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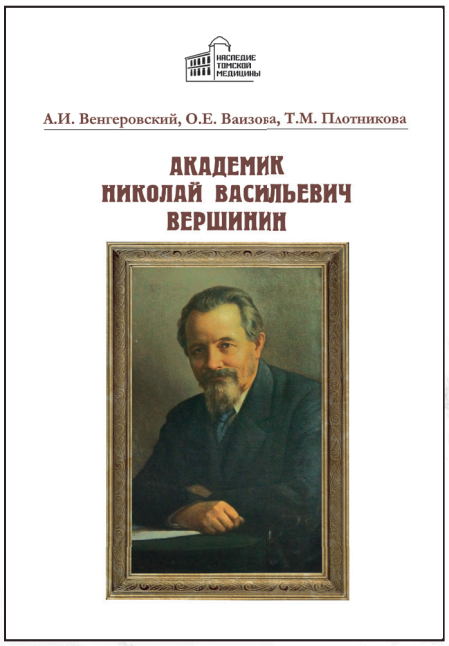

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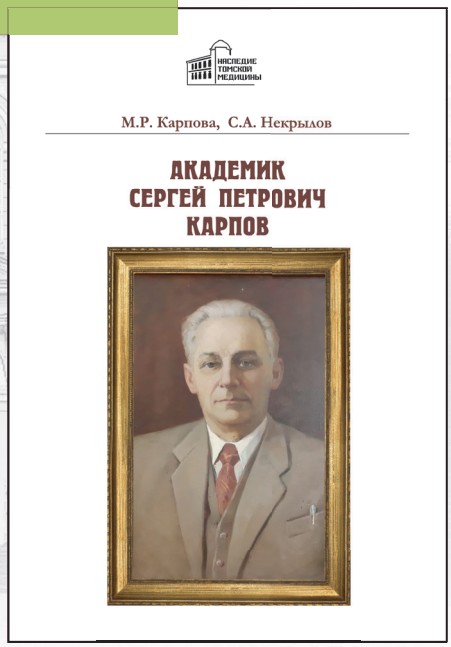

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СОДЕРЖАНИЕ

CONTENTS

ОРИГИНАЛЬНЫЕ СТАТЬИ

ORIGINAL ARTICLES

Dear authors and readers,

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We express our gratitude to the authors for their contributions to the development of our journal. Your efforts help us enhance the level of knowledge and professional qualifications of our readers.

We wish everyone inspiration and new ideas for further scientific research!

Sincerely yours,

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Corresponding Member of RAS
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Factors associated with the development of dynapenia in patients with ulcerative colitis

Bikbavova G.R.[1], Livzan M.A.[1], Lisyutenko N.S.[1], Romanyuk A.E.[1], Bondarenko A.A.[2]

[1] *Omsk State Medical University*
12, Lenina Str., Omsk, 644099, Russian Federation

[2] *Omsk Regional Clinical Hospital*
3, Berezovaya Str., Omsk, 644111, Russian Federation

ABSTRACT

Aim. To evaluate the association of insulin resistance and secretion of neuropeptide Y with dynapenia in patients with ulcerative colitis (UC).

Materials and methods. A single center, observational, cross-sectional study included 80 patients with UC. Participants were divided into two groups: patients with dynapenia and patients with normal hand grip strength. The body mass index (BMI), dietary habits, and stress levels were studied, patients underwent dynamometry. C-reactive protein (CRP), TNFα, interleukin-6, leptin, adiponectin, soluble leptin receptors (sOb-R), neuropeptide Y and peptide YY, insulin and glucose were measured in blood serum. We determined the index of insulin resistance HOMA-IR. Median (*Me*) of the upper and lower quartiles (P_{25}; P_{75}), proportion and standard error of the proportion were calculated. We also applied the Mann – Whitney and Kruskal – Wallis tests, Yates chi-squared test, and two-tailed Fischer's test. The Spearman's correlation coefficient was calculated.

Results. We found that 54 ± 5.6% of patients with dynapenia were overweight or obese. It should be noted that patients with dynapenia were relatively young (35 (32; 51) years). Dynapenia is associated with increased CRP levels, insulin resistance, and higher values of neuropeptide Y. We found a positive correlation between neuropeptide Y and the consumption of simple carbohydrates and alcoholic beverages. The study did not reveal a relationship between the concentration of neuropetide Y and the intensity of UC, the localization of the pathological process, and the course of the disease. A positive association between neuropeptide Y and the level of sOb-R, peptide YY, was established.

Conclusion. Long-lasting chronic inflammation leads to the premature development of dynapenia and insulin resistance in patients with UC at a young age. In patients with dynapenia, the level of neuropeptide Y is significantly higher than in patients without dynapenia, which is probably due to the regulation of energy balance, glucose, and insulin homeostasis.

Keywords: ulcerative colitis, sarcopenia, dynapenia, insulin resistance, neuropeptide Y

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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✉ *Bondarenko Anastasia A.,* kise-1995@mail.ru

Факторы, ассоциированные с развитием динапении у пациентов с язвенным колитом

Бикбавова Г.Р.[1], Ливзан М.А.[1], Лисютенко Н.С.[1], Романюк А.Е.[1], Бондаренко А.А.[2]

[1] *Омский государственный медицинский университет (ОмГМУ)*
Россия, 644099, г. Омск, ул. Ленина, 12

[2] *Областная клиническая больница (ОКБ)*
Россия, 644111, г. Омск, ул. Березовая, 3

РЕЗЮМЕ

Цель: оценить связь инсулинорезистентности и секреции нейропептида Y с динапенией у пациентов с язвенным колитом (ЯК).

Материалы и методы. В одноцентровое обсервационное кросс-секционное исследование включено 80 больных ЯК; участники разделены на две группы: пациенты с динапенией и пациенты с нормальной силой кистевого хвата. Исследован индекс массы тела (ИМТ), проведена динамометрия, изучены особенности питания и определен уровень стресса. В сыворотке крови исследованы С-реактивный белок (СРБ), ФНО-α, интерлейкин-6, лептин, адипонектин, растворимые рецепторы лептина (РРЛ), нейропептид Y, пептид YY, инсулин и глюкоза. Определялся индекс инсулинорезистентности HOMA-IR. Рассчитывалась медиана (*Me*) верхнего и нижнего квартилей (P_{25}; P_{75}); доля и стандартная ошибка доли; критерий Манна – Уитни; критерий Краскела – Уоллиса; χ^2 с поправкой Йетса; критерий Фишера, двусторонний вариант. Рассчитывался корреляционный критерий Спирмена.

Результаты. Имели избыточную массу тела либо ожирение 54 ± 5,6% пациентов с динапенией. Обращает на себя внимание относительно молодой возраст пациентов с динапенией (35 (32; 51) лет). Динапения связана с повышением уровня СРБ, инсулинорезистентностью и более высокими значениями нейропептида Y. Выявлена положительная корреляционная связь нейропептида Y с потреблением простых углеводов и алкогольных напитков. Связи между содержанием нейропетида Y с активностью ЯК, локализацией патологического процесса и характером течения заболевания не выявлено. Установлена положительная связь нейропептида Y с уровнем РРЛ, пептидом YY.

Заключение. Продолжительное хроническое воспаление приводит к преждевременному появлению динапении и развитию инсулинорезистентности у пациентов с ЯК в молодом возрасте. У пациентов с динапенией уровень нейропептида Y значимо выше, чем у пациентов без динапении, что, вероятно, связано с регуляцией энергетического баланса, гомеостазом глюкозы и инсулина.

Ключевые слова: язвенный колит, саркопения, динапения, инсулинорезистентность, нейропептид Y

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

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Соответствие принципам этики. Все лица подписали информированное согласие на участие в исследовании. Исследование одобрено локальным этическим комитетом ОмГМУ (протокол № 97 от 12.10.2017).

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INTRODUCTION

The number of patients with inflammatory bowel diseases (IBD) is increasing, the maximum rate has been observed recently in developing countries [1]. A common feature of geographically unrelated regions with a rapidly growing incidence of ulcerative colitis (UC) is the transition to the Western pattern diet, which includes processed foods, foods containing preservatives, animal fat and protein, and increased consumption of foods with a high glycemic index [2]. These dietary features not only trigger the

occurrence of IBD, but also promote the development of obesity [3, 4].

Recent studies show that from 15 to 40% of IBD patients are obese, and 20–40% are overweight [5]. Thus, a study conducted in Australia showed that within two years after the diagnosis of UC or Crohn's disease and the start of therapy, the proportion of obese patients increased from 23 to 31%, and these growth rates are higher than those of the country's population over a given period [6].

Modern therapeutic and diagnostic approaches have led to a significant pathomorphosis of IBD: the life expectancy of patients increases [7, 8] with the formation of a new type of associative multimorbidity with an increasing proportion of patients with metabolic syndrome, cardiovascular pathology [8, 9], and sarcopenic obesity [10]. It has been established that sarcopenia and sarcopenic obesity are not just conditions and a consequence of pathology, but a predictor of adverse outcomes of IBD [11]. The mechanisms of sarcopenia development in patients with UC include chronic inflammation, malnutrition, malabsorption with insufficient protein intake, as well as low physical activity [12].

Studies have demonstrated that insulin resistance contributes to the pathogenesis of sarcopenia and sarcopenic obesity, namely increased degradation of muscle mass, since insulin and insulin-like growth factor-1 are responsible not only for glucose uptake, but also for maintaining muscle mass by stimulating muscle protein synthesis and inhibiting its breakdown. The second mechanism of the association of insulin resistance and sarcopenia includes a number of pathogenetic events: insulin resistance – decreased absorption of cellular calcium – impaired muscle contraction [13].

Diagnosis of sarcopenia and sarcopenic obesity is a time-consuming multi-step process [14]. From a practical point of view, dynapenia (decrease in muscle strength) [15], one of the three criteria for sarcopenia, is not difficult to diagnose and at the same time is a significant indicator in predicting adverse outcomes for patients with UC. In a previously published article [16], we reported that dynapenia is present in 32.5% of UC patients, most of whom are women. Overweight or obesity were observed in 54 ± 5.6% of patients with dynapenia.

The aim of the study is to evaluate the association of insulin resistance and secretion of neuropeptide Y with dynapenia in patients with UC.

MATERIALS AND METHODS

A single-center, cross-sectional, observational study included 80 patients with UC. The diagnosis, treatment, and follow-up of patients were carried out according to the clinical guidelines for the diagnosis and treatment of UC of the Russian Gastroenterological Association and the Russian Association of Coloproctology [17]. The study was conducted on the basis of Omsk Regional Clinical Hospital (clinical base of the Department of Advanced-Level Therapy, Endocrinology of Omsk State Medical University) and on the basis of the Academic Medical Center of Omsk State Medical University.

The study included patients who were followed up on an inpatient and outpatient basis by a gastroenterologist at these healthcare facilities in 2020–2023. BMI was calculated using the formula: weight (kg) / height^2 (m^2). The World Health Organization classification was used to interpret the obtained BMI values. The presence or absence of dynapenia in patients was determined using dynamometry. The hand grip strength measured in Newtons was considered a dynapenia when it was less than 16N in women and less than 27N in men [15]. The study of dietary habits was carried out using a standardized questionnaire of the World Health Organization CINDI program [18]. The questionnaire includes 12 questions regarding the frequency and amount of consumption of meat, fruits, vegetables, and simple carbohydrates. The Reeder Stress Inventory was used to determine the stress level [19]. All the questions were combined into one questionnaire, and respondents were asked to complete it.

The blood serum of patients was examined on an iMark tablet photometer (Bio-Rad, USA) by enzyme immunoassay of inflammatory parameters: C-reactive protein (CRP), tumor necrosis factor α (TNFα), interleukin-6 (IL-6); adipose tissue hormones (adipokines) – leptin, adiponectin, soluble leptin receptors (sOb-R); peptides (neuropeptide Y and peptide YY); indicators of carbohydrate metabolism – insulin and glucose. The study of TNFα and IL-6 concentration was carried out using test systems (Vector-Best, Russia). Leptin was assessed using the ELISA test system (DBC, Canada), adiponectin using the ELISA test system (Mediagnost, Germany), neuropeptide Y – using the Cloud-Clone test system (China) and YY peptide – using the ELISA test system (VMA, Switzerland), insulin – using the

Vector-Best test systems (Vector-best, Russia). Insulin resistance was assessed by the HOMA-IR indicator (Homeostatic Model Assessment of Insulin Resistance) according to the formula HOMA-IR = fasting insulin, mcU/ml × fasting glycemia, mmol/l/ 22.5. The HOMA-IR value of more than 2.7 indicated the presence of IR.

The median (*Me*) age of all patients included in the study was 38 (32; 48.5) years, among whom there were 45 women, *Me* age was 34 (32; 45) years and 35 men, *Me* age 42 (34; 52) years. Acute course of UC was observed in 16 (20 ± 4.5%) patients, chronic recurrent course – in 45 (56 ± 5.5%) patients, 19 (24 ± 5.5%) patients had a chronic continuous course. Overweight and obesity in UC patients were observed in 46 ± 5.6% of patients.

Inclusion criteria were as follows: the presence of diagnosed UC, a signed informed consent to participate in the study. Exclusion criteria included participation in a clinical trial of unregistered medicines; age under 18 years; pregnancy; professional athletics; the presence of diseases of the musculoskeletal system and systemic connective tissue diseases.

The study is observational and does not involve additional medical interventions. To conduct the study, all participants were divided into two groups: 26 patients with dynapenia (32.5%) and 54 individuals (67.5%) with normal hand grip strength. The study was approved by the local Ethics Committee of Omsk State Medical University (Protocol No. 97 of 12.10.2017).

The Statistica 10.01.1011 program was used to analyze the results of the study. Median (*Me*) of the upper and lower quartiles (P_{25}, P_{75}) was calculated to describe quantitative features. The proportion and the standard error of the proportion were calculated to describe the frequency of occurrence of a binary feature. The Mann – Whitney test was conducted to compare the two groups by quantitative criteria. The Kruskal – Wallis test was used to compare several groups based on quantitative characteristics. Spearman's rank correlation (*R*) was calculated to identify a statistical relationship between quantitative features.

RESULTS

As can be seen from the Table, patients with dynapenia and patients without dynapenia did not differ in age, duration of the disease, and BMI (Table).

At the same time, 14 out of 26 patients with dynapenia had a body weight corresponding, according to WHO criteria, to excess weight or obesity (17.5 ± 4.2% of the total number of patients with UC, 54± 05.6% of patients with UC and dynapenia). In the subgroup of patients without dynapenia, 24 patients were overweight or obese (30 ± 5.1% of the total number, 37.5 ± 6.1% of patients without dynapenia).

Table

Comparison of patients with dynapenia and patients without dynapenia by age, duration of the disease, and BMI, *Me* (P_{25}; P_{75})

Parameter	Patients with dynapenia	Patients without dynapenia	*p* for the Mann – Whitney test
Age	35 (32; 51)	41 (34; 52)	0.237
Duration of the disease	4 (2; 8)	6.5 (2; 9)	0.642
BMI, kg/m^2	25.6 (20.0; 29.0)	24 (21.5; 28.4)	0.856

We studied the relationship of neuropeptide Y concentration with dietary habits (the amount of vegetables, fruits, meat consumed per day, g), alcohol consumption (g per week) and stress levels (the number of points according to the Reeder Stress Inventory) in patients with UC. The data obtained indicate that there is no connection with the amount of vegetables (Spearman's rank correlation coefficient $R = –0.008$; $p = 0.946$) and fruits ($R = 0.154$; $p = 0.170$), meat ($R = –0.177$; $p = 0.113$) consumed by patients. A positive correlation was established between the neuropeptide Y concentration with alcohol consumption ($R = 0.232$; $p = 0.037$) and simple carbohydrates ($R = 0.230$; p for $R = 0.039$). According to the results of questionnaires of patients with UC, the analysis of associations of neuropeptide Y concentration with stress levels did not confirm the relationship (in all statements, for the Kruskal – Wallis test $p > 0.05$).

The blood concentration of neuropeptide Y is significantly higher in patients with dynapenia (0.021 (0.019; 0.0237)) than in patients without dynapenia (0.019 (0.017; 0.021); for the Mann–Whitney test $p = 0.014$). The range of fluctuations in the neuropeptide Y concentration in UC patients did not exceed the reference values (0–10 ng/ml) and amounted to 0.014–0.050 ng/ml, 0.02 (0.018; 0.023). The level of neuropeptide Y was higher in young UC patients ($R = –0.251$; $p = 0.024$).

Gender differences in the neuropeptide Y concentration in patients with UC were revealed. Thus, the level of neuropeptide Y in women (0.021 (0.014; 0.043)) was significantly higher than in men

(0.019 (0.018; 0.022); for the Mann – Whitney test $p = 0.041$). There were no differences in the level of neuropetide Y in patients with varying degrees of UC activity (the Kruskal – Wallis test = 2.058; $p = 0.560$), localization of the pathological process in the colon (the Kruskal – Wallis test = 1.126; $p = 0.569$) and the course of the disease (the Kruskal – Wallis test = 2.342; $p = 0.310$). The study established a positive association of neuropeptide Y with sOb-R ($R = 0.331$; $p = 0.002$), peptide YY ($R = 0.529$; $p < 0.001$). The correlation analysis revealed a trend toward a higher neuropeptide Y concentration in patients with low levels of adiponectin, however, this relationship was not statistically significant ($R = p$ for $R = 0.068$). The study did not reveal a statistical relationship between the concentration of the studied neuropeptide hormones and inflammatory laboratory markers: peptide YY with TNFα ($R = 0.197$; $p = 0.234$), IL-6 ($R = –0.022$; $p = 0.892$), CRP ($R = 0.105$; $p = 0.524$); neuropeptide Y with TNFα ($R = 0.006$; $p = 0.824$), IL-6 ($R = 0.13$; $p = 0.430$), CRP ($R = –0.014$; $p = 0.898$).

The level of HOMA-IR in patients with dynapenia (0.8 (0.2; 1.7)) was significantly higher compared with patients without dynapenia (0.2 (0.1; 0.5); for the Mann – Whitney test $p = 0.026$).

As demonstrated in previous work [16], the CRP level in patients with dynapenia (10.7 (4.020; 14.400)) was significantly higher than in patients without dynapenia (3.430 (0.860; 11.198); for the Mann – Whitney test $p = 0.006$) (Figure).

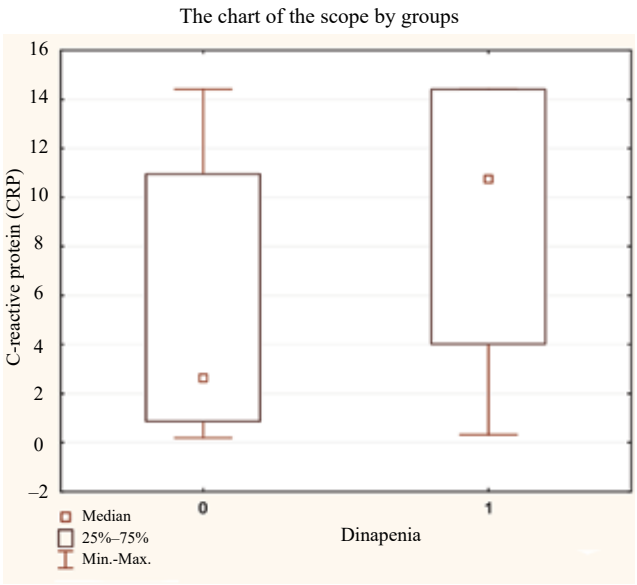

Figure. The concentration of C-reactive protein in UC patients with and without dynapenia

DISCUSSION

The study demonstrated that 54 ± 5.6% of patients with UC and dynapenia are overweight or obese, while among patients with dynapenia 65.4 ± 9.3% of patients were young according to the WHO criteria (younger than 45 years). Dynapenia in younger patients is associated with increased CRP levels, insulin resistance, and higher concentrations of neuropeptide Y. A positive correlation between neuropeptide Y and the consumption of simple carbohydrates and alcoholic beverages was revealed. The study did not reveal a dependence between the neuropetide Y concentration and the activity of UC, the localization of the pathological process in the colon and the course of the disease. A positive association of neuropeptide Y with the level of leptin receptors, peptide YY, has been established. The correlation analysis revealed a trend toward a higher neuropeptide Y concentration in patients with lower adiponectin, but this relationship was not statistically significant.

In the results of the previous study, we demonstrated a correlation between dynapenia in UC patients with malnutrition, low physical activity, formula in infancy and inflammation in the form of increased CRP levels [16]. The results of this study complement the previous one as it revealed the association of dynapenia with insulin resistance. We focus on the fact that most of the examined patients with dynapenia are overweight or obese. When patients suffer from the disease, their' physical activity decreases, which, in combination with an autoimmune inflammatory process, leads not only to the premature onset of dynapenia, but also to the possible development of insulin resistance. A major imbalance in energy exchange due to malnutrition, consumption of foods with a high glycemic index, decreased physical activity and chronic systemic inflammation trigger protein degradation mechanisms [21].

An increase in the level of neuropeptide Y in patients with UC and dynapenia, sarcopenia, and sarcopenic obesity requires research and substantiates the need for further comprehensive analysis, in particular, analysis of the contribution of neuropetide Y to the mechanisms of protein degradation, lipogenesis, and homeostasis of metabolism, which can further be used in effective strategies for the management and treatment of

patients. To date, it is known that neuropeptide Y is a powerful appetite stimulant and pro-inflammatory neurohormone/mediator, the secretion of which takes placet not only in the hypothalamus, but also in the peripheral nervous system and, in particular, in enteric neurons.

Literature describes the physiological effects of neuropeptide Y in reducing the energy expenditure of the body [22], reducing the motility of the gastrointestinal tract (neuropeptide Y and peptide YY are mediators of the ileal brake); inhibition of gastric, biliary, and pancreatic secretion; interaction of the immune and enteric nervous systems [23]. We assume that in patients with dynapenia, significantly higher levels of neuropeptide Y are associated with a body's need to reduce energy expenditure. The correlation between neuropetide Y in patients with dynapenia and the consumption of simple carbohydrates indicates the orexigenic effect of this peptide with the possibility of rapid recovery of energy balance.

Our study demonstrates that the serum neuropetide Y concentration does not exceed the reference values in patients with UC both with and without dynapenia. This is consistent with the results of a study by scientists from the Regensburg Hospital (Germany) [24], which indicate the absence of activation of the hypothalamus through the autonomic nervous system as a result of an autoimmune inflammatory process in UC.

Literature presents another point of view. For many years, a research group led by M. El-Salhy has studied the role of neuropeptide Y in the pathogenesis of functional and organic pathology of the colon [25, 26]. According to the researchers, changes in the expression of neuropeptides in IBD play a key role in the pathogenesis due to an increase in the density of neuropeptide Y-positive fibers and neurons of the enteric nervous system, which when interacting with immune cells has a pro-inflammatory effect. The researchers suggested that affecting the expression of neuropeptide Y may become an effective strategy for IBD therapy. A study conducted in Korea [27] demonstrated that an increase in the expression of neuropeptide Y in IBD may reflect a counterregulatory response to anorexia caused by inflammation, since neuropeptide Y is one of the most powerful orexigenic peptides. A review by M. Botelho provides data on the anti-inflammatory effect of neuropeptide Y [28].

CONCLUSION

This study demonstrated the association between dynapenia and insulin resistance in patients with UC for the first time. It is noteworthy that the majority of patients with UC and dynapenia were young, which indicates its premature development in this pathology. The relationship of neuropeptide Y with pro-inflammatory cytokines (CRP, TNFα, and IL-6), disease activity, localization of the pathological process, and the course of the disease has not been established.

