and to the development of human skin collagen pathology, in particular [55], is relevant. Yet, associative genetic studies on the genes responsible for collagen fiber function are currently insufficient to compile a complete and clear personalized algorithm for the management of such patients by cosmetologists and dermatologists. Therefore, doctors, to a greater extent, focus on the clinical presentation: increased flabbiness, hyper-elasticity, early manifestations of aging, and other signs indirectly indicating collagen pathology. Based on the clinical presentation, a treatment plan is designed aimed at protecting and improving synthesis of collagen fibers. Such recommendations, based on external and internal factors, may include lifestyle changes, additional intake of vitamins and minerals, and mesotherapy (biorevitalization) with amino acids and co-factors necessary for collagen synthesis. Taking into account the results of molecular genetic diagnosis of monogenic and multifactorial collagenopathies and transferring them into real clinical practice are very important. It can improve the effectiveness and safety of local and general therapy for normal and pathological skin aging.

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Potekaev N.N. – conception and design. Borzykh O.B. – review of publications on the topic under discussion, discussion of the study design, drafting of the article. Shnayder N.A. – final approval of the manuscript for publication. Petrova M.M. – substantiation of the manuscript. Karpova E.I. – review of publications on the topic under discussion, drafting of the article. Nasyrova R.F. – critical revision of the manuscript for important intellectual content

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ROSTISLAV S. KARPOV (devoted to the 85th birthday)

On September 8, 2022, we celebrate the 85th birthday of Rostislav S. Karpov, Doctor of Medical Sciences, Professor, Full Member of the Russian Academy of Sciences (RAS). Professor Karpov is a leading national scientist, physician, and cardiologist who has received well-deserved recognition in Russia and abroad. He has gained precious life experience and has invaluable experience in medicine, science, and teaching. He has been serving the good, his patients, students, and colleagues for over 60 years.

Rostislav S. Karpov successfully graduated from Tomsk Medical Institute in 1960 with a degree in General Medicine. He started his professional career as a physician in the Clinics of Tomsk Medical Institute (1962). Then he became the Head of Intermediate-Level Therapy Division with Clinical Pharmacology Course at Siberian State Medical University (1979–2018) and the Head of Cardiology Research Institute, Tomsk (1985–2015). He has been working as a Scientific Supervisor of Cardiology Research Institute of Tomsk National Research Medical Center (NRMC) since June 30, 2015 and as the Head of Research Direction at Tomsk NRMC since 2016 to the present.

Rostislav S. Karpov continued the traditions of Tomsk School of Internal Medicine established by M. S. Kurlov and D. D. Yablokov, Full Members of the Academy of Sciences of the Soviet Union and the Russian Academy of Medical Sciences. Professor Karpov shaped and developed new research areas, including rheumatology, cardiology, pediatric cardiology, and cardiovascular surgery, in Russia and the Tomsk region. Rostislav Karpov has been an organizer and consultant of Tomsk rheumatology service for many years. The scientific, educational, and clinical activities of Rostislav S. Karpov resulted in decreased morbidity, disability, and mortality of patients with rheumatic diseases. Professor Karpov was the founder and the first Dean of the Department for Advanced Medical Training at Tomsk Medical Institute (1979). Its establishment was of fundamental significance for the professional training of medical doctors in the region.

Together with Full Members of the Russian Academy of Medical Sciences E.I. Chazov and V.A. Almazov, Rostislav S. Karpov founded the USSR cardiological service and was responsible for its development in Siberia and the Far East. With direct participation of Professor Karpov, a branch of the All-Union Cardiology Center (which is Cardiology Research Institute today) was opened in Tomsk in 1980. Under his leadership, it became the largest research, clinical, and educational complex in the East of Russia with a branch in Tyumen. Cardiology Research Institute has been the leading institution in Siberia and the Far East in the fields of cardiology and pediatric cardiology for 36 years (1980–2016). Full Member of the Russian Academy of Sciences Rostislav S. Karpov initiated the opening of the first cardiology dispensary beyond the Urals in Tomsk with a capacity of 40 thousand visits per year. Under his leadership, for the first time in Russia, a mobile automated system for providing cardiac care for the scattered population of the Siberian region was developed and implemented in the practical healthcare

of the Tomsk region. Today, the system is being actively developed and continues to function on buses, trains, and river boats around the country.

In 1987, with the active participation and support of the Full Member of the Russian Academy of Medical Sciences V.V. Pekarskii, Rostislav S. Karpov organized one of the first cardiosurgical departments in Russia at the premises of Cardiology Research Institute. The clinic of Cardiology Research Institute has always received special attention and care from Rostislav S. Karpov. Currently, the Institute is a unique cardiovascular complex that allows to solve urgent problems of public healthcare in the fields of cardiology and cardiovascular surgery. The clinic is among top five federal medical institutions providing high-tech medical care for citizens of the Russian Federation.

From the very beginning, Rostislav S. Karpov has set the pace for the innovative and translational research at the Cardiology Research Institute. In order to develop innovative technologies, the Siberian Federal Arrhythmology Center was established at the premises of the Interventional Arrhythmology Department of Cardiology Research Institute in 1998. This department has been recognized as the leading regional center for arrhythmology, which largely determined the progress in the treatment of complex cardiac arrhythmias in people living in Siberia, the Far East, and the neighboring countries. In 2010, with the active support of Rostislav S. Karpov, the innovative Children's Heart Center was set up to help children and adolescents with cardiovascular diseases. It provides services from prenatal diagnosis to multi-stage hemodynamic and electrophysiology treatment of life-threatening congenital heart defects and heart rhythm disorders. Considering the range of interventions performed and outstanding results of treatment, the Children's Heart Center is currently considered one of the leading Russian clinics.