According to our study, the neuropeptide Y concentration in patients with dynapenia is higher than in patients without dynapenia, which is probably due to the regulation of energy balance, glucose and insulin homeostasis. The contribution of central metabolic regulation and expression of neuropetide Y to the pathogenesis of sarcopenia, dynapenia, and sarcopenic obesity in general and in patients with UC in particular requires further studying.

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Authors' contribution

Livzan M.A., Bikbavova G.R. – conception and design. Lisyutenko N.S., Bikbavova G.R., Romanyuk A.E., Bondarenko A.A. – analysis and interpretation of data. Livzan M.A., Bikbavova G.R. – substantiation of the manuscript or critical revision of the manuscript for important intellectual content. Livzan M.A. – final approval of the manuscript for publication.

Authors' information

Bikbavova Galiya R. – Cand. Sci. (Med.), Associate Professor, Department of Advanced-Level Therapy, Endocrinology, Omsk State Medical University, Omsk, galiya1976@mail.ru, https://orcid.org/0000-0001-9252-9152

Livzan Maria A. – Member of the Russian Academy of Sciences, Professor, Head of the Department of Intermediate-Level Therapy and Gastroenterology, Rector of Omsk State Medical University, Omsk, mlivzan@yandex.ru, https://orcid.org/0000-0001-6581-7017

Lisyutenko Natalia S. – Cand. Sci. (Med.), Department of Advanced-Level Therapy, Endocrinology, Omsk State Medical University, Omsk, n.labuzina@mail.ru, https://orcid.org/0000-0003-4088-240

Romanyuk Alisa E. – Student, General Medicine Faculty, Omsk State Medical University, Omsk, romalisa00@mail.ru, https://orcid.org/0000-0001-6308-4377

Bondarenko Anastasia A. – Doctor, Department of Gastroenterology, Omsk Regional Clinical Hospital, Omsk, kise-1995@mail.ru, https://orcid.org/0009-0009-9761-9101

(✉) **Bondarenko Anastasia A.,** kise-1995@mail.ru

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Radionuclide imaging of HER2/neu expression in metastatic axillary lymph nodes in breast cancer patients: comparing the efficacy of [99mTc]Tc-ADAPT6 and [^{99m}Tc]Tc-(HE)$_3$-G3

Bragina O.D.[1, 2], Tashireva L.A.[1], Garbukov E.Yu.[1], Vostrikova M.A.[1], Romanova A.A.[1], Deyev S.M.[2, 3, 5], Borodina M.E.[4], Chernov V.I.[1, 2, 5]

[1] *Cancer Research Institute, Tomsk National Research Medical Center (NRMC)*
5, Kooperativny Str., Tomsk, 634009, Russian Federation

[2] *National Research Tomsk Polytechnic University (NR TPU)*
30, Lenina Av., Tomsk, 634050, Russian Federation

[3] *Shemyakin – Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences, 16/10, Miklukho – Maklaya Str., Moscow, 117997, Russian Federation*

[4] *P. Hertsen Moscow Oncology Research Institute*
3, 2nd Botkinsky Proyezd Str., Moscow, 125284, Russian Federation

[5] *National Research Center «Kurchatov Institute»*
1, Akademika Kurchatova Str., Moscow, 123098, Russian Federation

ABSTRACT

Aim. To conduct a direct comparative analysis of single-photon emission computed tomography (SPECT-CT) with [^{99m}Tc]Tc-ADAPT6 and [^{99m}Tc]Tc-(HE)$_3$-G3 in patients with HER2-positive breast cancer (BC) with axillary lymph node metastases.

Materials and methods. The analysis included 8 patients with HER2-positive BC with axillary lymph node metastases before the systemic treatment. All patients were injected with [^{99m}Tc]Tc-ADAPT6 (500 μg) and [^{99m}Tc]Tc-(HE)$_3$-G3 (3,000 μg) with an interval of 3–4 days. The SPECT-CT scans of the chest and upper abdomen were performed after 2 hours for [^{99m}Tc]Tc-ADAPT6 and after 4 hours for [^{99m}Tc]Tc-(HE)$_3$-G3. The accumulation of radiopharmaceuticals was assessed by measuring the *maximum standardized uptake* values (SUV$_{max}$) in metastatic axillary lymph nodes, projections of the contralateral axillary lymph nodes, liver, latissimus dorsi muscle, and spleen. Additionally, mALN-to-background and mALN-to-reference organs ratios were calculated for each patient.

Results. Comparison of the mALN-to-background ratio revealed the advantage of [99mTc]Tc-ADAPT6 (38.93 (16.56–56.02)) over [^{99m}Tc]Tc-(HE)$_3$-G3 (19.39 (8.43–34.52)), p = 0.0391. The comparative analysis of the accumulation of the studied radiopharmaceuticals in the reference organs demonstrated higher SUV$_{max}$ for [^{99m}Tc]Tc-(HE)$_3$-G3 in the liver and spleen (4.44 (2.85–9.08) and 2.47 (1.28–4.41), respectively) than for [99mTc]Tc-ADAPT6 (2.98 (1.96–3.65) and 0.43 (0.14–0.62), respectively), p = 0.01 and p = 0.04. Comparison of the SUV$_{max}$ ratios in mALN and reference organs showed higher values of mALN / spleen for [99mTc]Tc-ADAPT6 (5.93 (1.04–11.85)) compared to [^{99m}Tc]Tc-(HE)$_3$-G3 (1.83 (0.46–4.54)), p = 0.02.

Conclusion. According to the results of the performed analysis, the diagnostic advantage of [99mTc]Tc-ADEPT6 for the detection of HER2/neu expression in metastatic lymph nodes in breast cancer patients was revealed.

Keywords: breast cancer, ADAPT6, DARPinG3, radionuclide diagnosis

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

✉ *Bragina Olga D.,* bragina_od@mail.ru

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Радионуклидная визуализация экспрессии HER2/NEU в метастатических аксиллярных лимфатических узлах у больных раком молочной железы: сравнение эффективности препаратов [^{99M}TC]TC-ADAPT6 и [^{99M}TC]TC-(HE)$_3$-G3

Брагина О.Д.[1, 2], Таширева Л.А.[1], Гарбуков Е.Ю.[1], Вострикова М.А.[1], Романова А.А.[1], Деев С.М.[2, 3, 5], Бородина М.Е.[4], Чернов В.И.[1, 2, 5]

Научно-исследовательский институт (НИИ) онкологии, Томский национальный исследовательский медицинский центр (НИМЦ) Российской академии наук
Россия, 634009, г. Томск, пер. Кооперативный, 5

[2] *Национальный исследовательский Томский политехнический университет (НИ ТПУ)*
Россия, 634050, г. Томск, пр. Ленина, 30

[3] *Институт биоорганической химии (ИБХ) им. акад. М.М. Шемякина и Ю.А. Овчинникова Российской академии наук (РАН)*
Россия, 117997, г. Москва, ГСП-7, ул. Миклухо-Маклая, 16/10

[4] *Московский научно-исследовательский онкологический институт (МНИИОИ) им. П.А. Герцена – филиал НМИЦ радиологии*
Россия, 125284, г. Москва, 2-й Боткинский пр-д, 3

[5] *Национальный исследовательский центр (НИЦ) «Курчатовский институт»*
Россия, 123098, г. Москва, пл. Академика Курчатова, 1

РЕЗЮМЕ

Цель**.** Провести прямой сравнительный анализ данных однофотонной эмиссионной компьютерной томографии с препаратами [^{99m}Tc]Tc-ADAPT6 и [^{99m}Tc]Tc-(HE)$_3$-G3 у больных раком молочной железы (РМЖ) с HER2-позитивными метастазами в аксиллярные лимфатические узлы.

Материалы и методы. В анализ включены восемь больных РМЖ с HER2-позитивными метастазами в аксиллярные лимфатические узлы (мАЛУ) до начала системного лечения. Всем больным последовательно проводилось введение препаратов [^{99m}Tc]Tc-ADAPT6 (500 мкг) и [^{99m}Tc]Tc-(HE)$_3$-G3 (3 000 мкг) с интервалом 3–4 дня. Однофотонная эмиссионная компьютерная томография органов грудной клетки и верхнего этажа брюшной полости проводилась через 2 ч для [^{99m}Tc]Tc-ADAPT6 и через 4 ч для [^{99m}Tc]Tc-(HE)$_3$-G3. Оценка накопления соединений выполнялась путем измерения максимального стандартного захвата (SUV_{max}) в метастатических аксиллярных лимфоузлах, проекции контралатеральной аксиллярной области, проекций печени, широчайшей мышцы спины и селезенки. Дополнительно у каждой больной рассчитывались такие параметры, как мАЛУ/фон и мАЛУ/референсные органы.

Результаты. Сравнение соотношения мАЛУ/фон выявило преимущество препарата [^{99m}Tc]Tc-ADAPT6 (38,93 (16,56–56,02)) над [^{99m}Tc]Tc-(HE)$_3$-G3 (19,39 (8,43–34,52)), p = 0,0391. Сравнительный анализ аккумуляции изучаемых радиофармпрепаратов в референсных органах продемонстрировал более высокий SUV_{max} в печени и селезенке для [^{99m}Tc]Tc-(HE)$_3$-G3 (4,44 (2,85–9,08) и 2,47 (1,28–4,41) соответственно), чем при использовании [^{99m}Tc]Tc-ADAPT6 (2,98 (1,96–3,65) и 0,43 (0,14–0,62) соответственно), p = 0,01 и p = 0,04. Сравнение соотношений SUVmax в мАЛУ и референсных органах показало более высокие

значения параметра мАЛУ/селезенка для препарата [^{99m}Tc]Tc-ADAPT6 (5,93 (1,04–11,85)) по сравнению с [^{99m}Tc]Tc-(HE)$_3$-G3 (1,83 (0,46–4,54)), p = 0,02.

Заключение. По результатам выполненного анализа выявлено диагностическое преимущество препарата [^{99m}Tc]Tc-ADAPT6 для детекции HER2 статуса в метастатических лимфатических узлах у больных РМЖ.

Ключевые слова: рак молочной железы, ADAPT6, DARPinG3, радионуклидная диагностика

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INTRODUCTION

Determining the status of regional lymph nodes is a mandatory step in the pre-hospital diagnosis of patients with breast cancer (BC). This information is primarily needed for planning the optimal scope of local and systemic treatment to achieve better overall and relapse-free survival rates [1]. Unfortunately, existing diagnostic methods, such as ultrasound (US), mammography, and computed tomography (CT), are not optimal and have a relatively high probability of false-positive and false-negative results [2–4].

For example, it has been proven that the sensitivity and specificity of US directly depend on the biological subtype of the tumor. According to R. Helfgott et al., the minimum sensitivity level of US in assessing the lymph node status was observed in patients with luminal HER2-negative BC (less than 40%), while the maximum sensitivity was noted for triple-negative and HER2-positive subtypes (68.8 and 71.4%, respectively) [3]. Moreover, rapidly evolving technologies and demands in clinical medicine create the necessity not only for anatomical detection, but also for the assessment of the molecular profile of the tumor to personalize systemic therapy in BC patients [1, 2].

Studying the molecular profile of identified metastatic changes is particularly relevant not only due to the need for additional invasive (sometimes difficult) diagnostic procedures, but also in light of existing intertumoral heterogeneity, which causes differences in the molecular characteristics of the primary tumor and metastatic foci [5]. According to the literature, the discrepancy in the receptor status between the primary tumor and regional lymph nodes can reach 30% for estrogen receptors, 20% for progesterone receptors, and 15% for HER2/neu [6].

One of the potential solutions to this clinical problem is exploring the capabilities of targeted radionuclide imaging for a specific molecular target [7]. Among “targeting” modules, alternative scaffold proteins have demonstrated the highest efficacy. These proteins are characterized by high specificity and affinity for the target antigen, low toxicity, and rapid clearance from the patient’s body after administration, thereby significantly reducing the time from the injection to the start of the diagnostic procedure [8]. One of the options for this targeted interaction could be human epidermal growth factor receptor 2 (HER2/neu), whose overexpression occurs in 20–30% of BC patients and requires the use of targeted therapy [9].

Phase II clinical trials with [^{99m}Tc]Tc-ADAPT6 (ClinicalTrials.gov Identifier: NCT05412446) and [^{99m}Tc]Tc-(HE)$_3$-G3 (ClinicalTrials.gov Identifier: NCT15122022) were conducted at the Department of Radionuclide Therapy and Diagnostics of Cancer Research Institute of Tomsk NRMC and assessed HER2/neu expression in metastatic axillary lymph nodes (mALNs) in patients with BC. The results indicated the efficacy of both agents ($p < 0.05$, Mann – Whitney test) [10, 11].

The aim of this study was to conduct a direct comparative analysis of SPECT-CT data using [^{99m}Tc]Tc-ADAPT6 and [^{99m}Tc]Tc-$(HE)_3$-G3 in patients with HER2-positive BC and mALNs.

MATERIALS AND METHODS

The analysis included 8 patients with HER2-positive BC and metastases in the axillary lymph nodes prior to the initiation of systemic treatment. All patients were injected with [^{99m}Tc]Tc-ADAPT6 and [^{99m}Tc]Tc-$(HE)_3$-G3 with an interval of 3–4 days.

Morphological and immunohistochemical studies of biopsy material obtained from the axillary lymph node tissue were performed in all patients. HER2/neu expression was considered positive if the immunohistochemistry (IHC) showed a score of 3+ or a score of 2+ with positive fluorescence *in situ* hybridization (FISH). Cases with receptor expression of 0 and 1+ by IHC were classified as negative, in accordance with the ASCO/CAP (American Society of Clinical Oncology and the College of American Pathologists) criteria from 2018 [12]. The size of the lymph nodes was measured using US before the initiation of systemic treatment and biopsy collection.

[^{99m}Tc]Tc-$(HE)_3$-G3 and [^{99m}Tc]Tc-ADAPT6 were prepared using the previously described tricarbonyl radiolabeling method under sterile conditions at the Department of Radionuclide Therapy and Diagnostics of Cancer Research Institute of Tomsk NRMC, immediately before intravenous administration. The dosage was 3,000 μg for [^{99m}Tc]Tc-$(HE)_3$-G3 and 500 μg for [^{99m}Tc]Tc-ADAPT6.

SPECT-CT of the chest and upper abdomen was performed in all patients 2 hours after the [^{99m}Tc]Tc-ADAPT6 injection and 4 hours after the [^{99m}Tc]Tc-$(HE)_3$-G3 injection. The accumulation of the radiopharmaceuticals was assessed by measuring the *maximum standardized uptake* values (SUV_{max}) in metastatic axillary lymph nodes and projections of the contralateral axillary lymph nodes and reference organs, such as liver, latissimus dorsi muscle, and spleen. Additionally, mALN-to-background and mALN-to-reference organs ratios were calculated for each patient. SUV_{max} was determined in the largest mALN, corresponding in anatomical location to the US description and biopsy material collection (Table).

Data analysis and visualization were performed using Prism 10 software (GraphPad, USA). The accumulation values of the agents were presented as the median and the interquartile range (Me (Q_1–Q_3)). The non-parametric Wilcoxon signed-rank test was used to determine the significance of differences between the accumulation values of the two agents. The differences were considered significant at $p < 0.05$.

Table

Accumulation of [^{99m}Tc]Tc-$(HE)_3$-G3 and [^{99m}Tc]Tc-ADAPT6 in metastatic HER2-positive axillary lymph nodes (SUV_{max}) and reference organs and mALN / reference organ ratios in patients with breast cancer

No.	SUV_{max} (mALN)	SUV_{max} (contralateral ALN)	mALNs/background	SUV_{max} (liver)	SUV_{max} (LDM)	SUV_{max} (spleen)	mALN/ liver	mALN/ LDM	mALN/ spleen
[^{99m}Tc]Tc-$(HE)_3$-G3									
1	1.8	0.3	6.7	9.1	0.3	4.0	0.2	6.2	0.5
2	2.6	0.2	15.2	5.2	0.3	2.5	0.5	8.6	1.0
3	2.2	0.2	13.5	3.0	0.3	1.3	0.7	6.2	1.7
4	10.7	0.3	33.3	4.7	0.4	2.5	2.3	26.0	4.3
5	8.7	0.3	34.9	5.7	0.4	2.1	1.5	21.3	4.2
6	2.4	0.4	5.9	4.1	0.2	1.7	0.6	10.9	1.5
7	14.0	0.3	41.2	2.9	0.5	3.1	4.9	25.9	4.5
8	8.7	0.4	23.5	3.4	0.3	4.4	2.6	27.2	1.9
[^{99m}Tc]^{99m}Tc-ADAPT6									
1	14.6	0.4	39.6	3.7	0.1	2.5	4.0	104.6	5.9
2	4.7	0.2	21.4	1.9	0.3	0.8	2.4	16.2	5.9
3	4.3	0.3	14.9	2.7	0.6	1.9	1.6	7	2.2
4	6.5	0.1	59.3	3.2	0.4	0.6	2.1	14.8	11.9
5	2.9	0.2	13.7	2.9	0.5	1.7	6	1.7	1.0
6	14.6	0.4	38.3	3.1	0.6	1.4	4.7	25.1	10.5
7	8.6	0.1	107.8	2.7	0.4	1.1	3.2	20.5	8.1
8	16.7	0.4	46.3	3.5	0.4	2.9	4.9	40.6	5.7

Note. mALN – metastatic axillary lymph node; LDM – latissimus dorsi muscle.

RESULTS

The results of the immunohistochemical analysis showed a HER2-positive status in the metastatic axillary lymph nodes of all patients included in the study. The obtained data were consistent with the results of the radionuclide studies with both agents. The average size of the lymph nodes was 20.5 ± 4.2 mm.

Comparing the accumulation of the agents showed comparable SUV_{max} levels in the metastatic axillary lymph nodes for [^{99m}Tc]Tc-ADAPT6 at 7.57 (4.43–14.62) and for [^{99m}Tc]Tc-$(HE)_3$-G3 at 5.65 (2.22–10.18) (p = 0.4609). The comparison of the mALNs / background ratio revealed an advantage of [^{99m}Tc]Tc-ADAPT6 (38.93 (16.56–56.02)) over [^{99m}Tc]Tc-$(HE)_3$-G3 (19.39 (8.43–34.52), p = 0.0391) (Fig. 1).

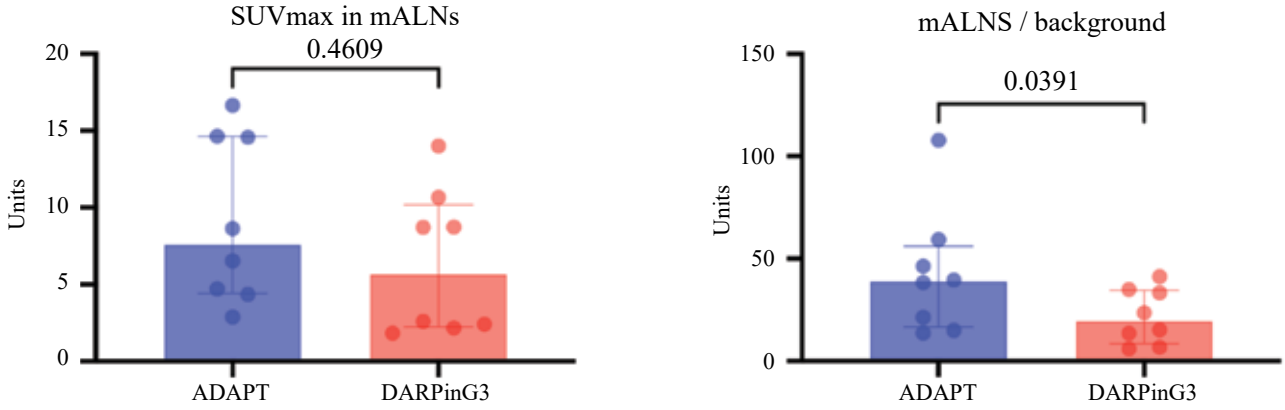

Fig. 1. SUV_{max} in mALNs and the mALNs / background ratio using [^{99m}Tc]Tc-ADAPT6 and [^{99m}Tc]Tc-$(HE)_3$-G3 in patients with HER2-positive breast cancer

The comparative analysis of the accumulation of the studied radiopharmaceuticals (RPs) in the reference organs demonstrated higher SUV_{max} in the liver and spleen for [^{99m}Tc]Tc-$(HE)_3$-G3 (4.44 (2.85–9.08) and 2.47 (1.28–4.41), respectively) than for [^{99m}Tc]Tc-ADAPT6 (2.98 (1.96–3.65) and 0.43 (0.14–0.62), respectively) (p = 0.01 and p = 0.04). The analysis of [^{99m}Tc]Tc-ADAPT6 (0.43 (0.14–0.6)) and [^{99m}Tc]Tc-$(HE)_3$-G3 (0.33 (0.22–0.54)) accumulation in the projection of the spleen did not reveal any significant differences (p = 0.5) (Fig. 2).

Comparison of the SUV_{max} ratios in the mALNs and reference organs showed higher values for mALNs / spleen for [^{99m}Tc]Tc-ADAPT6 (5.93 (1.04–11.85)) compared to [^{99m}Tc]Tc-$(HE)_3$-G3 (1.83 (0.46–4.54), p = 0.02). The comparison of the mALNs / liver (3.58 (1.58–6.00) and 1.12 (0.20–4.91), respectively) and mALNs / latissimus dorsi muscle ratios (18.37 (1.70–104.6) and 16.12 (6.17–27.22), respectively) did not show significant differences between the studied agents (p = 0.06 and p = 0.55, respectively) (Fig. 3).

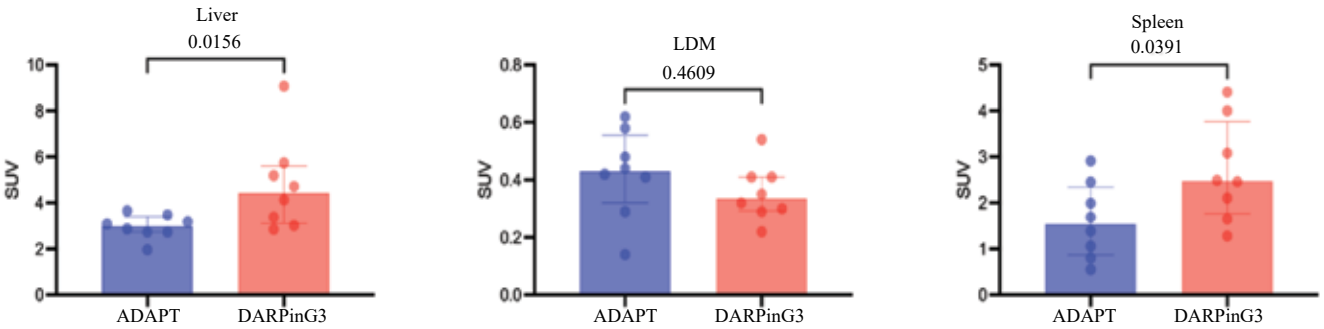

Fig. 2. SUV_{max} in the liver, latissimus dorsi muscle, and spleen using [^{99m}Tc]Tc-ADAPT6 and [^{99m}Tc]Tc-$(HE)_3$-G3 in patients with HER2-positive breast cancer

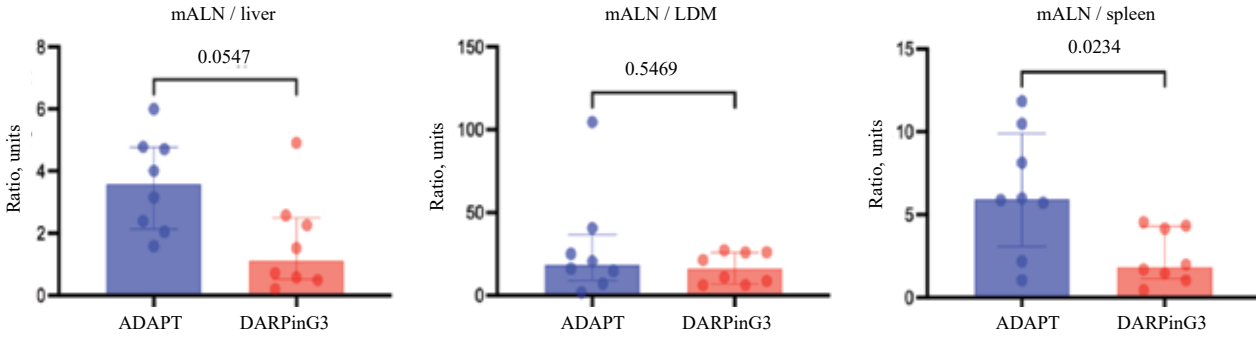

Fig. 3. Ratios of mALNs / liver, mALNs / latissimus dorsi muscle (LDM), and mALNs / spleen using [^{99m}Tc]Tc-ADAPT6 and [^{99m}Tc]Tc-(HE)$_3$-G3 in patients with HER2-positive breast cancer

DISCUSSION

Despite advancements in imaging technology, the challenge of assessing the status of regional lymph nodes in BC patients remains unresolved. This issue is particularly critical at the pre-hospital diagnostic stage, where obtaining the most accurate information is essential for determining appropriate local and systemic treatment strategies. One approach to anatomical detection and molecular typing of detected lesions (both primary tumors and metastatic sites) is to expand the use of radioisotope methods and focus on targeted molecular imaging. This approach, based on the use of RPs that are tropic to specific molecular targets, has gained significant popularity over the past 10 years. It was during this period that the active use of alternative scaffold proteins as "targeting" modules began, along with their clinical testing for the theranostics of cancers.

The Department of Radionuclide Therapy and Diagnostics at Cancer Research Institute of Tomsk NRMC has extensive experience in conducting clinical trials on the diagnosis of malignant tumors using labeled scaffold proteins [13]. Studies involving RPs targeting the human epidermal growth factor receptor 2 (HER2) have been particularly widespread. Phase I clinical trials of [^{99m}Tc]Tc-ADAPT6 (ClinicalTrials.gov Identifier: NCT03991260 and NCT05412446), [^{99m}Tc]Tc-(HE)$_3$-G3 (ClinicalTrials.gov Identifier: NCT05695859), and [^{99m}Tc]Tc -ZHER2:41071 (ClinicalTrials.gov Identifier: NCT05203497) were conducted in patients with BC in collaboration with Tomsk Polytechnic University (Tomsk), Shemyakin – Ovchinnikov Institute of Bioorganic Chemistry (Moscow), and Uppsala University (Sweden) and demonstrated the feasibility of determining the HER2/neu status in the primary tumor [14, 15].

The results obtained and the accumulated experience have allowed for the expansion of the scope of clinical characteristics studied with [^{99m}Tc]Tc-ADAPT6 and [^{99m}Tc]Tc-(HE)$_3$-G3. This expansion aims at defining diagnostic algorithms in the anatomical staging of metastatic axillary lymph nodes and assessing their molecular characteristics [10, 11].

The results obtained in this study almost completely replicate the direct comparison of [^{99m}Tc]Tc-ADAPT6 and [^{99m}Tc]Tc-(HE)$_3$-G3 performed within phase II clinical trials on the effectiveness of detecting the HER2/neu status in primary breast tumors [16, 17]. At the same time, it is obvious that the compound [^{99m}Tc]Tc-ADAPT6 has greater diagnostic accuracy in typing the HER2/neu status in primary tumors and metastases to regional lymph nodes, which can be widely used in clinical practice. In the meantime, the agent [^{99m}Tc]Tc-(HE)$_3$-G3 could be used for the dynamic assessment of the malignant process during neoadjuvant treatment, as it does not have competing characteristics with targeted agents, such as trastuzumab and pertuzumab.

CONCLUSION

Therefore, [^{99m}Tc]Tc-ADAPT6 has greater efficacy in determining the HER2/neu status in primary tumors and regional lymph node metastases. The clinical use of [^{99m}Tc]Tc-(HE)$_3$-G3, upon further study, may be possible for assessing tumor dynamics during preoperative treatment.

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Authors' contribution

Bragina O.D., Deyev S.M., Chernov V.I. – conception and design, analysis and interpretation of the data, justification of the manuscript, critical revision of the manuscript for important intellectual content, final approval of the manuscript for publication. Garbukov E.Yu., Vostrikova M.A., Romanova A.A., Borodina M.E. – collection of the clinical material, clinical adaptation. Tashireva L.A.– statistical processing of the data.

Authors' information

B**ragina Olga D.** – Dr. Sci. (Med.), Principal Researcher, Department of Nuclear Therapy and Diagnostics, Cancer Research Institute; Senior Researcher, Oncotheranostics Research Center, NR TPU, Tomsk, bragina_od@mail.ru, http://orcid.org/0000-0001-5281-7758

Tashireva Lyubov A. – Dr. Sci. (Med.), Head of the Laboratory for Molecular Cancer Therapy, Cancer Research Institute, Tomsk, tashireva@oncology.tomsk.ru, http://orcid.org/0000-0003-2061-8417.

Garbukov Eugenii Yu. – Cand. Sci. (Med.), Senior Researcher, General Oncology Department, Cancer Research Institute, Tomsk NRMC, Tomsk, jrmaximum9@gmail.com, http://orcid.org/0000-0002-6016-7078

Vostrikova Maria A. – Junior Researcher, General Oncology Department, Cancer Research Institute, Tomsk NRMC, Tomsk, vostrikova.m@mail.ru, http://orcid.org/0000-0002-0256-5342

Romanova Anastasiya A. – Junior Researcher, General Oncology Department, Cancer Research Institute, Tomsk NRMC, Tomsk, rom9133207716@yandex.ru, http://orcid.org/0009-0009-6426-9416

Borodina Mariya E. – Researcher, P. Hertsen Moscow Oncology Research Institute, Moscow, 6571544@mail.ru, http://orcid.org/0009-0002-2779-0746

Deyev Sergei M. – Dr. Sci. (Biology), Professor, Academician of the RAS, Head of Molecular Immunology Department, Shemyakin – Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences, Moscow, deev_sm@tpu.ru, http://orcid.org/0000-0002-3952-0631

Chernov Vladimir I. – Dr. Sci. (Med.), Professor, Corresponding Member of the RAS, Head of the Department of Nuclear Therapy and Diagnostics, Cancer Research Institute, Tomsk, chernov@tnimc.ru, http://orcid.org/0000-0002-5524-9546

(✉) **Bragina Olga D.,** bragina_od@mail.ru

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Features of integrin subunit β4 expression depending on clinical and morphological parameters of breast cancer

Zavyalova M.V.[1,2], Kuznetsov G.A.[2], Grigorieva E.S.[1], Tashireva L.A.[1], Zavyalov A.V.[2], Popova V.E.[2], Alifanov V.V.[1], Pismenny D.S.[1,2], Andryukhova E.S.[1,2], Perelmuter V.M.[1]

[1] *Cancer Research Institute, Tomsk National Research Medical Center, Russian Academy of Sciences*
5, Kooperativny Lane, Tomsk, 634009, Russian Federation

[2] *Siberian State Medical Universit (SSMU)*
2, Moscow Trakt, Tomsk, 634050, Russian Federation

ABSTRACT

Aim. To study the features of the expression of the integrin subunit β4 in primary tumor tissue depending on the clinical and morphological parameters of breast cancer.

Materials and methods. We examined biopsy samples from 49 patients with T1–4N0–3M0 breast cancer; the median age was 51.0 [44.0; 60.0] years. Patients did not receive neoadjuvant therapy. Surgical intervention involved resection of the mammary gland with axillary lymph node dissection or radical mastectomy. The expression of markers of estrogen receptor, progesterone receptor, c-erB-2 (Her2/neu), Ki67, CD104 (integrin subunit β4) was assessed using immunohistochemistry. Statistical processing of the results was carried out using the Statisctica 10.0 software package.

Results. In the group of patients with stage N3, cases with positive cytoplasmic/membrane colocalization of integrin subunit β4 expression were more frequently detected (45%), compared with observations where no such expression was found (8%; $p = 0.002$).

Conclusion. Positive cytoplasmic/membrane colocalization of integrin subunit β4 expression is associated with the prevalence of lymphatic metastasis, which corresponds to Stage N3.

Keywords: breast cancer, primary tumor, integrin subunit β4, metastases

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 individuals signed an informed consent to participate in the study. The study was approved by the local Ethics Committee at Siberian State Medical University (Protocol No. 8952 of 24.01.2022).

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✉ *Pismenny Dmitry S.,* pismenniy.dmitry@yandex.ru

Особенности экспрессии субъединицы интегрина β4 в зависимости от клинико-морфологических параметров рака молочной железы

Завьялова М.В.[1, 2], Кузнецов Г.А.[2], Григорьева Е.С.[1], Таширева Л.А.[1], Завьялов А.В.[2], Попова В.Е.[2], Алифанов В.В.[1], Письменный Д.С.[1, 2], Андрюхова Е.С.[1, 2], Перельмутер В.М.[1]

[1] Научно-исследовательский институт (НИИ) онкологии, Томский национальный исследовательский медицинский центр Российской академии наук
Россия, 634009, г. Томск, пер. Кооперативный, 5

[2] Сибирский государственный медицинский университет (СибГМУ)
Россия, 634050, г. Томск, Московский тракт, 2

РЕЗЮМЕ

Цель исследования – изучить особенности экспрессии субъединицы интегрина β4 в ткани первичной опухоли в зависимости от клинико-морфологических параметров рака молочной железы.

Материалы и методы. Изучался биопсийный материал от 49 больных раком молочной железы T1–4N0–3M0, средний возраст составил 51,0 [44,0; 60,0] год. Неоадъювантной терапии больные не получали. Оперативное вмешательство выполнялось в объеме резекции молочной железы с подмышечной лимфаденэктомией или радикальной мастэктомии. Экспрессия маркеров Estrogen receptor, Progesteron receptor, c-erB-2 (Her2/neu), Ki67, CD104 (субъединица интегрина β4) оценивалась иммуногистохимическим методом. Статистическая обработка результатов проводилась с применением пакета программ Statistica 10.0.

Результаты. В группе больных с N3 чаще (45%) обнаруживались случаи с позитивной цитоплазматической/мембранной колокализации экспрессии субъединицы интегрина β4 в сравнении с наблюдениями, когда подобной экспрессии не было (8%; $p = 0{,}002$).

Заключение. Позитивная цитоплазматическая/мембранная колокализация экспрессии субъединицы интегрина β4 ассоциирована с распространенностью лимфогенного метастазирования, соответствующей критерию N3.

Ключевые слова: рак молочной железы, первичная опухоль, субъединица интегрина β4, метастазы

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

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

Соответствие принципам этики. Все лица подписали информированное согласие на участие в исследовании. Исследование одобрено локальным этическим комитетом СибГМУ (протокол № 8952 от 24.01.2022).

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INTRODUCTION

Breast cancer is the number one cancer in patients with malignant neoplasms. It is also the leading cause of death among women, accounting for 16.2% [1]. Most often, mortality from malignant neoplasms is due to metastasis. The study of metastasis mechanisms is one of the key areas of modern oncology. For metastasis, the nature of intercellular and parenchymal-stromal interaction is important, which are largely mediated by integrins.

In this regard, studying the integrin profile of tumor cells seems promising. Integrins are transmembrane receptors that are macromolecules

consisting of two subunits – alpha and beta. Each subunit in turn has three parts: free extracellular N-terminal domain, a transmembrane segment, and intracellular tails.

In addition, there is evidence that the expression of some integrins can provide selectivity for distant metastasis. Thus, metastatic lung damage has been observed with the expression of integrin α6β4, and distant metastasis to the liver and brain – with the expression of integrin αvβ5 [2, 3]. Among the various integrins, much attention is paid to integrin β4 [4, 5]. This integrin is part of the heterodimer α6β4, which fixes epithelial cells to basement membranes. There is evidence linking the expression of α6β4 to metastasis and the invasion of lung cancer stem cells into the brain [2, 6].

The ability of integrin α6β4 to prevent the development of apoptosis (anoikis) of tumor cells that have detached from the basement membrane has been described in the literature [7, 8]. The expression of integrin α6β4 and laminin ligand underlies the matrix-independent existence of tumor cells. It is believed that expression of integrin α6β4 autocrinely activates laminin synthesis, then the integrin binds to the ligand, and a cascade of events is triggered, including increased cell proliferative activity, invasive growth, and metastasis [9–11]. Such cell subpopulations have the most pronounced resistance to anoikis and are most capable of becoming seed cells initiating regional and distant metastases.

The aim of this study was to investigate the expression patterns of the integrin subunit β4 in primary tumor tissue depending on the clinical and morphological parameters of breast cancer.

MATERIALS AND METHODS

All stages of the study comply with the legislation of the Russian Federation and regulatory documents of scientific organizations. All individuals signed a voluntary informed consent to participate in the study in accordance with the requirements of the local Ethics Committee of Siberian State Medical University (Protocol No. 8952 of January 24, 2022). We examined biopsy samples of primary tumor tissue from 49 patients with T1–4N0–3M0 breast cancer who received treatment as needed at the Cancer Research Institute of Tomsk National Research Medical Center from 2013 to 2020.

Biopsy samples were collected before patients began to receive therapy. The median age of the patients was 50.0 [44.0; 60.0] years. The patients did not receive neoadjuvant therapy. The surgical intervention involved resection of the mammary gland and axillary lymph node dissection or radical mastectomy. The primary tumor tissue and the removed lymph nodes were examined. The diagnosis was established according to the 2019 WHO classification and the TNM Classification of Malignant Tumors, 8th edition, of the Union for International Cancer Control.

Immunohistochemistry and histologic examination were performed using standard methods. Only cases with invasive ductal carcinoma of the mammary gland were included in the study. The degree of malignancy was determined using the Scarff – Bloom – Richardson histologic grading.

For immunohistochemistry, the following antibodies were used: progesterone receptor (clone PgR636, Dako), estrogen receptor (clone 1D5, Dako), c-erB-2 (Her2/neu) (Polyclonal Rabbit, Dako), Ki67 (clone SP6, Cell Marque), CD104 (integrin subunit β4, clone JM11-06, Invitrogen, dilution 1:200). Molecular subtypes of breast cancer were determined by assessing the expression of receptors to estrogen, progesterone, Ki67, and HER2. Luminal A, luminal B HER2 negative, luminal B HER2 positive, HER2 positive (non-luminal), and basal-like (triple negative) molecular subtypes have been established.

The whole slide image (WSI) method was used to digitize the histologic preparations using the Pannoramic Mirax Midi scanning microscope (Carl Zeiss, Germany). The analysis of the digitized sections was performed using the Panoramic Viewer 1.15.4.43061 software. The cytoplasmic expression and cytoplasmic/membrane colocalization of the expression of the integrin subunit β4 (CD104) were assessed in primary tumor cells (Figure).

The Statistica version 10.0 software package was used for statistical data processing. Due to the non-normal distribution of the studied variables, significant differences in medians between two independent samples were assessed using the non-parametric Mann – Whitney test. The t-test was used to compare the frequency of the detected features. The results were considered statistically significant at $p < 0.05$.

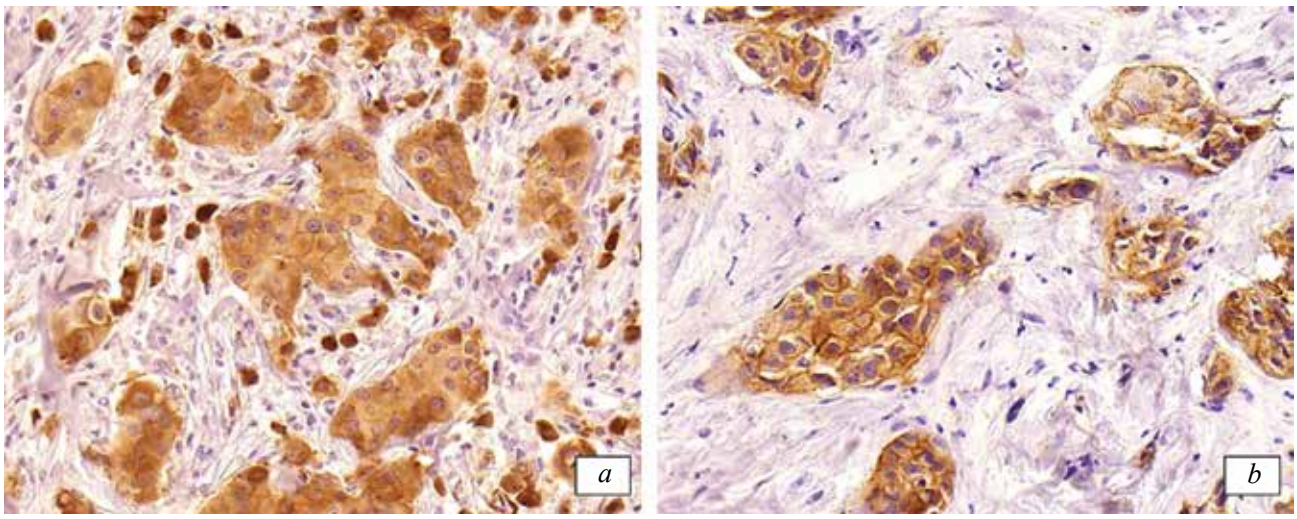

Figure. Expression of the integrin subunit *β*4 (CD104) in the primary tumor: *a* – cytoplasmic expression, *b* – cytoplasmic/membrane colocalization of expression. ×400

RESULTS

A study was conducted to examine the expression patterns of the integrin subunit β4 (CD104) in the cells of invasive ductal carcinoma of the mammary gland depending on various clinical and morphological manifestations of the tumor process.

The age of the patients did not differ depending on the presence or absence of positive cytoplasmic expression or cytoplasmic/membrane colocalization of the integrin subunit β4 expression. Positive and negative cytoplasmic expression of the integrin subunit β4 was detected with approximately the same frequency both in the group of patients with preserved menstrual function and in menopause. Positive cytoplasmic/membrane colocalization of integrin subunit β4 expression was detected more frequently (73%) in the group of patients with preserved menstrual function compared to cases without expression of this marker (42%; $p = 0.032$).

The presence of cytoplasmic or cytoplasmic/membrane colocalization of integrin subunit β4 expression was not associated with the characteristics of the primary tumor corresponding to different T criterion values. No significant differences were found in the frequency of cases with negative and positive expression of the studied marker in the groups of patients with T1, T2, T3 and T4 cancer.

The frequency of negative and positive cytoplasmic expression or cytoplasmic/membrane colocalization of integrin subunit β4 expression did not differ according to tumor grade G1, G2, or G3. There were also no differences in the percentage of cases with negative and positive cytoplasmic expression or cytoplasmic/membrane colocalization of integrin subunit β4 expression depending on the molecular subtype of breast cancer.

The prevalence of lymphatic metastasis, characterized by the N criterion, turned out to be associated with the features of expression of the integrin subunit β4 in the cells of invasive ductal breast cancer. Namely, in cases with the presence of metastases in the displaced axillary lymph nodes on the affected side, corresponding to the N1 criterion, positive cytoplasmic/membrane colocalization of the expression of the integrin subunit β4 was detected less frequently compared with cases without such localization of studied marker expression.

In cases with diffuse lymphatic metastasis with metastatic infraclavicular lymphadenopathy on the affected side, or with a combination of the internal mammary lymphadenopathy with metastases to the axillary lymph nodes or with metastases to the supraclavicular lymph nodes on the affected side, corresponding to stage N3, positive cytoplasmic/membrane colocalization of expression of the integrin subunit β4 was detected more often (45%) compared with cases without this type of expression localization (3/38 (8%; $p = 0.002$). The frequency of negative and positive cytoplasmic expression of the integrin subunit β4 did not differ between groups with stage N0, N1, N2, and N3 (Table).

Table

Features of cytoplasmic and cytoplasmic/membrane colocalization of integrin subunit β4 expression depending on the clinical and morphological parameters of invasive ductal carcinoma

Parameter	Cytoplasmic expression of the integrin subunit β4		Cytoplasmic/membrane colocalization of integrin subunit β4 expression	
	No (n = 28)	Yes (n = 21)	No (n = 38)	Yes (n = 11)
	1	2	3	4
Age, *Me* [Q_1; Q_3]	51.0 [45.0; 60.0]	48.0 [42.0; 56.0] p_{1-2} = 0.464	51.0 [45.0; 61.0]	47.0 [35.0; 56.0] p_{3-4} = 0.143
Menstrual function status, abs. (%)				
Preserved	12/28 (43%)	12/21 (57%) p_{1-2} = 0.166	16/38 (42%)	8/11 (73%) p_{3-4} = 0.032
Menopause	16/28 (57%)	9/21 (43%) p_{1-2} = 0.166	22/38 (58%)	3/11 (27%) p_{3-4} = 0.032
Characteristics of the primary tumor node, abs. (%)				
T1	7/28 (25%)	5/21 (24%) p_{1-2} = 0.468	9/38 (24%)	3/11 (27%) p_{3-4} = 0.419
T2	15/28 (54%)	8/21 (38%) p_{1-2} = 0.133	19/38 (50%)	4/11 (37%) p_{3-4} = 0.223
T3	1/28 (4%)	3/21 (14%) p_{1-2} = 0.104	3/38 (8%)	1/11 (9%) p_{3-4} = 0.458
T4	5/28 (17%)	5/21 (24%) p_{1-2} = 0.272	7/38 (18%)	3/11 (27%) p_{3-4} = 0.256
Cancer Grade, abs. (%)				
G1	1/28 (3,5%)	1/21 (5%) p_{1-2} = 0.359	1/38 (3%)	1/11 (9%) p_{3-4} = 0.195
G2	26/28 (93%)	19/21 (90%) p_{1-2} = 0.353	36/38 (94%)	9/11 (82%) p_{3-4} = 0.107
G3	1/28 (3,5%)	1/21 (5%) p_{1-2} = 0.359	1/38 (3%)	1/11 (9%) p_{3-4} = 0.195
Molecular genetic type, abs. (%)				
Luminal A	6/28 (21%)	3/21 (14%) p_{1-2} = 0.264	8/38 (21%)	1/11 (9%) p_{3-4} = 0.182
Luminal B HER2 negative	15/28 (54%)	9/21 (43%) p_{1-2} = 0.223	20/38 (53%)	4/11 (36%) p_{3-4} = 0.160
Luminal B HER2 positive	3/28 (11%)	4/21 (19%) p_{1-2} = 0.215	4/38 (10,5%)	3/11 (27%) p_{3-4} = 0.150
HER2 overexpression	2/28 (7%)	4/21 (19%) p_{1-2} = 0.102	4/38 (10,5%)	2/11 (18%) p_{3-4} = 0.234
Triple negative	2/28 (7%)	1/21 (5%) p_{1-2} = 0.386	2/38 (5%)	1/11 (9%) p_{3-4} = 0.310
Characteristics of lymphatic metastases, abs. (%)				
N0	13/28 (46.5%)	8/21 (38%) p_{1-2} = 0.288	16/38 (42%)	5/11 (45%) p_{3-4} = 0.429
N1	11/28 (39%)	6/21 (29%) p_{1-2} = 0.233	17/38 (45%)	0/11 (0%) p_{3-4} = 0.003
N2	1/28 (3.5%)	2/21 (10%) p_{1-2} = 0.154	2/38 (5%)	1/11 (10%) p_{3-4} = 0.271
N3	3/28 (11%)	5/21 (23%) p_{1-2} = 0.129	3/38 (8%)	5/11 (45%) p_{3-4} = 0.002

DISCUSSION

A study of the expression patterns of the integrin subunit β4 in invasive breast carcinoma cells revealed a relationship between positive cytoplasmic/membrane colocalization of integrin subunit β4 expression and the prevalence of lymphatic metastasis corresponding to stage N3.