Being a supporter of a patient-centered approach in medicine, Professor Karpov was among the initiators of merging six Tomsk research medical institutes (the research institutes of cardiology, oncology, medical genetics, psychiatry, pharmacology and regenerative medicine, obstetrics, gynecology and perinatology) into a single Tomsk National Research Medical Center of the Russian Academy of Sciences in 2016. After five years, the Center became the largest scientific medical organization in the country. It is ranked first among organizations of the Ministry of Science and Higher Education in the field of clinical medicine.

Rostislav S. Karpov created a well-known therapeutic and cardiological school. His scientific works adequately reflect the most significant stages in solving the pressing problems of modern medical science and healthcare. The main areas of his research are rheumatology, cardiology, clinical pharmacology, clinical and population epidemiology, cardiovascular disease prevention, and public health and healthcare.

He is the author of over 1,000 scientific papers, including 38 monographs and 43 patents for inventions. Under his supervision, 42 dissertations for the degree of Candidate of Sciences and 81 dissertations for the degree of Doctor of Sciences were completed and defended. Students of Professor Karpov successfully work in internal medicine, cardiology, rheumatology, and clinical cardiology in over 40 leading organizations in the country and around the world.

Rostislav S. Karpov was awarded numerous state and public awards for highest excellence.

Rostislav S. Karpov devoted his entire life to serving people: his patients, students, and colleagues. He remains professionally active and generously shares his wisdom and invaluable human and professional experience with those around him.

The administration and the entire team of Cardiology Research Institute of Tomsk NRMC cordially congratulate the Teacher, Friend, and Colleague Rostislav S. Karpov on his 85th birthday and wish him excellent health, joy, positive mood, and active longevity! May the interest in life never fade away, and may positive emotions inspire new achievements!

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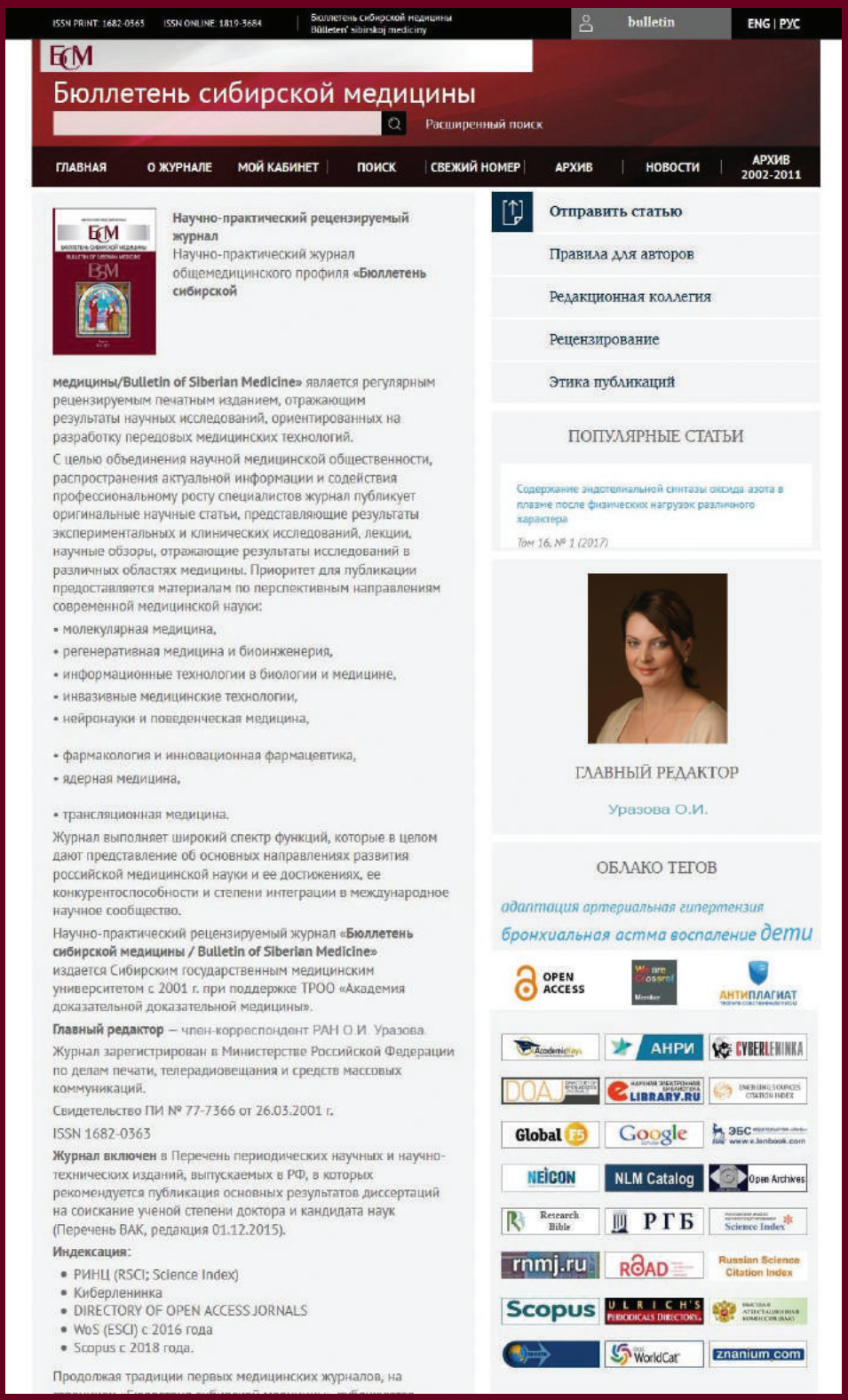

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