Determining the localization of expression was important. Given that integrins consist of three parts

(free extracellular N-terminal domain, transmembrane segment, and intracellular tails), we decided to differentiate between cytoplasmic expression and cytoplasmic/membrane colocalization of the integrin subunit β4 expression when determining expression. Only membrane/cytoplasmic colocalization of integrin subunit β4 expression was found to be associated with the most advanced lymphatic metastasis corresponding to stage N3.

The study did not reveal significant associations between the frequency of positive cytoplasmic expression and cytoplasmic/membrane colocalization of integrin subunit β4 expression and other clinical and morphological patterns of breast cancer.

The integrin subunit β4, a member of the integrins that recognize laminin, plays the main role in maintaining epithelial differentiation and structure. Under physiological conditions, loss of epithelial cell contact with the basement membrane results in cessation of proliferation and induction of apoptosis, known as anoikis. However, in malignant tumors, cells can adapt to a lack of adhesion, allowing them to avoid anoikis and contributing to their invasiveness and metastatic potential [12]. One of the mechanisms is the synthesis of basement membrane molecules. Such surrogate substitution of the basement membrane bond restores the ability to proliferate and avoids anoikis [13].

As a result, such tumor cells acquire the ability to grow independent of attachment to the stroma. The integrin subunit β4 mediates tumor growth independent of the matrix by activating the Shp2-Src signaling pathway. It is believed that acquisition of matrix-independent growth ability promotes metastatic spread [14, 15]. Apparently, the ability for matrix-independent existence is one of the necessary factors that ensures metastasis. Studying the key processes responsible for the role of integrin subunit β4 in lymphatic metastasis can be useful both for understanding the metastasis mechanisms and for identifying potential targets for targeted therapy.

CONCLUSION

The obtained data indicate the role of integrin subunit β4 expression in the mechanisms of lymphatic metastasis development in breast cancer. Positive cytoplasmic/membrane colocalization of integrin subunit β4 expression is associated with the predominance of lymphatic metastasis corresponding to stage N3 cancer.

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Authors' contribution

Zavyalova M.V., Perelmuter V.M. – conception and design. Alifanov V.V., Andryukhova E. S. – collection and processing of material. Kuznetsov G.A., Zavyalov A.V., Popova V.E., Pismenny D.S. – drafting of the manuscript. Grigorieva E.S., Tashireva L.A. – editing the manuscript.

Authors' information

Zavyalova Marina V. – Dr. Sci. (Med.), Professor, Leading Researcher, Department of General and Molecular Pathology, Cancer Research Institute, Tomsk NRMC; Head of the Pathological Anatomy Division, SSMU SibMed, Tomsk, zavyalovamv@mail.ru, http://orcid.org/0000-0001-9429-9813

Kuznetsov Gleb A. – Postgraduate Student, Pathological Anatomy Division, SSMU SibMed, Tomsk, kuznetsov.gleb199710@gmail.com, http://orcid.org/0000-0002-9443-7224

Grigorieva Evgenia S. – Cand. Sci. (Med.), Senior Researcher, Laboratory of Molecular Cancer Therapy, Senior Researcher, Laboratory of Molecular Oncology and Immunology, Cancer Research Institute, Tomsk NRMC, Tomsk, grigoryeva.es@gmail.com, http://orcid.org/0000-0003-4737-8951

Tashireva Lyubov A. – Dr. Sci. (Med.), Head of the Laboratory of Molecular Cancer Therapy, Cancer Research Institute, Tomsk NRMC, Tomsk, lkleptsova@mail.ru, http://orcid.org/0000-0003-2061-8417

Zavyalov Alexander V. – Student, General Medicine Department, SSMU SibMed, Tomsk, zavyalov.av@ssmu.ru. http://orcid.org/0009-0009-0266-6707

Popova Victoria E. – Graduate Student, Pathological Anatomy Division, SSMU SibMed, Tomsk, popova.ve@ssmu.ru, http://orcid.org/0009-0001-9740-7776

Alifanov Vladimir V. – Cand. Sci. (Med.), Junior Researcher, Department of General and Molecular Pathology, Cancer Research Institute, Tomsk NRMC, Tomsk, alifanov.vl@yandex.ru, http://orcid.org/0000-0002-3025-4445

Pismenny Dmitry S. – Cand. Sci. (Med.), Doctor of Clinical Laboratory Diagnostics, Department of General and Molecular Pathology, Cancer Research Institute, Tomsk NRMC; Assistant of the Pathological Anatomy Division, SSMU SibMed, Tomsk, pismenniy.dmitry@yandex.ru, http://orcid.org/0000-0001-8973-8439

Andryukhova Elena S. – Junior Researcher, Department of General and Molecular Pathology, Cancer Research Institute, Tomsk NRMC; Graduate Student, Pathological Anatomy Division, SSMU SibMed, Tomsk, elenasergeevna9607@gmail.com, http://orcid.org/0000-0003-0909-9206

Perelmuter Vladimir M. – Dr. Sci. (Med.), Professor, Honored Scientist of the Russian Federation, Chief Researcher, Department of General and Molecular Pathology, Cancer Research Institute, Tomsk NRMC, Tomsk, pvm@ngs.ru, http://orcid.org/0000-0002-7633-9620

(✉) **Pismenny Dmitry S.,** pismenniy.dmitry@yandex.ru

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Association of single nucleotide variants of the *SLCO1B1* gene with the Gilbert syndrome phenotype

Ivanova A.A.[1], Apartseva N.E.[1], Kashirina A.P.[1], Nemtsova E.G.[2], Ivanova Ju.V.[1], Kruchinina M.V.[1], Kurilovich S.A.[1], Maksimov V.N.[1]

[1] *Research Institute of Internal and Preventive Medicine – Branch of the Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences*
175/1, B.Bogatkova Str., Novosibirsk, 630089, Russian Federation

[2] *North-Western State Medical University named after I.I. Mechnikov*
41, Kirochnaya Str., Saint Petersburg, 191015, Russian Federation

ABSTRACT

The aim of the study is to investigate the association of rs2306283 and rs4149056 variants of the *SLCO1B1* gene with benign unconjugated hyperbilirubinemia.

Materials and methods. A case-control study design was employed. The group with the Gilbert syndrome (GS) phenotype comprised 414 individuals (mean age 36.7 ± 15.9 years, 49.8% men). The control group consisted of 429 individuals (mean age 38.5 ± 14.3 years, 52.2% men) randomly selected from DNA banks of MONICA project participants, young adults aged 25–44 years, and participants in a cross-sectional study of schoolchildren in Novosibirsk. Genotyping of the groups for nucleotide sequence variants rs2306283 and rs4149056 of the *SLCO1B1* gene was performed using real-time polymerase chain reaction.

Results. No statistically significant differences were found between the GS and control groups regarding the frequencies of genotypes and alleles of rs2306283 ($p > 0.05$). Carriers of the TT rs4149056 genotype were less common (OR = 0.67, 95% CI 0.51–0.89, $p = 0.005$), while carriers of the TC genotype were more prevalent (OR = 1.46, 95% CI 1.1–1.94, $p = 0.009$) in the GS group compared to the control group. The frequency of the C allele rs4149056 was higher in the GS group compared to the control group (OR = 1.35, 95% CI 1.07–1.7, $p = 0.012$). These differences persisted for carriers of the 6TA/7TA genotype but not for the 6TA/6TA and 7TA/7TA genotypes of rs3064744 in the *UGT1A* gene.

Conclusion. The single nucleotide variant rs2306283 of the *SLCO1B1* gene is not associated with benign unconjugated hyperbilirubinemia. The TC genotype and C allele of the single nucleotide variant rs4149056 of the *SLCO1B1* gene are the genotype and risk allele of Gilbert syndrome, while the TT variant genotype exhibits a protective effect against the development of the syndrome, particularly for carriers of the 6TA/7TA genotype of rs3064744 in the *UGT1A* gene.

Keywords: Gilbert syndrome, gene, rs2306283, rs4149056, *SLCO1B1*, unconjugated hyperbilirubinemia

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 participants of the study signed an informed consent. The study was approved by the local Ethics committee of the Research Institute of Internal and Preventive Medicine – Branch of the Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences (Protocol No. 4 of 14.02.2023).

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✉ *Ivanova Anastasiya A.,* ivanova_a_a@mail.ru

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Ассоциация однонуклеотидных вариантов гена *SLCO1B1* с фенотипом синдрома Жильбера

Иванова А.А.[1], Апарцева Н.Е.[1], Каширина А.П.[1], Немцова Е.Г.[2], Иванова Ю.В.[1], Кручинина М.В.[1], Курилович С.А.[1], Максимов В.Н.[1]

[1] Научно-исследовательский институт терапии и профилактической медицины (НИИТПМ) – филиал Института цитологии и генетики Сибирского отделения Российской академии наук (ИЦиГ СО РАН) Россия, 630089, г. Новосибирск, ул. Б. Богаткова, 175/1

[2] Северо-Западный государственный медицинский университет (СЗГМУ) им. И.И. Мечникова Россия, 191015, г. Санкт-Петербург, ул. Кирочная, 41

РЕЗЮМЕ

Цель исследования – проверка ассоциации rs2306283 и rs4149056 гена *SLCO1B1* с доброкачественной неконъюгированной гипербилирубинемией.

Материалы и методы. Дизайн исследования «случай – контроль». Группа с фенотипом синдрома Жильбера (СЖ) включала 414 человек (средний возраст 36,7 ± 15,9 лет, 49,8% мужчин). Группа контроля (429 человек, средний возраст 38,5 ± 14,3 лет, 52,2% мужчин) – случайная выборка лиц из банков ДНК участников проекта MONICA, скрининга молодых людей 25–44 лет и одномоментного исследования школьников г. Новосибирска. Генотипирование групп по вариантам нуклеотидной последовательности rs2306283 и rs4149056 гена *SLCO1B1* выполнено методом полимеразной цепной реакции в режиме реального времени.

Результаты. По частотам генотипов и аллелей rs2306283 не найдено статически значимых различий между группой СЖ и контрольной группой ($p > 0,05$). Носители генотипа TT rs4149056 встречаются реже (отношение шансов (ОШ) = 0,67, 95%-й доверительный интервал (95%ДИ) 0,51–0,89, $p = 0,005$), а носители генотипа TC чаще (ОШ = 1,46, 95%ДИ 1,1–1,94, $p = 0,009$) в группе СЖ по сравнению с контрольной группой, частота аллеля C rs4149056 больше в группе СЖ по сравнению с контрольной группой (ОШ = 1,35, 95%ДИ 1,07–1,7, $p = 0,012$). Полученные различия сохраняются для носителей генотипа 6TA/7TA, но не генотипа 6TA/6TA и 7TA/7TA rs3064744 гена *UGT1A*.

Заключение. Однонуклеотидный вариант rs2306283 гена *SLCO1B1* не ассоциирован с доброкачественной неконъюгированной гипербилирубинемией. Генотип TC, аллель C однонуклеотидного варианта rs4149056 гена *SLCO1B1* являются генотипом и аллелем риска синдрома Жильбера, а генотип TT – протективный в отношении развития синдрома, прежде всего для носителей генотипа 6TA/7TA rs3064744 гена *UGT1A*.

Ключевые слова: синдром Жильбера, ген, rs2306283, rs4149056, *SLCO1B1*, неконъюгированная гипербилирубинемия

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

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Соответствие принципам этики. Все участники подписали добровольное информированное согласие на участие в исследовании. Исследование одобрено этическим комитетом НИИТПМ – филиал ИЦиГ СО РАН (протокол № 4 от 14.02.2023).

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INTRODUCTION

The most common genetic cause of benign unconjugated hyperbilirubinemia (Gilbert syndrome, GS) in adults is an increase in the number of TA repeats in the promoter of the *UGT1A1* gene (rs3064744) to 7 in the homozygous state (7TA/7TA). However, some individuals with clinical symptoms of GS do not have an increased number of TA repeats in the homozygous state (6TA/6TA), or are heterozygous carriers of the rs3064744 variant (6TA/7TA), which may indicate the contribution of other singlenucleotide gene variants to the development of pathology [1].

In a genome-wide association study on the Mayo Genome Consortium cohort, two loci were identified that are associated with total bilirubin levels – 2q37 (corresponding to the *UGT1A1* gene) and 12p12 (corresponding to the *SLCO1B1* gene) [2]. A meta-analysis of three genome-wide association studies showed a strong genetic effect on serum bilirubin levels of the *UGT1A1* gene and the 12p12.2 locus. At the same time, the peak signal in the 12p12.2 region was a variant of rs4149056 in the *SLCO1B1* gene, which leads to the replacement of the amino acid valine with alanine, which leads to a decrease in the activity of a carrier protein in the liver with a known affinity for bilirubin [3]. Therefore, we suggested that variants of the *SLCO1B1* gene may be associated with benign unconjugated hyperbilirubinemia. To test this hypothesis, we selected two variants of the *SLCO1B1* gene, rs2306283 and rs4149056, which were the most studied in relation to bilirubin concentration, hyperbilirubinemia.

MATERIALS AND METHODS

The study design is a case-control study. The group of people with the Gilbert syndrome (GS) phenotype (n = 414; average age 36.7 ± 15.9 years, 49.8% were men) was formed by gastroenterologists and included people with unconjugated hyperbilirubinemia who underwent a standard clinical examination. Individuals with known causes of unconjugated hyperbilirubinemia, except for genetic ones, were excluded from the group. DNA was isolated from venous blood using either phenol chloroform extraction or the express method (PREP-RAPID-GENETICS, DNA-Technology LLC, Moscow).

The control group (n = 429; average age 38.5 ± 14.3 years, 52.2% were men) was a random sample of individuals from DNA banks of participants in the MONICA project (Multinational MONItoring of trends and determinants in CArdiovascular disease), a screening of young people aged 25–44 years, and a cross-sectional study of schoolchildren in Novosibirsk. Information on the level of total or unconjugated bilirubin, liver and gallbladder diseases, and diagnosis of GS was not available for these studies, which is a limitation of this study since isolated cases of the diagnosed or undiagnosed GS may be present in the control group. The DNA of the individuals included in the control group was isolated from venous blood using phenol chloroform extraction. The GS group and the control group did not differ in terms of gender and age.

In previous studies, we determined the genotypes of the rs3064744 variant (the number of TA repeats in the promoter) of the *UGT1A1* gene for individuals included in the GS group and the control group. In the GS group, the distribution of genotypes according to rs3064744 of the *UGT1A1* gene was: 73.3% – 7TA/7TA, 20.3% – 6TA/7TA, 5.8% – 6TA/6TA, 0.2% – 5TA/7TA, 6TA/8TA, 7TA/8TA. In the control group, the distribution of genotypes according to rs3064744 of the *UGT1A1* gene was: 11.7% – 7TA/7TA, 42.9% – 6TA/7TA, 45.0% – 6TA/6TA, 0.2% – 5TA/6TA, 6TA/9TA.

Genotyping of groups according to variants of the nucleotide sequences rs2306283 and rs4149056 of the *SLCO1B1* gene was carried out by real-time polymerase chain reaction using kits from NPF SINTOL LLC (Russia) on Light Cycler 96 (Roche, Switzerland/ Germany) (rs4149056) and CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) (rs2306283).

Comparison of groups by frequencies of genotypes and alleles and calculation of a relative risk for a specific allele or genotype were carried out using cross tables, the Pearson chi-square criterion ($\chi 2$), and the exact two-sided Fisher criterion with Yates correction for continuity. The normality of the distribution of the level of total and unconjugated bilirubin was checked using the Kolmogorov – Smirnov test, and then the Kruskall – Wallis test and the Mann – Whitney test were used. Quantitative data are presented as median and interquartile range Me [Q_{25}–Q_{75}]; $p < 0.05$ was also used as the significance level.

All participants in the study signed a voluntary informed consent. The study was approved by the Ethics Committee of IIPM – Branch of IC&G SB RAS (Protocol No. 4 of 14.02.2023).

RESULTS

The obtained frequencies of genotypes and alleles of variants rs2306283 and rs4149056 of the *SLCO1B1* gene are presented in Table 1.

Table 1

The frequencies of genotypes and alleles of variants rs2306283 and rs4149056 of the *SLCO1B1* gene in the GS and control groups

Single nucleotide variant	Genotype/ allele	GS group		Control group		*p*
		n	%	*n*	%	
rs2306283	TT	181	43.7	184	42.9	0.25
	TC	148	35.7	173	40.3	
	CC	85	20.6	72	16.8	
	T	510	61.6	541	63.1	0.54
	C	318	38.4	317	36.9	
rs4149056	TT*	228	55.1	277	64.6	0.02
	TC*	164	39.6	133	31.0	
	CC	22	5.3	19	4.4	
	T	620	74.9	687	80.1	0.01
	C	208	25.1	171	19.9	

Note: *n* – number of individuals, *p* – significance of differences between groups. * – statistically significant differences when using the genotype 1 vs genotype 2 + genotype 3 model. No differences were found in the frequencies of rs2306283 genotypes and alleles between the GS group and the control group ($p > 0.05$) (Fig. 1).

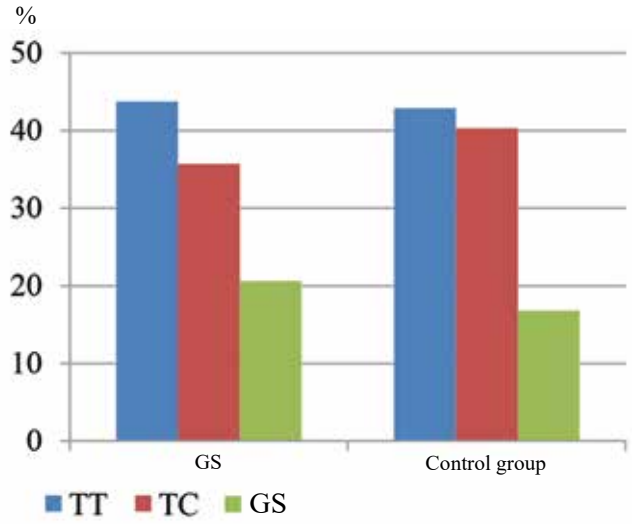

Fig. 1. The frequencies of rs2306283 genotypes of the *SLCO1B1* gene in the GS group and the control group

However, we found differences in the frequencies of rs4149056 genotypes ($p = 0.02$) between the two groups: carriers of the TT genotype were less common (TT vs TC+CC: odds ratio (OR) = 0.67, 95% CI 0.51–0.89, $p = 0.005$), and carriers of the TC genotype were more common (TC vs TT+CC: OR = 1.46, 95% CI 1.1–1.94, $p = 0.009$) in the GS group compared to the control group (Fig. 2). Significant differences were also found in allele frequencies. C allele was more frequent in the GS group (0.25) compared to the control group (0.2) (OR = 1.35, 95% CI 1.07–1.7, $p = 0.012$).

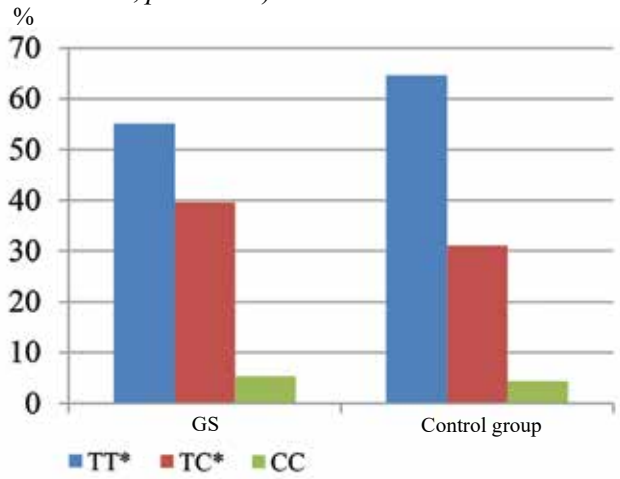

Fig. 2. The frequencies of rs4149056 genotypes of the *SLCO1B1* gene in the GS group and the control group

When dividing the GS group and the control group by genotypes of the rs3064744 variant of the *UGT1A1* gene (excluding rare genotypes 5TA/7TA, 6TA/8TA, 5TA/6TA, 6TA/9TA, 7TA/8TA) into three subgroups – carriers of the genotype 6TA/6TA, 6TA/7TA, and 7TA/7TA – significant differences in the frequencies of the rs4149056 genotype of the *SLCO1B1* gene were only observed in the subgroup of the 6TA/7TA genotype carriers ($p = 0.005$). In carriers of the 6TA/7TA rs3064744 genotype of the *UGT1A1* gene, the TT rs4149056 genotype of the *SLCO1B1* gene is protective against GS (TT vs TC+CC: OR = 0.41, 95% CI 0.24–0.71, $p = 0.002$), while the TC genotype and the C allele are the genotype and allele that increase risk of GS (TC vs TT+CC: OR = 2.36, 95% CI 1.35–4.14, $p = 0.004$; C vs T: OR = 1.91, 95% CI 1.23–2.97, $p = 0.005$, respectively).

There was no association between the genotypes of the rs4149056 variant and the concentration of total or unconjugated bilirubin ($p > 0.05$, Table 2).

Table 2

The concentration of total and unconjugated bilirubin based on the genotype rs4149056, *Me* (Q_{25}–Q_{75}), umol/l

Genotype rs4149056	Total bilirubin	Unconjugated bilirubin
TT	36.8 (27.0–31.3)	30.0 (20.6–35.0)
TC	35.0 (26.2–40.2)	27.9 (20.5–33.6)
CC	36.8 (27.9–40.2)	30.1 (21.3–40.2)

The concentration of total or unconjugated bilirubin with which the association was searched is a randomly detected concentration with which the patient went to see a doctor. During the life of patients, more severe hyperbilirubinemia could be observed. The average concentration of total bilirubin in the GS group was 36.9 umol/l (27.2–42.2), and unconjugated bilirubin level was 29.9 umol/l (21.0–36.1). At the same time, there is a statistically significant difference in the concentration of bilirubins based on the genotypes of the variant rs3064744 of the *UGT1A1* gene. The concentration of total ($p < 0.01$) and unconjugated ($p < 0.01$) bilirubin is higher in carriers of the *UGT1A1* gene genotype 7TA/7TA rs3064744 compared to carriers of the genotypes 6TA/6TA and 6TA/7TA (carriers of rare genotypes were not included in the calculations) (Table 3). Thus, even the randomly detected concentration of total and unconjugated bilirubin is higher in carriers of the 7TA/7TA rs3064744 genotype of the *UGT1A1* gene, which suggests that if there was an association of bilirubin concentration with the genotypes of the rs4149056 variant, it would have been detected.

Table 3

The concentration of total and unconjugated bilirubin based on the genotype rs3064744 of the *UGT1A1* gene, *Me* (Q_{25}–Q_{75}), umol/l

Genotype rs3064744	Total bilirubin	Unconjugated bilirubin
7TA/7TA	38.2 (28.1–44.6)	31.1 (22.2–37.9)
6TA/6TA + 6TA/7TA	31.7 (22.2–37.9)	24.9 (18.5–28.7)

DISCUSSION

The *SLCO1B1* gene (solute carrier organic anion transporter family member 1B1, 12p12.1) encodes a transmembrane receptor specific to liver cells, which mediates the sodium-independent absorption of numerous endogenous compounds, including bilirubin, and participates in the excretion of medicinal compounds, such as statins, bromosulfophthalein, and rifampicin from the blood into hepatocytes [4].

Both of the studied variants belong to the missense variants resulting in the substitution of amino acids in the amino acid sequence of the protein (rs2306283 – c.388A>G, p.Asn130Asp; rs4149056 – c.521T>C, p.Val174Ala).

The obtained frequencies of rare alleles of the studied variants in the control group do not differ from the GnomAD data: the frequency of the rare allele C rs2306283 according to GnomAD data for the European population is 0.37, rs4149056 is 0.2.

The rs2306283 variant according to ClinVar is benign for GS, and Rotor syndrome. It has been shown that the expression of the SLCO1B1 protein is significantly associated with the rs2306283 variant [5]. Studies of the rs2306283 variant have been conducted regarding changes in the metabolism of certain drugs (statins, sorafenib, rocuronium and others) [6–10]. In China, an association of rs2306283 with the risk of pulmonary tuberculosis was found in a group of women [11]. The association of rs2306283 with hyperbilirubinemia was not detected in newborns in China [12]. Our study also did not find an association of the rs2306283 variant with the GS phenotype.

According to ClinVar, the rs4149056 variant is benign for Rotor syndrome, pathogenic for GS, and is related to the metabolism of simvastatin, atorvastatin, and rosuvastatin. There is evidence that the rs4149056 variant reduces the transport activity of the SLCO1B1 protein, which increases the plasma concentration of a number of substances whose transport is associated with this protein [13]. Numerous studies have been conducted on the association of the rs4149056 variant with the development of statin-induced myopathy [14, 15]. In Korea, the relationship of the variant with the pharmacokinetics of rifampicin used in the treatment of tuberculosis has been shown [16]. Carriers of the rs4149056 C allele have an increased risk of bleeding compared to carriers of the TT genotype when taking the drug edoxaban [17]. A study on patients with HIV infection showed the effect of rs4149056 on the concentration of lopinavir (an antiretroviral drug) [18]. Another study found an association between the level of SLCO1B1 protein, allele C and genotype TC rs4149056 with exudative age-related macular degeneration [19]. A Chinese study revealed the association of the CC genotype and C allele of rs4149056 with the risk of pulmonary tuberculosis in women [11]. According to a large meta-analysis in 2009, rs4149056 affects the level of total bilirubin, explaining about 1% of the variability [3]. However, a study in Chile did not find a correlation between rs4149056 and total bilirubin levels or the phenotype of the GS [20].

According to our data, the allele with rs4149056 is a risk allele for unconjugated hyperbilirubinemia, which was also observed in a study in newborns with neonatal hyperbilirubinemia in India. In this case-control design study, carriers of rare alleles of the studied variants, including the rs4149056

variant, were more common in the case group compared to the control group. At the same time, the carriage of more than three of the studied variants (rs4124874, rs8175347 of the *UGT1A1* gene, rs2306283 and rs4149056 of the *SLCO1B1* gene) was also more common in the case group, and the average levels of total bilirubin in the blood and the need for phototherapy increased with the number of coexpressed variants [21]. The association between rs4149056 of the *SLCO1B1* gene and neonatal hyperbilirubinemia was also shown in Chinese newborns: carriers of the CC genotype had a higher risk of neonatal hyperbilirubinemia compared to carriers of other genotypes [22]. According to our data, the TC genotype is a risk genotype for benign unconjugated hyperbilirubinemia.

CONCLUSION

Thus, according to the results of the study, the single nucleotide variant rs2306283 of the *SLCO1B1* gene is not associated with benign unconjugated hyperbilirubinemia. The TC genotype and allele C of the single nucleotide variant rs4149056 of the *SLCO1B1* gene are the genotype and allele with a risk for GS. The TT genotype of the *SLCO1B1* gene is conditionally protective against the development of the syndrome, primarily for carriers of the 6TA/7TA rs3064744 genotype of the *UGT1A* gene.

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Authors' contribution

Ivanova A.A. – conception and design, molecular genetic analysis, data interpretation. Apartseva N.E. – formation of the GS and control groups, molecular genetic analysis. Kashirina A.P. – molecular genetic analysis. Nemtsova E.G. – formation of the GS group. Ivanova Yu.V. – molecular genetic analysis. Kurilovich S.A. – formation of the GS group. Kruchinina M.V. – formation of the GS group. Maximov V.N. – critical revision of the manuscript for important intellectual content, and final approval of the manuscript for publication.

Authors' information

Ivanova Anastasiya A. – Dr. Sci. (Med.), Senior Researcher at the Laboratory of Molecular Genetic Studies of Therapeutic Diseases, Research Institute of Internal and Preventive Medicine – Branch of the Institute of Cytology and Genetics, Siberian Branch of Russian Academy of Sciences, Novosibirsk, ivanova_a_a@mail.ru, http://orcid.org/0000-0002-9460-6294.

Apartseva Natalia E. – PhD student, Junior Researcher at the Laboratory of Genetic and Environmental Determinants of the Human Life Cycle, Research Institute of Internal and Preventive Medicine – Branch of the Institute of Cytology and Genetics, Siberian Branch of Russian Academy of Sciences, Novosibirsk, tusya_evdokimova@mail.ru, http://orcid.org/0000-0003-3772-1058.

Kashirina Anastasiia P. – PhD student, Junior Researcher at the Laboratory of Genetic and Environmental Determinants of the Human Life Cycle, Research Institute of Internal and Preventive Medicine – Branch of the Institute of Cytology and Genetics, Siberian Branch of Russian Academy of Sciences, Novosibirsk, kashirina_a_p_91@mail.ru, http://orcid.org/0000-0002-1968-9712.

Nemcova Elena G. – Cand. Sci. (Med.), Associate Professor of the Department of Propaedeutics of Internal Diseases, Gastroenterology and Dietology named after S. M. Ryss, North-Western State Medical University named after I.I. Mechnikov, Saint Petersburg, neg-85@yandex.ru, http://orcid.org/0000-0003-1501-6796.

Ivanova Julija V. – Resident, Research Institute of Internal and Preventive Medicine – Branch of the Institute of Cytology and Genetics, Siberian Branch of Russian Academy of Sciences, Novosibirsk, juliaivanovvaa@yandex.ru, http://orcid.org/0000-0002-1251-4610.

Kruchinina Margarita V. – Dr. Sci. (Med.), Associate Professor, Leading Researcher, Head of the Gastroenterology Laboratory, Research Institute of Internal and Preventive Medicine – Branch of the Institute of Cytology and Genetics, Siberian Branch of Russian Academy of Sciences, Novosibirsk, kruchmargo@yandex.ru, http://orcid.org/0000-0003-0077-3823.

Kurilovich Svetlana A. – Dr. Sci. (Med.), Professor, Research Institute of Internal and Preventive Medicine – Branch of the Institute of Cytology and Genetics, Siberian Branch of Russian Academy of Sciences, Novosibirsk, kurilovich@yandex.ru, http://orcid.org/0009-0000-7764-7513.

Maksimov Vladimir N. – Dr. Sci. (Med.), Professor, Chief Researcher of the Laboratory of Molecular Genetic Studies of Therapeutic Diseases, Research Institute of Internal and Preventive Medicine – Branch of the Institute of Cytology and Genetics, Siberian Branch of Russian Academy of Sciences, Novosibirsk, medik11@mail.ru, http://orcid.org/0000-0002-7165-4496.

(✉) **Ivanova Anastasiya A.,** ivanova_a_a@mail.ru

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Statistical modeling to determine severity of respiratory sarcoidosis and parameters associated with cardiac sarcoidosis: as a way to stratify the risk of developing pulmonary hypertension

Kalacheva T.P.[1], Denisova O.A.[1], Brazovskaya N.G.[1], Fedosenko S.V.[1], Karnaushkina M.A.[2], Ostanko V.L.[1], Chernyavskaya G.M.[1], Kalyuzhina E.V.[1], Chernogoryuk G.E.[1], Palchikova I.A.[3], Romanov D.S.[1], Purlik I.L.[1], Kulumaeva K.A.[1], Kalyuzhin V.V.[1]

[1] *Siberian State Medical University (SSMU)*
2, Moscow Trakt, Tomsk, 634050, Russian Federation

[2] *Patrice Lumumba Peoples' Friendship University (RUDN University)*
6, Miklukho-Maklaya Str., Moscow, 117198, Russian Federation

[3] *Tomsk Regional Clinical Hospital*
96, I. Chernykh Str., Tomsk, 634063, Russian Federation

ABSTRACT

Aim. Using statistical modeling techniques, we aim to develop a model that optimizes the prediction of severity of sarcoidosis that affects the respiratory system (SRS) based on the identification and determination of signs (anamnestic, clinical, laboratory, instrumental examination data, etc.) associated with disease severity and subsequent stratification of the long-term risk for pulmonary hypertension (PH) development.

Materials and methods. The 12-year observational cohort comparative study included 298 participants, both male and female, who had SRS. More than 200 different patient examination parameters were analyzed. The models were built using logistic regression and linear discriminant analysis. The quality of the models was assessed by constructing a classification matrix, calculating sensitivity and specificity as well as calculating the area under ROC curve.

Results. As a result of the study, optimal classification models were developed for predicting SRS severity, constructed using various methods of statistical modeling. The models demonstrated that several characteristics, including parameters of echocardiography examination of patients (including indicators that allow for indirect diagnosis of PH), are associated with disease severity. A set of characteristics associated with particular sarcoidosis severity will allow for its prediction upon confirmation of diagnosis (individual prognosis), as well as patient management (observation or requiring the prescription of pathogen-specific immunosuppressive therapy).

Conclusion. Such a complex model for predicting disease severity in patients with non-cardiac diseases (SRS) is of great importance for risk stratification in terms of PH development in patients with severe sarcoidosis. Further analysis of the features identified during model construction can help clinicians to contribute to more accurate predictions of SRS severity in real-world clinical practice.

Keywords: pulmonary hypertension, respiratory sarcoidosis, transthoracic echocardiography, prognosis of pulmonary hypertension

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✉ *Kalacheva Tatiana P.,* tatyana-kalachyova@yandex.ru

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Статистическое моделирование для определения типа течения саркоидоза органов дыхания и параметров, ассоциированных с поражением сердца, как способ стратификации риска развития легочной гипертензии

Калачева Т.П.[1], Денисова О.А.[1], Бразовская Н.Г.[1], Федосенко С.В.[1], Карнаушкина М.А.[2], Останко В.Л.[1], Чернявская Г.М.[1], Калюжина Е.В.[1], Черногорюк Г.Э.[1], Пальчикова И.А.[3], Романов Д.С.[1], Пурлик И.Л.[1], Кулумаева К.А.[1], Калюжин В.В.[1]

[1] *Сибирский государственный медицинский университет (СибГМУ)*
Россия, 634050, г. Томск, Московский тракт, 2

[2] *Российский университет дружбы народов им. Патриса Лумумбы (РУДН)*
Россия, 117198, г. Москва, Миклухо-Маклая, 6

[3] *Томская областная клиническая больница (ТОКБ)*
Россия, 634063, г. Томск, И. Черных, 96

РЕЗЮМЕ

Цель. С помощью методов статистического моделирования разработать оптимальную модель прогнозирования типа течения саркоидоза органов дыхания (СОД), основанную на выявлении и определении признаков (анамнестических, клинико-лабораторных, данных инструментального обследования и других), ассоциированных с тяжестью течения заболевания и последующей стратификацией долгосрочного риска развития легочной гипертензии (ЛГ).

Материалы и методы. В 12-летнее наблюдательное когортное сравнительное исследование включено 298 больных СОД обоего пола. Проанализировано более 200 различных параметров обследования пациентов. Модели построены методами логистической регрессии и линейного дискриминантного анализа. Качество моделей оценивалось с помощью построения матрицы классификации и расчета чувствительности и специфичности, а также построения и расчета площади под ROC-кривой.

Результаты. Разработаны оптимальные классификационные модели прогнозирования типа течения СОД, построенные с применением разных методов статистического моделирования. Модели продемонстрировали, что ряд характеристик, включая параметры эхокардиографического обследования пациентов (в том числе показатели, позволяющие косвенно диагностировать ЛГ), имеют связь с типом течения заболевания. Совокупность характеристик, ассоциированных с типом течения саркоидоза, позволит прогнозировать тип течения СОД уже при подтверждении диагноза (индивидуальный прогноз), а также тактику ведения пациентов с данной патологией (наблюдательная или требующая назначения патогенетической иммуносупрессивной терапии).

Заключение. Подобная комплексная модель прогнозирования типа течения заболевания у больных некардиологического профиля (СОД) имеет важное значение в отношении стратификации риска развития ЛГ для пациентов с неблагоприятным типом течения саркоидоза. Дальнейший анализ выделенных при построении моделей признаков может помочь клиницистам в реальной клинической практике способствовать более точному прогнозированию типа течения СОД.

Ключевые слова: легочная гипертензия, саркоидоз органов дыхания, трансторакальная эхокардиография, прогнозирование легочной гипертензии

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

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INTRODUCTION

Pulmonary hypertension (PH) is a hemodynamic and pathophysiological condition that complicates the course of various respiratory and cardiovascular disorders [1]. The issue of predicting PH course in various nosological subtypes at different stages is a significant challenge, which is particularly true when employing clinical, laboratory, and instrumental examination techniques available to clinicians [2–4].

Pulmonary hypertension in sarcoidosis of the respiratory system (SRS) belongs to clinical group V according to the clinical classification of pulmonary hypertension (2020), which includes unclear and/or multifactorial mechanisms of the disease as well as pulmonary hypertension developed in patients with systemic and metabolic disorders. At the same time, sarcoidosis can be caused not only by damage to the lung parenchyma but also by heart damage (pathology of left chambers) and granulomatous arteriopathy [2].

Right heart catheterization (RHC) is necessary to determine the pressure in the pulmonary artery (PA) and is the basis for the diagnosis of pulmonary hypertension. The diagnosis of PH is set when the mean pressure in the pulmonary artery (mPAP) is more than 25 mm Hg at rest and more than 30 mm Hg during exercise [2, 5]. The assessment of hemodynamic parameters, as well as the diagnosis of PH, is most often one of the main indications for RHC. Despite the fact that the RHC is a minimally invasive procedure that requires careful medical monitoring to ensure patient safety, its use is limited in clinical routine.

The limitations include the availability of specialized equipment and units in a hospital, as well as the mandatory hospitalization of the patient 4–6 hours prior to heart catheterization; limited research due to a lack of staff qualified in interventional cardiology with specific research skills; invasive risks and potential development of a number of complications (including risk of infection at catheter insertion sites, bleeding, etc.); patient's refusal to undergo the procedure, etc. Literature does not present a unanimous opinion on the use of the RHC in the monitoring of patients with PH over time [2, 4–6].

In this regard, a worthy alternative to the RHC is transthoracic echocardiography (echo), whose doppler techniques make the most significant contribution to the assessment of PH. According to the 2020 clinical guidelines on PH of the Ministry of Healthcare of the Russian Federation, echocardiography is the only widely available non-invasive method which makes it possible to assess blood pressure in patients with suspected PH [2]. All patients at risk of developing PH should undergo thorough examination using echocardiography, which makes it possible to non-invasively assess intracardiac hemodynamics and calculate blood pressure in the chambers of the heart and in the PA in real time [4–7]. Echocardiography helps not only assess the systolic blood pressure in the PA, but also obtain important information on the cause and complications of PH. This diagnostic method helps exclude the heart valve damage, myocardial diseases, and congenital heart disorders with left-to-right cardiac shunts leading to the development of PH [4, 8].

It is still difficult to predict the life expectancy of a patient with PH since the disease may progress very quickly, leading to sudden deterioration and death, or slowly over several years. It can only be noted that usually the patient's life expectancy depends on the type and causes of PH [9–11].

Sarcoidosis is a systemic inflammatory disease of unknown etiology with a wide range of clinical manifestations involving immune system activity. Given the absence of specific clinical manifestations of this pathology, its diagnosis is rather complicated [12]. A characteristic feature of sarcoidosis is the formation of non-caseating granulomas, affecting almost all organs, causing their dysfunction and altering the tissue structure [11–13].

The number of patients with severe sarcoidosis and complications is increasing every year. A specific treatment strategy is chosen based on SRS severity. When sarcoidosis is mild, drug treatment is not required, while the prescription of systemic glucocorticoids (SGCS) and cytostatics is necessary for patients with severe sarcoidosis. The degree of activity of this pathology can be different and depends on both the severity of the general inflammatory symptoms and on the number of organs involved in the pathological process and the degree of structural and functional disorders [14–16].

It is highly important to predict the severity of the pathological process, which implies that a promising approach is to analyze the obtained data on clinical and laboratory tests and instrumental examinations and identify signs that directly affect sarcoidosis severity [16]. As a rule, PH in sarcoidosis is associated with severe disease. Despite the fact that it is most common in patients with later stages of sarcoidosis, it can sometimes develop without lung parenchyma damage. Among patients with end-stage sarcoidosis awaiting lung transplantation, PH occurs in approximately 75% of cases, which indicates a high mortality rate. PH may be an early manifestation of sarcoidosis in patients without indications for transplantation. This is the reason for significant interest in the interdisciplinary problem of PH formation associated with mortality in SRS [17, 18].

The aim was to develop optimal classification models for predicting SRS severity based on the identification and determination of signs (anamnestic, clinical, laboratory, instrumental examination data, etc.) associated with the severity of sarcoidosis and subsequent risk stratification of PH development.

MATERIALS AND METHODS

The study was carried out in 2007–2019 in Tomsk Regional Clinical Hospital. We analyzed 298 sarcoidosis cases in patients aged 19 to 74 years (107 (35.9%) men and 191 (64.1%) women) at the pulmonary hospital and in the outpatient sarcoidosis room of the consultative and diagnostic polyclinic. At the time of diagnosis, 145 patients underwent echocardiography.

The average age of the SRS onset was 42 (34; 52) years. The average duration of sarcoidosis at the time of inclusion in the study was 5.6 ± 0.2 years, with the longest history of the disease in the patient aged 30 years. The diagnosis of sarcoidosis was established based on the criteria set by the statement of the World Association for Sarcoidosis and Other Granulomatous Disorders (1999), confirmed morphologically and/or in the presence of a typical clinical presentation and radiological data, provided that other diseases with similar manifestations are excluded [19].

According to the results of the assessment of the features of chronic sarcoidosis, two groups of patients were formed: group 1 included 163 patients with mild sarcoidosis; group 2 – 135 patients with severe sarcoidosis. Mild sarcoidosis was determined in the presence of spontaneous regression of the disease, including spontaneous, without SGCS or during short-term administration of small doses of SGCS, in the absence of relapses of the disease, weight loss, and generalized sarcoidosis. Patients with progression and recurrence of sarcoidosis or its generalized forms have severe sarcoidosis [4, 7, 8]. When describing the nature of the disease severity, we used concepts such as the active phase (progression), the regression phase (spontaneous or after treatment), and the stabilization phase (inpatient), which are mentioned in the Federal Clinical Guidelines for the Diagnosis and Management of Sarcoidosis 2022 [4].

The work analyzes more than 200 different parameters and characteristics obtained during examination of patients. Thus, the following data were collected and analyzed from all patients with SRS: complaints, medical history, assessment of laboratory parameters over time (complete blood count and blood biochemistry, general urinalysis, Diaskintest), data from functional and instrumental examination (spirometry, radiography, and high-resolution computed tomography (CT scan) of the chest and lungs, abdominal and kidney ultrasound examination, ECG, transthoracic echocardiography), and the presence of granulomas in internal organs. Histologic examination of biopsy samples from the affected lesions in the lungs and/or intrathoracic lymph nodes was used to confirm the diagnosis of sarcoidosis.

Statistical data analysis was performed using the RStudio v. 4.3.1 software package and the Statistica 13.3 software package. Descriptive statistics of quantitative parameters that do not fit a normal distribution are presented as the median and the upper and lower quartiles of *Me* (Q_1; Q_3). The comparison of differences between the groups was performed using the nonparametric Mann – Whitney test. The results were considered statistically significant at $p < 0.05$. The mathematical forecasting model was developed using the logistic regression with stepwise selection. The quality of the models was assessed by constructing a classification matrix, calculating sensitivity and specificity as well as calculating the area under ROC curve [9].

RESULTS AND DISCUSSION

Among all types of sarcoidosis severity, the combination of changes in the lungs with cardiac involvement is a difficult issue in managing this patient group due to severe SRS and difficulties encountered at the stage of diagnosis of the disease [13, 14]. Currently, diagnosing sarcoidosis is rather difficult, as the etiology, pathogenesis, and risk factors for the development of the disease itself are not fully understood [15, 16]. Literature contains different opinions about the dependence of the frequency of PH detection on the stage of sarcoidosis and the activity of the pathological process [8, 15]. The intravital diagnosis of cardiac sarcoidosis is quite difficult due to the low specificity of clinical signs and the low (20–30%) sensitivity of endomyocardial biopsy results [18].

According to the results of of Doppler echocardiography, changes in the morphometric parameters of the left and right sides of the heart were noted in patients with SRS. Thus, dilation of the left atrium (LA) was noted in 10.3% of patients and the left ventricle (LV) in 3.4% of cases. To diagnose dilation of the right ventricular outflow tract, trunk, and branches of the LA, measurements were performed from a parasternal approach along a short axis at the level of the aortic root [5, 6]. LV dilation was detected in 17.9% of patients, of which the majority (65.3%) belonged to the group with severe sarcoidosis. The analysis did not reveal any statistically significant differences between the groups in terms of parameters reflecting the functions of the right heart, as well as when comparing them with those in the control group, with the exception of mPAP.

According to the standard echocardiography protocol, the generally accepted diagnostic criterion, which makes it possible to diagnose PH, is the predicted value of the mean pulmonary artery pressure (pmPAP) > 35 mm Hg [1, 6–8]. As a result of the study, a predicted value of mPAP > 35 mm Hg was detected in 11 (7.6%) patients with SRS, which was 2.7% of SRS cases with mild sarcoidosis and 12.7% (more than 4 times more often) in the group with severe disease ($\chi^2 = 409.5$; $p = 0.01$). On average, patients were diagnosed with PH after 2.6 years of SRS. To assess the pressure in the LA during Doppler echocardiography, the tricuspid regurgitation rate (TR) was assessed. The breakpoint in this case is > 2.8 m/s. [1, 5, 6, 8]. The TR value in group 1 (2.4 m/s) did not differ from that in group 2 (2.6 m/s), $p = 0.18$.

Given the absence of specific PH symptoms in patients without cardiac involvement, its diagnosis requires strict adherence to a diagnostic algorithm with a gradual transition from the most common causes of PH to the rarer ones in order to consistently eliminate them. In this regard, the main purpose of a comprehensive examination of a patient with suspected PH is to diagnose it at an early stage, as well as to assess the functional and hemodynamic status [14, 16].

We compared patients with mild and severe sarcoidosis to develop models for predicting its severity. Next, a model was developed based on the parameters of transthoracic echocardiography, which indirectly reflect the damage to the heart in sarcoidosis, which is difficult to verify in clinical practice [1, 5, 6, 8].

The following predictors were used in the model: gender, age at the onset of the disease, the presence of extrapulmonary localizations of sarcoidosis, computed tomography (CT) data: quantitative assessment of the lung parenchyma lesion, areas of pronounced pulmonary fibrosis, new elements of the disseminated sarcoidosis, interstitial component, lymphopenia, predominant lesion area, and skin manifestations.

The model was obtained by binary logistic regression using stepwise elimination of predictors. The final model included: the presence of extrapulmonary localizations in the patient (odds ratio (OR) 2.99, 95% confidence interval (CI) (1.32; 7.03)), skin manifestations (OR 3.19, 95% CI (1.12; 9.46)), severe pulmonary fibrosis (OR 4.50, 95% CI (1.38; 16.24)), new elements of the disseminated sarcoidosis (OR 8.84, 95% CI (3.32; 26.72)), interstitial component (OR 3.76, 95% CI

(1.20; 12.55)), lymphopenia (OR 1.88, 95% CI (0.87; 4.15)), as well as quantitative assessment of changes in the lungs (OR 1.65, 95% CI (1.21; 2.31)), gender (OR 2.51, 95% CI (1.06; 6.32)).

The sensitivity of the model was 84%, the specificity was 86%, and the percentage of correct solutions was 85%. The area under ROC curve was 0.9, which indicates the high quality of the model.

The equation of the model was the following:

$$p = \frac{1}{1+e^{-(-4.916 + 1.097 * \text{EP} + 1.159 * SM + 1.504 * SPF + 2.180 * \text{NEDS} + 1.329 * IC + 0.633 * L + 0.500 * QACL + 0.919 * \text{Gender})}}$$

If the value of the function for the variable p is greater than 0.5, then severe sarcoidosis is predicted, if less – mild sarcoidosis.

Then, a model was developed based on the parameters of transthoracic echocardiography, radiographic findings, and CT scan. We chose the binary logistic regression method to develop the model. The model included: hypokinetic zones detected during echocardiography, the LV posterior wall thickness (LV PWT, mm), stage of sarcoidosis based on the pattern of chest radiographic findings, and the presence of few lesions in the lungs based on the high-resolution CT scan (Table).

Table

Predictors of sarcoidosis severity

Parameter	Ratio	OR	CI		*p*
			2.5%	97.5%	
Intercept term	–3.9				<0.025
LV PWT, mm	0.1	1.16	0.84	1.62	0.383
Few lesions in the lungs	–0.8	0.43	0.16	0.91	0.049
Hypokinetic zones based on echocardiography	1.3	3.51	0.52	69.28	0.265
Stage of sarcoidosis based on the pattern of chest radiographic findings	1.4	4.15	1.79	10.94	0.002

The logistic regression equation is as follows:

$$p = \frac{1}{1+e^{-(-3.886 + 1.255 * HZ + 0.145 * \text{LVPWT} + 1.423 * RS - 0.839 * FLL)}}, \quad (1)$$

where HZ is hypokinetic zones identified based on the echocardiography data (yes / no); LV PWT is the LV posterior wall thickness (mm); RS is the stage of sarcoidosis based on the pattern of chest radiographic findings (stage 0, 1, 2, 3); FLL is the presence of few lesions in the lungs (yes / no).

The standard value of 0.5 is used as the cut-off point. The decision rule (1) is as follows: if the probability p, calculated by formula (1), is greater than 0.5, then the disease is classified as severe, if less than 0.5, then mild.

Example of applying model 1. Patient M. had verified stage 3 SRS based on the patterns of radiological findings, absence of hypokinetic zones based on echocardiography, no lesions in the lungs; LV PWT was 11 mm. The calculated p value was 0.99, which is more than 0.5. Therefore, sarcoidosis is classified as severe. The predicted value is the same as the actual value set after 13 months of follow-up.

The quality of the model was assessed using a test sample. The sensitivity was 80%, the specificity was 73%, and the percentage of correct solutions was 77%. The area under ROC curve was 0.81.

Most of the parameters assessed in patients in clinical practice relate to signs that are determined by physicians. Therefore, models developed using quantitative features can be assumed to have a greater diagnostic value. Linear discriminant analysis is the method of statistical modeling, in which quantitative features are predictors.

Using this method, a model was developed that included the following predictors: age at the time of the onset of the disease, blood calcium levels, the Tiffeneau – Pinelli index (prebronchodilatory), as well as some echocardiographic parameters.: LV PWT (mm), interventricular septal thickness (IST, mm), volume of the right ventricle, LV ejection fraction (LVEF) (%), and AST level (U/l).

All the listed predictors of the model are very diverse and non-specific. The size of the right ventricle may indicate the development of a chronic pulmonary heart. In addition, the detection of structural changes in the heart, primarily in its right side (enlarged right atrium and ventricle), is important in the echocardiographic diagnosis of PH [5, 6]. The presence of hypokinetic zones, LVEF of less than 50%, and LV IST (≤ 4 mm at a distance of 10 mm from the aortic fibrous ring or its hypertrophy in the basal parts) may also indicate the onset of early signs of PH [4, 16].

These data also allow us to imply heart damage in sarcoidosis, which is very difficult to prove in clinical practice, given that the intravital diagnosis of SRS in the heart is quite difficult due to the non-specific clinical signs [19]. The AST level can also indicate damage to the heart muscle or myositis (clinically rarely diagnosed), as well as liver damage in sarcoidosis.

The resulting model is statistically significant (Wilks' lambda is 0.83; F (8.143) = 3.7; $p < 0.001$).

The equation was as follows:

$$y = 0.380*Ca^{2+}+0.096*RV-0.941*TPI+ \\ +0.349*LV\ PWT - 0.173*IST-0.098*LVEF+ \\ +0.407*AST+0.063*A, \quad (2)$$

where Ca^{2+} is the calcium level in the blood (mmol/l); RV is the volume of the right ventricle (ml); TPI is the the Tiffeneau – Pinelli index (%); LV PWT is the LV posterior wall thickness (mm); IST is the interventricular septal thickness (mm); LVEF is the LV ejection fraction (%), AST (U/l); A is the age of the onset of the disease (years).

The decision rule (2) is the following: if the y value calculated by formula (2) is less than 0.01, then the disease is classified as severe, if it is greater than 0.01, then mild.

Example of applying model 2. Patient K. was diagnosed with SRS and was 50 years old (0.56) when the diagnosis was verified, had the following indicators: blood calcium level of 2.54 mmol/l (0.36), the Tiffeneau – Pinelli index (prebronchodilatory) of 100% (–3.21), LV PWT – 10 mm (–0.40), IST – 9 mm (–0.21), the RV volume was 22 ml (1.06), LVEF was 69% (–0.20), and the AST level was 36 U/l (–0.71). The calculated value of y = 2.93, which is greater than –0.01, therefore, the disease is classified as mild. The predicted value is the same as the actual value set after 10 months of follow-up.

The quality of the model was assessed using a test sample. The sensitivity was 86%, the specificity was 87%, and the percentage of correct solutions was 85%.

CONCLUSION

To date, it is very difficult to identify the phenotype in SRS, as well as to assess disease severity. According to various literature data, it may take at least 1 to 10 years to establish the duration and assess severity of sarcoidosis in patients due to completely different reasons, including anything from the lack of a universal marker of sarcoidosis activity to a tendency to chronic intermittent course [20, 21].

As a result of the study, models were developed using various statistical modeling methods to predict sarcoidosis severity.

1. Patients with severe SRS developed PH more than 4 times more often than those with mild one ($\chi^2 = 409.5$; $p = 0.01$).

2. The first model, based on the signs used in clinical practice, has a high quality of prediction and includes predictors such as gender, age of the onset, the presence of extrapulmonary localization of sarcoidosis, CT data, lymphopenia, predominant lung lesion area, and skin manifestations. Model 1 is characterized by 84% sensitivity and 86% specificity.

4. The second model, based on the parameters of transthoracic echocardiography, including indicators that indirectly assess the development of PH and heart damage in sarcoidosis, depending on its severity, has a sensitivity of 80% and a specificity of 73%.

Both models include different groups of features that allow us to assess the relationship of various factors with SRS severity from different points of view. The application of the proposed models in clinical practice will make it possible to predict SRS severity already upon confirmation of the diagnosis, which may be useful for deciding on the patient management strategy (observation or requiring the prescription of pathogenetic immunosuppressive therapy). In addition, the set of characteristics associated with sarcoidosis severity includes some echocardiographic indicators (LV PWT, IST, RV volume, LVEF), taking into account the higher risk of developing PH in patients with severe sarcoidosis.

Thus, complex prediction models combining a variety of parameters associated with sarcoidosis severity are crucial for making prognosis regarding disease severity, including taking into account the higher risk of developing PH.

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Authors' contribution

Kalacheva T.P., Denisova O.A., Chernogoryuk G.E., Kalyuzhina E.V., Purlik I.L. – conception and design; data collection, analysis and interpretation, justification of the manuscript and critical revision for important intellectual content, final approval of the manuscript for publication. Fedosenko S.V., Karnaushkina M.A., Kalyuzhin V.V., Chernyavskaya G.M. – critical revision for important intellectual content, final approval of the manuscript for publication. Ostanko V.L., Palchikova I.A., Romanov D.S., Kulumaeva K.A. – data collection, analysis, and interpretation. Brazovskaya N.G. – statistical processing, analysis, and interpretation of data.

Authors' information

Kalacheva Tatiana P. – Cand. Sci. (Med.), Associate Professor of the General Medical Practice and Outpatient Therapy Division, SibMed, Tomsk, tatyana-kalachyova@yandex.ru, http://orcid.org/0000-0002-4292-7723

Denisova Olga A. – Cand. Sci. (Med.), Assistant of the Advanced Therapy Division with Rehabilitation Training, Physiotherapy and Sports Medicine, SibMed, Tomsk, oadeni@yandex.ru, http://orcid.org/0000-0003-1652-9622

Brazovskaya Natalia G. – Cand. Sci. (Med.), Associate Professor of the Medical and Biological Cybernetics Division, SibMed, Tomsk, brang@mail.ru, http://orcid.org/0000-0002-0706-9735

Fedosenko Sergey V. – Doctor of Medical Sciences, Professor of the General Medical Practice and Outpatient Therapy Division, SibMed, Tomsk, s-fedosenko@mail.ru, http://orcid.org/0000-0001-6655-3300

Karnaushkina Maria A. – Dr. Sci. (Med.), Professor of the Department of Internal Medicine with a Course in Cardiology and Functional Diagnostics named after Academician V.S. Moiseev, RUDN University, Moscow, kar3745@yandex.ru, http://orcid.org/0000-0002-8791-2920

Ostanko Valentina L. – Cand. Sci. (Med.), Associate Professor, Associate Professor of the Advanced Therapy Division with Rehabilitation Training, Physiotherapy and Sports Medicine, SibMed, Tomsk, valala@yandex.ru, http://orcid.org/0000-0002-9950-721X

Chernyavskaya Galina M. – Dr. Sci. (Med.), Professor of the Advanced Therapy Division with Rehabilitation Training, Physiotherapy and Sports Medicine, SibMed, Tomsk, chernyavskayag@gmail.com, http://orcid.org/0000-0003-0105-2307

Kalyuzhina Elena V. – Dr. Sci. (Med.), Professor of the Advanced Therapy Division with Rehabilitation Training, Physiotherapy and Sports Medicine, SibMed, Tomsk, kalyuzhina.e@mail.ru, http://orcid.org/0000-0002-7978-5327

Chernogoryuk Georgy E. – Dr. Sci. (Med.), Professor of the Advanced Therapy Division with Rehabilitation Training, Physiotherapy and Sports Medicine, SibMed, Tomsk, chernogoryuk@yandex.ru, http://orcid.org/0000-0001-5780-6660

Palchikova Inna A. – Pulmonologist, Rheumatologist, Rheumatology Unit, Tomsk Regional Clinical Hospital, Tomsk, pial83@mail.ru, http://orcid.org/0000-0003-4968-1110

Romanov Dmitry S. – Assistant, Propaedeutics of Internal Diseases Division with Therapy Course of the Pediatrics Department, SibMed, Tomsk, romanov.ds@ssmu.ru, http://orcid.org/0009-0002-2028-4963

Purlik Igor L. – Dr. Sci. (Med.), Professor of the Pathological Anatomy Division, SibMed, Tomsk, igor0812@rambler.ru, http://orcid.org/0000-0003-3757-0173

Kulumaeva Karina A. – Resident, Advanced Therapy Division with Rehabilitation, Physiotherapy and Sports Medicine Course, SibMed, Tomsk, karina.kulumaeva12@mail.ru, http://orcid.org/0009-0002-5933-3873

Kalyuzhin Vadim V. – Dr. Sci. (Med.), Professor, Head of the Advanced Therapy Division with Rehabilitation Training, Physiotherapy and Sports Medicine, SibMed, Tomsk, kalyuzhinvv@mail.ru, http://orcid.org/0000-0001-9640-2028

(✉) **Kalacheva Tatiana P.,** tatyana-kalachyova@yandex.ru

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The impact of the hypothalamic-pituitary-thyroid axis hormone levels on suicide risk in patients with schizophrenia

Kornetova E.G.[1], Galkin S.A.[1], Lobacheva O.A.[1], Mednova I.A.[1], Kornetov A.N.[2], Bokhan N.A.[1]

[1] *Mental Health Research Institute, Tomsk National Research Medical Center (NRMC), Russian Academy of Sciences 4, Aleutskaya Str., Tomsk, 634014, Russian Federation*

[2] *National Research Tomsk State University 36, Lenin Av., Tomsk, 634050, Russian Federation*

ABSTRACT

Aim. To assess the impact of thyroid stimulating hormone (TSH), free triiodothyronine (FT3), and free thyroxine (FT4) concentrations in the blood serum of patients with schizophrenia with suicide risk.

Materials and methods. A total of 120 patients with schizophrenia (75 women and 45 men) were examined. Suicide risk was assessed using the Beck Hopelessness Scale (BHS). Serum levels of FT3, FT4, and TSH in patients with schizophrenia were determined using enzyme immunoassay kits. Multiple linear regression analysis and one-way analysis of variance (ANOVA) were used to identify the relationships between the studied indicators.

Results. Among 120 patients with schizophrenia, 11 patients (9.2%) had elevated serum TSH values (> 4.0 mU / l), 108 (90%) had decreased FT3 levels, (< 4.0 pmol / l), 42 (35%) had decreased FT4 levels (< 10.3 pmol / l). The study revealed statistically significant differences in the level of hopelessness between the groups of patients with normal and elevated TSH ($F(1.118) = 5.160$, $p = 0.025$), as well as with normal and decreased FT3 ($F(1.118) = 4.568$, $p = 0.035$).

Conclusion. It was found that TSH and FT3 concentrations in blood serum significantly affect the level of hopelessness assessed using the Beck scale in patients with schizophrenia. The results of this study confirm the need for regular dynamic monitoring of hormone levels of the hypothalamic–pituitary–thyroid axis in patients with schizophrenia in order to maintain its normal functioning, as well as prevent adverse effects in the form of suicide attempts and suicide.

Keywords: schizophrenia, thyroid hormones, TSH, hopelessness, suicide risk

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✉ *Kornetov Alexander N.,* alkornetov@gmail.com

Влияние уровней гормонов гипоталамо-гипофизарно-тиреоидной оси на суицидальный риск у пациентов с шизофренией

Корнетова Е.Г.[1], Галкин С.А.[1], Лобачева О.А.[1], Меднова И.А.[1], Корнетов А.Н.[1], Бохан Н.А.[1, 2]

[1] Научно-исследовательский институт (НИИ) психического здоровья, Томский национальный исследовательский медицинский центр (НИМЦ) Российской академии наук
Россия, 634014, г. Томск, ул. Алеутская, 4

[2] Национальный исследовательский Томский государственный университет (НИ ТГУ)
Россия, 634050, г. Томск, пр. Ленина, 36

РЕЗЮМЕ

Цель. Оценить влияние уровней тиреотропного гормона (ТТГ), свободного трийодтиронина (T_3св.) и свободного тироксина (T_4св.) в сыворотке крови пациентов с шизофренией с суицидальным риском.

Материалы и методы. Обследовано 120 больных (75 женщин и 45 мужчин). Суицидальный риск оценивался по шкале безнадежности Бека (Beck Hopelessness Scale, BHS). Уровни T_3св., T_4св. и ТТГ в сыворотке крови у пациентов определяли с помощью наборов для иммуноферментного анализа. Для выявления связей между исследуемыми показателями использовался множественный линейный регрессионный анализ и однофакторный дисперсионный анализ (ANOVA).

Результаты. Среди 120 пациентов с шизофренией у 11 (9,2%) были выявлены повышенные значения ТТГ в сыворотке крови (>4,0 мМЕ/л), у 108 (90%) снижены показатели T_3св. (<4,0 пкмоль/л), у 42 (35%) – снижены показатели T_4св. (<10,3 пкмоль/л). Обнаружены статистически значимые различия уровня безнадежности между группами пациентов с нормальным и повышенным показателем ТТГ ($F(1,118) = 5,160$, $p = 0,025$), а также с нормальным и сниженным показателем T_3 св. ($F(1,118) = 4,568$, $p = 0,035$).

Заключение. Установлено, что на уровень безнадежности по шкале Бека у больных шизофренией оказывают значимое влияние показатели ТТГ и T_3св. в сыворотке крови. Результаты данного исследования подтверждают необходимость регулярного динамического мониторинга показателей гормонов гипоталамо-гипофизарно-тиреоидной оси у пациентов с шизофренией с целью сохранения ее нормального функционирования, а также предотвращения неблагоприятных последствий в виде суицидальных попыток и суицидов.

Ключевые слова: шизофрения, тиреоидные гормоны, ТТГ, безнадежность, суицидальный риск

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

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INTRODUCTION

Schizophrenia is a severe mental disorder that often begins with a catastrophe experienced by the patient. It usually has a chronic course, is accompanied by anhedonia, leads to disability and a significant decrease in the duration and quality of patient life [1, 2]. The comorbid somatic-symptom disorder (cardiovascular diseases, obesity, diabetes mellitus, etc.) and suicide are the main causes of early mortality and reduce the life expectancy of patients with schizophrenia by an average of 10 years [3, 4]. Despite the fact that the pathophysiological mechanisms of schizophrenia are still poorly understood, studies have shown that neuroendocrine disorders can play an important role in its development, determine its course, clinical manifestations, as well as in the occurrence of concomitant pathology, complications, and adverse effects of antipsychotics [5–7].

The functional state of the hypothalamic–pituitary–thyroid axis is of great importance for the development and normal functioning of the brain [6]. Previous studies have shown a connection between fluctuations in thyroid hormone levels and various manifestations of mental disorders and response to therapy [5–9]. Our recent studies revealed a significant decrease in serum levels of thyroid hormones and thyroid-stimulating hormone (TSH) in patients with schizophrenia compared to healthy individuals [7, 10].

The role of various thyroid conditions in the formation of psychopathological symptoms as part of schizophrenia has already been reflected in the literature [6, 7, 10, 11]. This fact once again emphasizes the multifactorial nature of not only the disorder itself, but also the leading symptoms in the clinical pattern. Thus, the predictive role of thyroid hormones and TSH in relation to the schizophrenia prognosis is now obvious. For example, there is a strong negative correlation between negative symptoms that have an unfavorable course and the TSH level [12]. In addition, changes in the thyroid hormone level are associated with antipsychotic treatment, in which subclinical hypothyroidism often develops [13]. However, the role of the hypothalamic–pituitary–thyroid axis in the development of suicidal behavior in patients with schizophrenia remains poorly understood, and the available data are ambiguous.

For example, it was found that individuals with and without a history of suicide attempts differed only in the level of free triiodothyronine (FT3) [14]. Moreover, patients with a history of suicide attempts were more likely to have low levels of free T3 [14]. Another study showed that suicidal thoughts were more common in patients with schizophrenia only with higher levels of free thyroxine (free T4) [11]. It is also assumed that low TSH levels may be associated with a predisposition to depression and suicidal behavior [15, 16]. This led us to the hypothesis of a "pessimistic" relationship between TSH, thyroid hormones, schizophrenia, and suicide risk.

The aim of the study was to evaluate the TSH, free T3, and free T4 concentrations in the blood serum of patients with schizophrenia at a risk of suicide.

MATERIALS AND METHODS

The study was conducted according to the protocol approved by the local Bioethics Committee at the Mental Health Research Institute of Tomsk National Research Medical Center (Protocol No. 147 of 22.11.2021). During the study, we examined 120 patients with schizophrenia (75 (62.5%) women and 45 (37.5%) men, F20.0 according to ICD-10) aged 43 [36; 52] years and with a disease duration of 15 [9; 23] years, who were treated at the clinic of the Mental Health Research Institute. The inclusion criteria for this study were as follows: age 18–55 years, confirmed diagnosis of schizophrenia according to ICD-10 criteria, signed voluntary consent to participate in the study. The exclusion criteria were the following: clinically evident dependence on psychoactive substances except for tobacco, mental retardation or dementia, no known neurological disorders (brain injury, stroke), thyroid disease, hormone replacement therapy.

At the beginning of the study, all patients received basic therapy, 95 (79.2%) of them were receiving first-generation antipsychotics: haloperidol, zuclopenthixol, chlorpromazine, chlorprothixene, 25 (20.8%) – second-generation antipsychotics: quetiapine, clozapine, olanzapine, risperidone in therapeutic doses approved by the Russian Ministry of Healthcare, which were recalculated to chlorpromazine equivalent (CPZeq). This recalculation made it possible to calculate the median of the total antipsychotic load, which ultimately amounted to 482.5 [271; 758.5] mg/day, while the duration of therapy was 11 [5; 19] years.

Individual registration cards were filled out for all patients included in the study. The card included

general information, a set of sociodemographic, clinical, and therapeutic characteristics, as well as psychometric examination data. An objective assessment of the severity of psychopathological manifestations was performed using the Positive and Negative Syndrome Scale (PANSS) [17] in the adapted Russian version – SCI-PANSS [18]. The total PANSS score for the entire sample was 107 [96; 116], the severity of positive symptoms was 25 [22; 28] points vs. 24 [22; 29] negative with general psychopathological symptoms – 56 [51; 61]. Suicide risk was assessed using the Beck Hopelessness Scale (BHS) [19], which measures the severity of negative attitude towards one's own perceived future and makes it possible to indirectly determine suicide risk [20]. Studies using this scale have shown that it can be used to assess suicide risk [21], including in patients with schizophrenia [22].

Blood was collected from fasting patients in the morning from the cubital vein into Vacuette vacuteiner tubes, and the blood serum was obtained by centrifugation at 2,000 rpm for 30 minutes. Concentrations of TSH, FT3, and FT4 in the blood serum were determined by solid-phase enzyme immunoassay using Vector-Best reagent kits (Novosibirsk, Russia). In accordance with the manual of the kits, the reference intervals for FT3, FT4, and TSH in the blood serum were 4.0–8.6 pmol / l, 10.3–24.5 pmol / l, and 0.4–4.0 mU / l, respectively.

The statistical analysis was performed using the Statistica 12.0 software package (Dell). The Shapiro–Wilk test was used to check for the normality of the data sample. It showed that all the data we obtained did not fit the normal distribution. Therefore, the quantitative data are presented as the median (*Me*) of the lower and upper quartiles [Q_1; Q_3]. Qualitative data are presented as frequency indicators in absolute (*n*) and relative units (%). Multiple linear regression analysis and one-way analysis of variance (ANOVA) were used to identify the relationships between the studied indicators. The results were considered statistically significant at $p = 0.05$.

RESULTS

Using multiple linear regression, we verified the effect of each hormone on the level of hopelessness in patients (Table). It turned out that the model obtained during the calculations was statistically insignificant (F (3.116) = 1.166, $p = 0.325$). R^2 was 0.029, which indicates that TSH and thyroid hormone levels explain 2.9% of the variability in the level of hopelessness according to the Beck scale. All studied predictors of hopelessness (TSH ($t = –1.676$, $p = 0.096$), FT3 ($t = –0.607$, $p = 0.544$), and FT4 ($t = –0.224$, $p = 0.822$)) were statistically insignificant.

Table

Values of the coefficients in the multiple linear regression model for the dependence of hopelessness on TSH and thyroid hormone levels

Indicator	Coefficient (B)	Standard error	*t*-test	*p*
Constant	9.012	2.711	3.323	0.001
TSH	–0.426	0.254	–1.676	0.096
FT3	–0.397	0.654	–0.607	0.544
FT4	–0.045	0.203	–0.224	0.822

Then, TSH and thyroid hormone levels were transformed into categorical variables (0 – normal, 1 – abnormal) based on the reference values. Thus, among 120 patients with schizophrenia, 11 patients (9.2%) had elevated serum TSH levels (> 4.0 mU / l), 108 (90%) had decreased FT3 levels (< 4.0 pmol / l), and 42 (35%) had decreased FT4 level (<10.3 pmol / l). Accordingly, based on the categories obtained, the effect of the hypothalamic–pituitary–thyroid axis hormone levels on the level of hopelessness was assessed using one-way ANOVA (Fig.).

The results of one-way ANOVA test revealed statistically significant differences in the level of hopelessness between the groups of patients with normal and elevated TSH (F (1.118) = 5.160, $p = 0.025$), as well as with normal and reduced FT3 (F (1.118) = 4.568, $p = 0.035$). The presented groups of patients were comparable in terms of sociodemographic, clinical, and therapeutic indicators ($p > 0.05$). Thus, the level of hopelessness according to the Beck scale in patients with schizophrenia was significantly affected by TSH and FT3 concentrations in the blood serum.

DISCUSSION

The study assessed the effect of the hypothalamic–pituitary–thyroid axis hormone levels on the risk of suicide in patients with schizophrenia. During the analysis of variance, we found a significant effect of serum TSH and FT3 in these patients on the level of hopelessness, which confirms the role of thyroid function in the suicidal behavior of patients with schizophrenia.

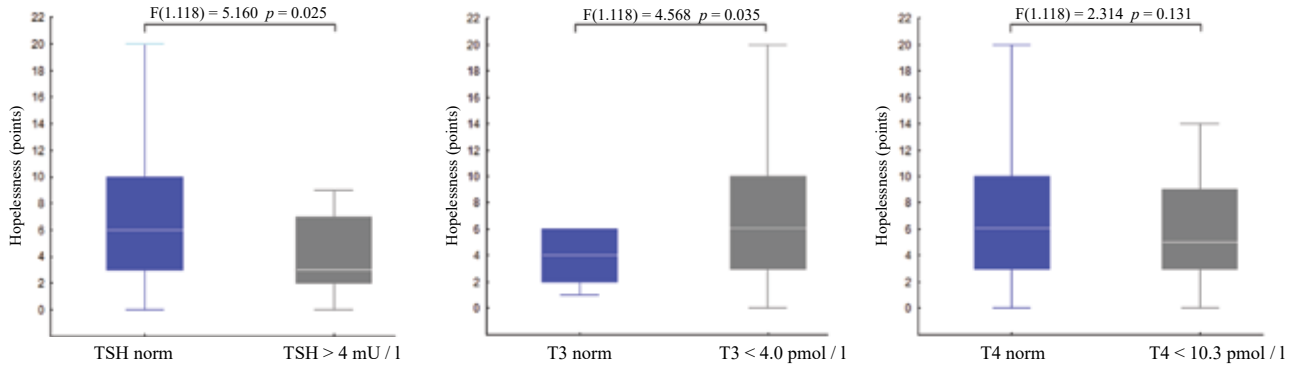

Figure. Boxplots of the level of hopelessness depending on the indicators of the hypothalamic–pituitary–thyroid axis hormones

Many authors associate TSH with psychotic symptoms, anxiety and depression [15, 16]. TSH can increase the risk of suicide by exacerbating anxiety, depression, and psychotic symptoms [23]. However, our study showed that patients with elevated TSH values (subclinical hypothyroidism) had a lower level of hopelessness and, as a result, a lower risk of suicide. On the other hand, in patients with reduced FT3, we observed a significantly more pronounced level of hopelessness. Our findings regarding the effect of triiodothyronine on suicide risk in patients with schizophrenia comply with the results of previous studies presented in the systematic review and meta-analysis of F.J.K. Toloza et al. [24].

As is known, TSH and thyroid hormones interact with each other using a negative feedback mechanism [25]. A decrease in thyroid hormones (hypofunction) entails an increase in the synthesis of TSH by the pituitary gland. Thus, we assume that patients with schizophrenia and hypothyroidism have increased synthesis of TSH, which has a compensatory effect on thyroid function and on the concentrations of FT3 and FT4, in particular, and can also contribute to reducing the suicide risk.

CONCLUSION

This study has shown the need for continuous dynamic monitoring of the hypothalamic–pituitary–thyroid axis hormone level in individuals with schizophrenia in order to preserve and maintain normal neuroendocrine balance, as well as to prevent suicide attempts and suicide.

It should be emphasized that our study does not directly assess the cause-and-effect relationships between suicide risk and hypothyroidism, which dispels the initial pessimistic assumptions, however, in accordance with the results obtained, monitoring of these indicators is extremely important for patients with schizophrenia, especially in regions with iodine deficiency.

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Authors' contribution

Kornetova E.G. – conception and design, review of publications on the topic of the article, drafting of and editing the manuscript. Galkin S.A. – review of publications on the topic of the article, drafting of and translating the manuscript, statistical analysis. Lobacheva O.A. – review of publications on the topic of the article, sample survey. Mednova I.A. – sample survey, database formation. Kornetov A.N. – conception and design, review of publications on the topic of the article, drafting of and editing the manuscript, translating the manuscript. Bokhan N.A. – final approval of the manuscript topic.

Authors' information

Kornetova Elena G. – Dr. Sci. (Med.), Head of the Department of Endogenous Disorders, Mental Health Research Institute, Tomsk NRMC, Tomsk, ekornetova@outlook.com, http://orcid.org/0000-0002-5179-9727.

Galkin Stanislav A. – Cand. Sci. (Med.), Researcher, Mental Health Research Institute, Tomsk NRMC, Tomsk, s01091994@yandex.ru, http://orcid.org/0000-0002-7709-3917.

Lobacheva Olga A. – Dr. Sci. (Med.), Senior Researcher of Laboratory of Clinical Psychoneuroimmunology and Neurobiology, Mental Health Research Institute, Tomsk NRMC, Tomsk, oalobacheva@mail.ru, http://orcid.org/0000-0002-7477-6296.

Mednova Irina A. – Cand. Sci. (Med.), Researcher, Mental Health Research Institute, Tomsk NRMC, Tomsk, irinka145@yandex.ru, http://orcid.org/0000-0002-8057-3305.

Kornetov Alexander N. – Dr. Sci. (Med.), Mental Health Research Institute, Tomsk NRMC, Tomsk; alkornetov@gmail.com, http://orcid.org/0000-0002-2342-7504.

Bokhan Nikolay A. – Dr. Sci. (Med.), Professor, Academician, Director of the Mental Health Research Institute, Tomsk NRMC, National Research Tomsk State University, Tomsk, bna909@gmail.com, http://orcid.org/0000-0002-1052-855X

(✉) **Kornetov Alexander N.,** alkornetov@gmail.com

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Mechanisms of the cardioprotective effect of lithium

Mukhomedzyanov A.V.[1], Plotnikov E.V.[2,3,4], Maslov L.N.[1], Chernov V.I.[2,5], Naryzhnaya N.V.[1], Slidnevskaya A.S.[1], Yusubov M.S.[2], Larkina M.S.[2,3], Artamonov A.A.[6], Belousov M.V.[2,3]

[1]*Cardiology Research Institute, Tomsk National Research Medical Center (NRMC), Russian Academy of Sciences 111 A, Kievskaya Str., Tomsk, 634012 Russian Federation*

[2] *National Research Tomsk Polytechnic University (NR TPU) 30, Lenina Av., Tomsk. 634050, Russian Federation*

[3] *Siberian State Medical University 2, Moscow Trakt, Tomsk, 634050, Russian Federation*

[4] *Mental Health Research Institute, Tomsk National Research Medical Center (NRMC), Russian Academy of Sciences 4, Aleutskaya Str., Tomsk, 634021, Russian Federation*

[5] *Cancer Research Institute, Tomsk National Research Medical Center (NRMC), Russian Academy of Sciences 5, Kooperativny Str., Tomsk, 634009, Russian Federation*

[6] *Institute of Biomedical Problems, Russian Academy of Sciences 76 A, Khoroshevskoe Highway, Moscow, 123007, Russian Federation*

ABSTRACT

Background. A study of the mechanisms of infarct size-limiting effects of lithium chloride (LiCl) on a model of myocardial infarction *in vivo*. Myocardial infarction is one of the main causes of death among the adult working population in economically developed countries. Currently, there are no drugs in clinical practice that would effectively protect the myocardium against ischemia – reperfusion injury, so there is a need to develop new drugs that can limit infarct size and reduce mortality.

Aim. To study the mechanisms of infarct size-limiting effects of lithium chloride.

Materials and methods. A study was performed in anesthetized Wistar rats with coronary artery occlusion (45 min) and reperfusion (120 min). The molecular mechanism of the protective effects of LiCl was examined in this model using appropriate blockers, including non-selective and selective blockers of ATP-sensitive potassium channel and NO synthase.

Results. Administration of LiCl before ischemia significantly reduced infarct size as well as the as the incidence of ventricular arrhythmias. Administration of LiCl after ischemia also promoted a decrease in infarct size. NO synthase, cycloxigenase-2, protein kinase C, and endogenous opioids were not involved in the cardioprotective effect of lithium. The cardioprotective effect of LiCl is mediated via sarcolemmal ATP-sensitive potassium channel ($sarcK_{ATP}$ channel) opening.

Conclusion. LiCl reduced infarct size and prevented reperfusion cardiac injury. The main cellular ways of the infarct size-limiting effects of LiCl are mediated through the $sarcK_{ATP}$ channel opening.

Keywords: lithium, myocardial infarction, cardioprotection, potassium channels, ischemia/reperfusion

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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✉ *Plotnikov Evgenii V.,* Plotnikov.e@mail.ru

Conformity with the principles of ethics. All experiments were performed in compliance with the principles of humanity set forth in the European Community directives (86/609/EEC) and the Declaration of Helsinki and approved by the Ethics Committee at the Cardiology Research Institute of TNIMC, Protocol No. 207 of 23.12.2020). The equipment of the Center for Collective Use "Medical Genomics" of Tomsk NIMC was used in the work.

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Механизмы кардиопротекторного эффекта лития

Мухомедзянов А.В.[1], Плотников Е.В.[2, 3, 4], Маслов Л.Н.[1], Чернов В.И.[2, 5], Нарыжная Н.В.[1], Слидневская А.С.[1], Юсубов М.С.[2], Ларькина М.С.[2, 3], Артамонов А.А.[6], Белоусов М.В.[2, 3]

[1] Научно-исследовательский институт (НИИ) кардиологии, Томский национальный исследовательский медицинский центр (НИМЦ) Российской академии наук
Россия, 634012, г. Томск, Киевская, 111а

[2] Национальный исследовательский Томский политехнический университет (НИ ТПУ)
Россия, 634050, г. Томск, пр. Ленина, 30

[3] Сибирский государственный медицинский университет (СибГМУ)
Россия, 634050, г. Томск, Московский тракт, 2

[4] Научно-исследовательский институт (НИИ) психического здоровья, Томский национальный исследовательский медицинский центр (НИМЦ) Российской академии наук
Россия, 634021, Россия, Томск, ул. Алеутская, 4

[5] Научно-исследовательский институт (НИИ) онкологии, Томский национальный исследовательский медицинский центр (НИМЦ) Российской академии наук
Россия, 634009, г. Томск, пер. Кооперативный, 5

[6] Институт медико-биологических проблем (ИМБП) Российской академии наук (РАН)
Россия, 123007, г. Москва, Хорошёвское шоссе, 76а

РЕЗЮМЕ

Цель. Исследование механизмов инфаркта – лимитирующего действия хлорида лития (LiCl) на модели инфаркта миокарда *in vivo*. Инфаркт миокарда является одной из основных причин смерти взрослого трудоспособного населения в экономически развитых странах. В настоящее время в клинической практике не существует препаратов, которые с высокой эффективностью защищали бы миокард от ишемии/реперфузии, поэтому имеется необходимость в разработке новых лекарственных средств, способных ограничить размер инфаркта и снизить смертность.

Материалы и методы. Моделирование инфаркта миокарда проводили на крысах линии Вистар путем окклюзии коронарной артерии (45 мин) и реперфузии (120 мин). Молекулярный механизм защитного действия хлорида лития исследовали на модели инфаркта с помощью соответствующих блокаторов, в том числе неселективных и селективных блокаторов АТФ-чувствительного калиевого канала и NO-синтазы.

Результаты. Введение LiCl до ишемии значительно уменьшало размер инфаркта, при этом NO-синтаза, циклоксигеназа-2, протеинкиназа С, эндогенные опиоиды не были вовлечены в кардиопротекторный эффект лития. Кардиопротекторный эффект LiCl опосредован через открытие сарколеммального АТФ-чувствительного калиевого канала (саркКАТФ-канала).

Заключение. Хлорид лития может предотвратить ишемическое и реперфузионное повреждение сердца. Основные клеточные пути инфаркт-лимитирующего действия LiCl реализуются в основном через открытие саркКАТР-каналы.

Ключевые слова: литий, инфаркт миокарда, кардиопротекция, калиевые каналы, ишемия/реперфузия

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

Источник финансирования. Программа «Приоритет 2030».

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INTRODUCTION

Acute myocardial infarction (AMI) is one of the main causes of death among patients with cardiovascular diseases [1, 2]. Lethality in ST-segment elevation myocardial infarction (STEMI) is 4.6–6.8% [3, 4], and in patients with cardiogenic shock within 30 days, it exceeds 20% [5, 6]. The probability of death in patients with AMI is primarily related to infarct size, concomitant rhythm disturbances, and the time of pharmacologic or surgical reperfusion. Reperfusion provides restoration of coronary blood flow, but also contributes to reperfusion damage of the heart.

Currently in clinical practice, there are no drugs that can significantly increase cardiac resistance to reperfusion and, consequently, reduce infarct size. The most effective method of treatment of AMI is percutaneous coronary intervention; nevertheless, mortality remains at a high level [3, 4, 7, 8]. Therefore, there is an urgent need to develop new drugs that can reduce infarct size. Lithium (Li^+) and its salts are of particular interest in this regard. Lithium has a wide range of biological activities, including normothymic, cytoprotective, antioxidant, and antiapoptotic effects [9–11].

It has been previously reported that Li^+ can increase the resistance of rat heart to ischemia – reperfusion injury *in vitro* [12, 13]. However, it was unclear whether Li^+ could limit infarct size *in vivo*. The receptor and signaling mechanisms of the infarct size-limiting effect of Li^+ have not yet been studied in detail. A number of studies have shown that the cardioprotective effect of LiCl depends on the activation of cyclooxygenase [12, 13]. There are data that indicate the participation of opioid receptors (ORs) in the antinociceptive effect of Li^+, and that Li^+ is able to activate ATP-sensitive K^+ -channels (K_{ATP}-channels) [14, 15]. K_{ATP}-channels, ORs, protein kinase C (PKC), cyclooxygenase-2 (COX-2), and NO synthase (NOS) are known to play important roles in the mechanisms of cardiac tolerance to ischemia – reperfusion [16–20]. In the present study, we investigated the infarct size-limiting effect of lithium and determined the molecular mechanism of cardioprotection in *in vivo* models.

MATERIALS AND METHODS

The study design was planned according to ARRIVE guidelines 2.0 for reporting animal studies [21]. Outbred male Wistar rats weighing 250–300 g were used. One animal was considered as one experimental unit. The animals were maintained under standardized conditions at 24 ± 2 °C. The rats received pellets of normal feed and drinking water *ad libitum*. Division of animals into groups was done randomly. Simple randomization was used, with animals numbered and assigned to groups by selecting a set of numbers for each group using a random number generator. Twelve experimental animals were included in each experimental group and were processed by parametric statistical methods.

The control group (ischemia – reperfusion injury model without pharmacologic treatment) consisted of 12 experimental rats. The study was conducted using the blinding strategy. Surgical procedures and infarct size measurements were performed by different specialists who did not know to which group the animal belonged. Experimental procedures were performed in accordance with Directive 2010/63/EU of the European Parliament and the Guidelines for the Care and Use of Laboratory Animals. Pharmacologic effects in the experimental groups included the use of lithium salts and other pharmacologic agents described in detail below. LiCl solution was administered intravenously as a bolus in a volume of 1 mL of saline solution. Lithium solution

was administered 15 minutes before coronary occlusion. Other pharmacologic agents were administered intravenously 25 min before coronary artery occlusion.

The animals were anesthetized with α-chloralose (50 mg / kg, intraperitoneally) and connected to a SAR-830 ventilator (CWE Inc., Ardmore, USA). Blood pressure (BP) was recorded using an SS13L pressure transducer (Biopac System Inc., Goleta, USA) paired with an MP35 electrophysiologic study device (Biopac System Inc., Goleta, USA). This device was also used for ECG recording. Coronary occlusion (45 min) was performed according to the method of Neckar et al [22]. The animals underwent thoracotomy and pericardium removal, then ligature was applied to the coronary artery. After 45 minutes of ischemia, the ligature was removed, the restoration of blood flow was confirmed by the appearance of epicardial hyperemia. The duration of reperfusion was 2 hours.

After completion of the reperfusion period, the hearts were removed and flushed with saline solution through the aorta. The risk zone was determined by staining the myocardium through the aorta with 5% potassium permanganate solution. Then slices perpendicular to the longitudinal axis of the heart (1 mm thick) were made. The area of necrosis was distinguished from the area at risk by staining with 1% 2,3,5-triphenyltetrazolium chloride solution [22]. Heart slices were scanned and infarct area was calculated using the ImageJ program (NIH, USA). Myocardial infarct size was expressed as a percentage from the risk area size as the ratio of infarct area to risk area.

The compounds used were lithium chloride, L-NAME, glibenclamide, naltrexone, 5-GD and celecoxib (Sigma-Aldrich, USA), chelerythrine (MedChemExpress, USA), and hydroxypropyl β-cyclodextrin (Tocris Bioscience, UK). HMR 1098 was synthesized and provided by Sanofi-Aventis Deutschland GmbH (Frankfurt, Germany). Drug solutions for administration were prepared in saline *ex tempore*. Water-insoluble compounds (glibenclamide, chelerythrine, celecoxib) were first dissolved in 0.1 ml of dimethyl sulfoxide, and then 0.9 ml of 20% hydroxypropyl β-cyclodextrin was added.

We have previously found that hydroxypropyl β-cyclodextrin has no effect on animal hemodynamics, heart rhythm, and infarct size. Lithium chloride was used at doses of 40 mg / kg and 200 mg / kg intravenously. The other drugs were also administered intravenously. Naltrexone (a nonselective OR antagonist) was administered at a dose of 5 mg / kg. Chelerythrine (a selective PKC inhibitor) was administered at a dose of 5 mg / kg. The NOS inhibitor L-NAME was administered at a dose of 10 mg / kg. The non-selective K_{ATP} -channel blocker glibenclamide was administered at a concentration of 1 mg / kg. 5-Hydroxydecanoate (5-HD, a blocker of mitochondrial K_{ATP} ($mitoK_{ATP}$) channels) was administered at a dose of 5 mg / kg. HMR 1098 (a selective blocker of sarcolemmal ($sarK_{ATP}$) channels) was administered at a dose of 3 mg / kg. The selective COX-2 inhibitor celecoxib was administered at a concentration of 0.24 mg / kg. A.M. Stevens et al. demonstrated that celecoxib at this dose inhibits COX-2 [23].

The results of the study were processed using the Statistica 13.0 program (Stat Soft, USA). Data were presented as the mean and the standard deviation $M \pm \sigma$. Normality was checked by the Shapiro – Wilk test. One-factor analysis of variance (ANOVA) with the Bonferroni correction was used to compare differences between the groups. The differences between the groups were considered statistically significant at $p < 0.005$.

RESULTS

No significant changes in hemodynamic parameters were detected in animals of the control group (Table). L-NAME increased systolic blood pressure and decreased heart rate. The other drugs had no effect on hemodynamic parameters (Table).

Table

HR (beats / min) and BP (mm.Hg) in rats during coronary occlusion (45 min) and reperfusion (120 min)

Group	Dose, mg / kg	Before ischemia		45 min of ischemia		30 min reperfusion		120 min reperfusion	
		HR	AD	HR	AD	HR	AD	HR	AD
Control		363 ± 4	124 ± 3	357 ± 4	120 ± 3	352 ± 5	117 ± 3	342 ± 6	113 ± 4
LiCl	200	364 ± 4	126 ± 3	359 ± 4	121 ± 4	355 ± 3	116 ± 4	346 ± 5	111 ± 6
LiCl	40	361 ± 4	122 ± 3	355 ± 5	118 ± 3	350 ± 4	114 ± 3	341 ± 5	110 ± 6
Celecoxib	0.24	364 ± 3	121 ± 3	357 ± 4	117 ± 3	350 ± 4	113 ± 3	340 ± 5	109 ± 4

Table (continued)

Group	Dose, mg / kg	Before ischemia		45 min of ischemia		30 min reperfusion		120 min reperfusion	
		HR	AD	HR	AD	HR	AD	HR	AD
L-NAME	10	364 ± 4	125 ± 3	334 ± 5*	145 ± 3*	326 ± 6*	147 ± 4*	320 ± 6*	149 ± 6*
Chelerythrine	5	363 ± 4	123 ± 3	357 ± 3	120 ± 4	353 ± 4	115 ± 5	340 ± 6	109 ± 4
Glibenclamide	1	367 ± 3	122 ± 4	363 ± 4	119 ± 3	357 ± 4	115 ± 5	347 ± 4	110 ± 4
HMR 1098	3	365 ± 4	122 ± 3	359 ± 5	119 ± 4	355 ± 5	120 ± 4	347 ± 5	112 ± 5
5-HD.	5	362 ± 5	124 ± 4	358 ± 3	120 ± 3	353 ± 4	117 ± 4	341 ± 4	114 ± 5
Naltrexone	5	365 ± 4	126 ± 4	361 ± 4	122 ± 3	356 ± 3	118 ± 4	345 ± 4	113 ± 5

Note: LiCl – lithium chloride; 5-HD – 5-hydroxydecanoic acid; HR – heart rate, BP –blood pressure; * $p < 0.005$ compared to the control group.

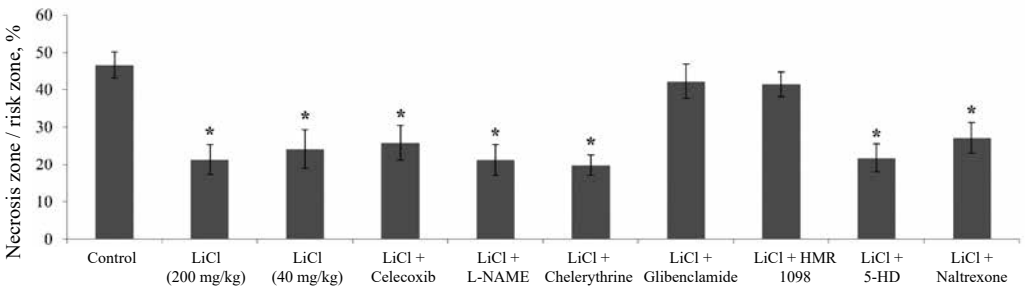

Fig. 1. Participation of COX-2, NO synthase, protein kinase C, K_{ATP}-channels, and opioid receptors in the infarct size-limiting effect of LiCl. Here and in Fig.2, the data are shown as M ± σ. * $p < 0.005$ compared to the control group

Pre-injection of LiCl (40 mg / kg) statistically significantly ($p = 0.00045$) reduced the necrosis zone / risk zone ratio by 48% compared to the controls (Fig. 1). Increasing the dose of lithium chloride to 200 mg / kg did not significantly enhance the cardioprotective effect (Fig. 1). Therefore, in further studies, lithium chloride was used at a concentration of 40 mg / kg.

As shown in Fig. 1, the COX-2 inhibitor celecoxib and the NOS inhibitor L-NAME did not affect the infarct size-limiting effect of LiCl, i.e., there was a statistically significant reduction in the infarct size compared to the controls ($p = 0.00088$ and $p = 0.00021$, respectively). A similar result was observed with the PKC inhibitor chelerythrine ($p = 0.00011$). However, glibenclamide (a non-selective blocker of K_{ATP}-channels) and HMR 1098 (a selective blocker of sarcK_{ATP}-channels) abolished the cardioprotective effect of lithium chloride (Fig. 1), in both cases the infarct zone size was not significantly different from the control values ($p = 0.303$ and $p = 0.206$, respectively). Pre-administration of the selective mitoK_{ATP}-channel inhibitor 5-HD did not affect lithium-induced cardiac tolerance to ischemia – reperfusion injury (a statistically significant reduction in infarct area was observed, $p = 0.00041$). The OR antagonist naltrexone also did not affect the infarct size-limiting effect of lithium chloride ($p = 0.0035$) (Fig. 1). All inhibitors used had no significant effect on the size of the infarct area (Fig. 2)

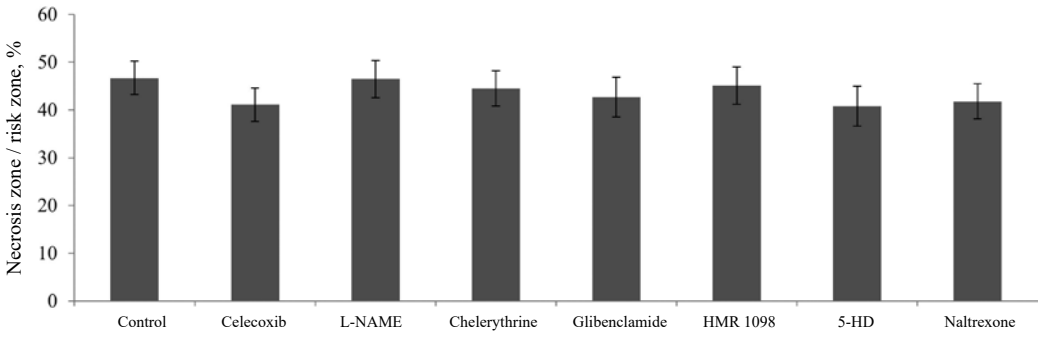

Fig. 2. Estimation of the intrinsic effect of the drugs used (inhibitors) on myocardial infarction size

DISCUSSION

The cardioprotective effect of lithium was previously shown in *ex vivo* studies [12, 13]. Cyclooxygenase may be involved in the realization of the infarct size-limiting effect of lithium [13]. Several studies have shown that COX-2 activation improves cardiac tolerance to ischemia [19, 20, 24]. We found that celecoxib (COX-2 inhibitor) did not eliminate the cardioprotective effect of LiCl. This may be due to the fact that M.Faghihi et al. conducted their studies on the isolated rat heart and used the non-selective COX inhibitor indomethacin, which also inhibits COX-1 [13].

We hypothesized that COX-1 is involved in the cardioprotective effect of LiCl. However, this hypothesis contradicts the current opinion that COX-2 activation increases cardiac tolerance to ischemia [19, 20]. Activation of PKC definitely plays an important role in the cardioprotective effect of ischemic preconditioning [16] and opioids [25]. Therefore, it was surprising that PKC is not involved in the infarct size-limiting effect of LiCl, since chelerythrine exposure did not affect the efficacy of lithium. NOS has been reported to be involved in the development of delayed (24 h) ischemic preconditioning [20]. However, the NOS inhibitor L-NAME did not affect the LiCl-induced increase in cardiac tolerance to ischemia – reperfusion.

The results of our study are in agreement with the data of Y. Terashima et al. who showed that the infarct size-limiting effect of LiCl *ex vivo* is independent of PKC activation [12]. Our data are in agreement with the work of M. Faghihi et al. [13], where it was shown that L-NAME does not attenuate lithium cardioprotection *in vitro.*

K_{ATP}-channels are known to be involved in cardioprotection due to ischemic pre- and postconditioning [16, 17]. It has been reported that LiCl can induce the opening of K_{ATP}-channels [15]. Summarizing the obtained results, we concluded that the infarct size-reducing effect of LiCl is partially realized through K_{ATP}-channels. This version is confirmed by the revealed effects of non-selective blocker of K_{ATP}-channels glibenclamide and selective blocker of sarcK_{ATP}-channels HMR 1098, which completely eliminated the cardioprotective effect of LiCl. Hence, sarK_{ATP}-channels are involved in the infarct size-limiting effect of LiCl. In this case, it remains unclear whether activation of the sarK_{ATP}-channel is the result of a direct action of Li^+ on this channel or whether its opening is mediated by the effect of kinases.

In our study, lithium chloride demonstrated an infarct size-limiting effect. The antinociceptive effect of lithium chloride depends on the release of endogenous opioids [17]. However, the results showed that endogenous opioids were not involved in the cardioprotective effect of LiCl. Lithium carbonate is used orally in psychiatry with achieving blood concentrations of 0.4–1.2 mmol / l in patients [26]. This concentration is comparable to the dose of LiCl used in our study. Consequently, these results indicate the feasibility of conducting a clinical trial on the treatment of AMI with lithium salts.

CONCLUSION

It has been shown that lithium chloride reduces the myocardial infarction zone *in vivo* and increases cardiac resistance to ischemia – reperfusion. The infarct size-limiting effect of LiCl is associated with the opening of sarK_{ATP}-channels. PKC, NOS, COX-2, endogenous opioids, and mitoK_{ATP}-channels are not involved in the cardioprotective effect of LiCl. The obtained data indicate the need for further study of the mechanism of cardioprotective effect of lithium.

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Authors' contribution

Mukhomedzyanov A.V. – performance of in vivo operations. Plotnikov E.V. – conception, carrying out of research. Maslov L.N. – research design, interpretation of the results. Chernov V.I. – analysis and interpretation of the data. Naryzhnaya N.V. – analysis of hemodynamics. Slidnevskaya A.S. – processing of heart slices. Yusubov M.S. – analysis of lithium compounds, processing of the results. Larkina M.S. – statistical analysis and data processing. Artamonov A.A. – graphic design of the results. Belousov M.V. – analysis and interpretation of the research results. All authors contributed to drafting of the article and final approval of the manuscript for publication.

Authors' information

Mukhomedzyanov Alexander V. – Cand. Sci. (Med.), Researcher, Laboratory for Experimental Cardiology, Cardiology Research Institite, Tomsk NRMC, Tomsk, sasha_m91@mail.ru, http://orcid.org/0000-0003-1808-556X

Plotnikov Evgenii V. – Cand. Sci. (Chemistry), Associate Professor, Research School of Chemical and Biomedical Technologies, NR TPU, Tomsk, plotnikov.e@mail.ru, http://orcid.org/0000-0002-4374-6422

Maslov Leonid N. – Dr. Sci. (Med.), Professor, Head of the Laboratory for Experimental Cardiology, Cardiology Research Institite, Tomsk NRMC, Tomsk, Maslov@cardio-tomsk.ru, http://orcid.org/0000-0002-6020-1598

Naryzhnaya Natalya V. – Dr. Sci. (Med.), Principal Researcher, Laboratory for Experimental Cardiology, Cardiology Research Institite, Tomsk NRMC, Tomsk, nataalynaar@yandex.ru, http://orcid.org/0000-0003-2264-1928

Slidnevskaya Alisa S. – Senior Laboratory Assistant, Laboratory for Experimental Cardiology, Cardiology Research Institite, Tomsk NRMC, Tomsk, alisaslidnevskaa@gmail.com, http://orcid.org/0009-0004-2215-5414

Chernov Vladimir I. – Corresponding Member of the Russian Academy of Sciences, Dr. Sci. (Med.), Professor, Deputy Director for Research and Innovation, Tomsk NRMC; Head of the Department of Radionuclide Diagnostics, Cancer Research Institute, Tomsk NRMC, Tomsk, chernov@tnimc.ru, http://orcid.org/0000-0002-5524-9546

Yusubov Mehman S. – Dr. Sci. (Chemistry), Professor, Research School of Chemical and Biomedical Technologies, NR TPU, Tomsk, yusubov@mail.ru, http://orcid.org/0000-0001-9233-1824

Larkina Maria S. – Dr. Sci. (Pharmaceut.), Professor, Division of Pharmaceutical Analysis, Siberian State Medical University, Tomsk, larkina.ms@ssmu.ru, http://orcid.org/0000-0003-1176-2441

Artamonov Anton A. – Senior Researcher, Institute of Medical and Biological Problems, Russian Academy of Sciences, Moscow, anton.art.an@gmail.com, http://orcid.org/0000-0002-7543-9611

Belousov Mikhail V. – Dr. Sci. (Pharmaceut.), Professor, Head of the Pharmaceutical Analysis Division, Siberian State Medical University, Tomsk, belousov.mv@ssmu.ru, http://orcid.org/0000-0002-2153-7945

(✉) **Plotnikov Evgenii V.,** Plotnikov.e@mail.ru

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Interleukin-4 and interferon gamma in bronchial remodeling in asthma patients with cold airway hyperresponsiveness

Pirogov A.B., Prikhodko A.G., Pirogova N.A., Gassan D.A., Naumov D.E., Perelman J.M.

Far Eastern Scientific Center of Physiology and Pathology of Respiration
22, Kalinina Str., Blagoveshchensk, 675000, Russian Federation

ABSTRACT

Interleukin-4 (IL-4) and interferon gamma (IFNγ) are key participants in the polarization of the immune response toward Th1 or Th2 types in bronchial asthma. However, their role in bronchial remodeling in patients with asthma and cold airway hyperresponsiveness (CAHR) remains unclear.

Aim. To study the involvement of IL-4 and IFNγ in the disorganization of bronchial epithelium and the regulation of airway remodeling in asthma with CAHR.

Materials and methods. A total of 47 patients with mild persistent asthma were examined. Induced sputum collection, blood sampling for biochemical studies, spirometry, and the isocapnic hyperventilation test with cold (-20 °C) air (IHCA) were performed. The sputum was analyzed for cellular composition (in %), and the cytokine profile (IL-4 and IFNγ in pg / ml) was evaluated in peripheral blood.

Results. The patients were divided into groups with CAHR (group 1, 17 patients) and without cold-induced bronchoconstriction (group 2, 30 patients). Forced expiratory volume in 1 sec. (FEV_1) and maximal mid-expiratory flow (MMEF) in group 1 were lower compared to group 2: 84.0[83.0; 93.0]% and 99.0 [85.0; 105.0]% ($p = 0.012$); 55.0[51.0;67.0]% and 76.0[59.0;88.0]% ($p = 0.021$), respectively. The blood content of IL-4 and IFNγ in group 1 was 11.48[10.82;22.48] pg / ml and 26.98[17.24; 73.5] pg / ml, while in group 2, it was 1.88 [0.66; 5.96] ($p = 0.003$) and 7.24[1.5; 26.98] pg / ml ($p = 0.047$), respectively. In group 1, an association was found between blood IL-4 and IFNγ levels ($Rs = 0.65$; $p = 0.016$), between FEV_1 and the number of epithelial cells in sputum ($Rs = –0.74$; $p = 0.0003$), and between IL-4 and airway response (ΔFEV_1/Vital Capacity) after the IHCA ($Rs = –0.70$; $p = 0.007$).

Conclusion. The escalation of the proinflammatory and pro-oxidant function of IFNγ indicates a shift from Th2 immune response activation, regulated by IL-4, toward a Th1 response, which stimulates bronchial remodeling in patients with asthma and CAHR.

Keywords: bronchial asthma, cold airway hyperresponsiveness, IL-4, IFNγ, bronchial epithelium, airway remodeling

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 local Bioethics Committee at the Far Eastern Scientific Center of Physiology and Pathology of Respiration (Protocol No. 148 of 24.05.2023).

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✉ *Perelman Juliy M.,* jperelman@mail.ru

Интерлейкин-4 и интерферон-гамма в ремоделировании бронхов у больных бронхиальной астмой с холодовой гиперреактивностью дыхательных путей

Пирогов А.Б., Приходько А.Г., Пирогова Н.А., Гассан Д.А., Наумов Д.Е., Перельман Ю.М.

Дальневосточный научный центр физиологии и патологии дыхания (ДНЦ ФПД)
Россия, 675000, г. Благовещенск, ул. Калинина, 22

РЕЗЮМЕ

Интерлейкин-4 (IL-4) и интерферон-гамма (IFNγ) – одни из основных участников поляризации иммунного ответа по Th1 или Th2 типу при бронхиальной астме (БА). Неизвестна их роль в ремоделировании бронхов у больных БА с холодовой гиперреактивностью дыхательных путей (ХГДП).

Цель. Изучение путей участия IL-4 и IFNγ в дезорганизации бронхиального эпителия и регуляции ремоделирования дыхательных путей при БА с ХГДП.

Материалы и методы. Обследованы 47 пациентов с легкой персистирующей БА. Проводился сбор индуцированной мокроты, забор крови для биохимических исследований, выполнялись спирометрия и бронхопровокационная проба изокапнической гипервентиляции холодным (–20 °С) воздухом (ИГХВ). В мокроте исследовали клеточный состав (в %), в периферической крови – цитокиновый профиль (IL-4, IFNγ, в пг/мл).

Результаты. Пациенты разделены на группы с холодовой гиперреактивностью дыхательных путей (1-я группа, 17 человек) и с отсутствием холодовой бронхоконстрикции (2-я группа, 30 человек). Объем форсированного выдоха за 1 с ($ОФВ_1$) и средняя объемная скорость выдоха ($СОС_{25–75}$) на уровне 25–75% жизненной емкости легких (ЖЕЛ) в 1-й группе были ниже по сравнению со 2-й группой: 84,0 [83,0;93,0] и 99,0 [85,0;105,0]% (p = 0,012); 55,0 [51,0;67,0] и 76,0 [59,0;88,0]% (p = 0,021) соответственно. Содержание в крови IL-4 и IFNγ в 1-й группе составляло 11,48 [10,82;22,48] и 26,98 [17,24;73,5] пг/мл, во 2-й группе 1,88 [0,66;5,96] (p = 0,003) и 7,24 [1,5;26,98] пг/мл (p = 0,047) соответственно. В 1-й группе найдена связь между содержанием в крови IL-4 и IFNγ (Rs = 0,65; p = 0,016), между $ОФВ_1$ и количеством эпителиоцитов в мокроте (Rs = –0,74; p = 0,0003), а также между IL-4 и реакцией дыхательных путей ($\Delta ОФВ_1$/ЖЕЛ) в ответ на пробу ИГХВ (Rs = –0,70; p = 0,007).

Заключение. Эскалация провоспалительной и прооксидантной функции IFNγ свидетельствует о смещении баланса активации Th2 иммунного ответа, регулируемого сигналами IL-4, в сторону Th1 иммунного ответа, стимулирующего ремоделирование бронхов у больных БА с ХГДП.

Ключевые слова: бронхиальная астма, холодовая гиперреактивность дыхательных путей, IL-4, IFNγ, бронхиальный эпителий, ремоделирование дыхательных путей

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

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

Соответствие принципам этики. Все пациенты подписали информированное согласие на участие в исследовании. Исследование одобрено локальным комитетом по биомедицинской этике ДНЦ ФПД (протокол № 148 от 24.05.2023).

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INTRODUCTION

Airway remodeling in patients with bronchial asthma (BA) appears as a change in the structural and functional organization of the parenchymal and stromal elements of the bronchi, induced by damage and impaired restoration of the epithelial barrier. Under the influence of various triggers, such as allergens, viruses, alarmins, and low temperatures, signaling pathways of inflammation are initiated in the disrupted epithelium, involving immunocompetent cells. Epithelial – mesenchymal units of the bronchi

are activated and proinflammatory cytokines are secreted, leading to persistent chronic inflammation, airway hyperresponsiveness, and obstruction [1–4].

The sources of inflammatory mediators in the bronchi of patients with BA are granulocytes, lymphocytes, macrophages, mast cells, interstitial cells, and smooth muscle cells. However, primary producers of cytokines and growth factors are damaged parenchymal cells. Activated epithelium generates alarmins, such as TSLP, IL-25, and IL-33, which stimulate the polarization of naive T-helper cells into Th2, the expression of IL-4, IL-5, and IL-13, and eosinophilic inflammation. IL-5 and GM-CSF together with eotaxins, CCL5/RANTES and MCP, regulate the production, maturation, recruitment, and activation of eosinophils. IL-9 and IL-13 induce the metaplasia of ciliated epithelial cells into secretory mucocytes. CCL17/TARC and CXCL8/IL-8 recruit Th17 cells and neutrophils, respectively. Proinflammatory cytokines and chemokines, such as IL-1β, IL-2, IL-6, IL-12, IL-18, IL-36, TNFα, CXCL5, CCL20, CCL22, CCL5/RANTES, CXCL10, interferons I (IFNα/β), III (IFNλ1, 2, and 3), and IFNγ, are secreted [1, 3, 5, 6].

The expression of CXCL2, CXCL8, IL-12, CCL20, IFNγ, IL-6, IL-18, IL-36, and TNFα is associated with activation of epithelium by viruses and other infectious agents, leading to the mobilization of neutrophils, neutrophil-macrophage infiltration of the bronchi, and neutrophil response to IL-12 and IFNγ signals in the form of proinflammatory cytokine release [6]. This Th1 variant of the immune response is characteristic of cold airway hyperresponsiveness (CAHR), which is associated with a mixed pattern of bronchial inflammation, neutrophil destruction, and cytolysis, accompanied by the escalation of proinflammatory cytokine synthesis and structural signs of epithelial dysfunction [7]. Clinically, this manifests as uncontrolled asthma with increased symptoms during the cold season, requiring higher doses of medication and/or the inclusion of systemic glucocorticoids in therapy [7].

Given that the central cytokine responsible for the differentiation, growth, and effector functions of Th1 cells, polarizing the immune response toward the Th1 type, is IFNγ [8–10] and IL-4 is one of the main activators of the Th2 immune response and allergic inflammation in the bronchi [5, 11], a study was planned to investigate the involvement of IL-4 and IFNγ in the disorganization of bronchial epithelium and the regulation of airway remodeling in patients with BA and CAHR.

MATERIALS AND METHODS

The study included 47 patients who sought outpatient care at the clinic of the Far Eastern Scientific Center of Physiology and Pathology of Respiration (FSCPPR) with a diagnosis of mild persistent BA [12] and who had not previously received inhaled glucocorticoid therapy on a regular basis.

This clinical study was conducted with the approval of the local Bioethics Committee of FSCPPR (Protocol No. 148 of 24.05.2023). All patients were familiarized with the clinical study protocol, the procedure for functional testing was explained, and they signed an informed consent to participate in the study.

The study design included a period to assess the patient's clinical condition and asthma severity, and a visit for induced sputum collection (day 1), blood sampling for biochemical studies and the isocapnic hyperventilation test with cold air (day 2). Patients were then divided into groups based on the presence or absence of CAHR (group 1 and group 2, respectively).

Inclusion criteria for the study were: forced expiratory volume in one second (FEV_1) > 75% of the predicted value according to spirometry; absence of a documented cold allergic reaction as confirmed by an allergist (Douglas method).

Patients with obstructive ventilatory disorders (FEV_1 < 75% of the predicted value), concomitant respiratory diseases (acute bacterial or viral infections at the time of testing, COPD, etc.), clinically significant comorbidities in other organs and systems, pregnant women, as well as those taking medications that could affect the interpretation of study results were excluded from the study.

Instrumental testing was performed by qualified medical staff in the Laboratory of Functional Research of the Respiratory System at FSCPPR.

Induced sputum collection was performed using a standard method under the control of FEV_1, which was evaluated by spirometry at the beginning of the collection and after each inhalation of 3, 4, and 5% sodium chloride (NaCl) solution. Before each sputum collection procedure, the patient rinsed their mouth with distilled water. The sputum samples were analyzed no later than two hours after the collection. Sputum smears were dried (5–10 minutes at 37 °C)

in a TM-2 thermostat, fixed in formaldehyde vapors (40% solution, 10 minutes), and stained with aqueous Romanowsky – Giemsa stain (4–5%, pH 6.8). A light optical immersion microscope was used to analyze the cellular composition by counting at least 400 cells in the fields of view (central and peripheral regions); the number of cellular elements was expressed as a percentage of the total content. In order to differentiate goblet epithelial cells, a cytochemical reaction was performed by staining formalin-fixed preparations with Alcian blue, which selectively binds mucins (acidic glycosaminoglycans) present in the cytoplasm of goblet cells [13].

Blood samples were collected from the median cubital vein in the morning hours (9:00 AM) into a vacutainer (5 ml) and stored frozen at –80 °C until the biological sample analysis was performed. The cytokine profile (IL-4, IFNγ, in pg / ml) was studied using a flow cytometer (BD FACSCanto II, BD, USA) with LEGENDplex HU Essential Immune Response Panel kits (BioLegend, USA), following the manufacturer's protocols precisely.

All tests involving spirometry were performed using the Easy on-PC device (NDD Medizintechnik AG, Switzerland). The following lung function parameters were measured: vital capacity (VC), forced expiratory volume in one second (FEV_1, in liters), mean mid-expiratory flow at 25–75% of forced VC (MMEF, % of predicted), and maximal expiratory flow rates at 50% (MEF_{50}, % of predicted) and 75% (MEF_{75}, % of predicted) of forced VC. Predicted values according to ECSC standards were used for individuals of European descent older than 18 years.

Isocapnic hyperventilation cold air test (IHCA) was conducted in a mode of submaximal hyperventilation (60% of the predicted maximum ventilation) with an air mixture containing 5% CO_2 for three minutes with individual selection of breathing depth and frequency during the load. Before and after IHCA (at 1 and 5 minutes), FEV_1 was recorded (in liters). The maximum changes in this parameter after IHCA relative to baseline were analyzed. The difference between the obtained values was expressed as a percentage of the baseline (ΔFEV_1, %). A decrease in FEV_1 by 10% or more indicated the presence of CAHR in the patient [14].

Statistical analysis of the obtained results included testing for normality of distribution using the Kolmogorov – Smirnov and Pearson – von Mises criteria. Variables with normal (Gaussian) distribution were compared using the Student's *t*-test (when homogeneity of group variances was confirmed by the Fisher's test). Variables with non-Gaussian distribution were compared by the Mann – Whitney test. Quantitative variables were presented as $M \pm m$ (M – arithmetic mean, m – standard error of the mean) or as $Me\ [Q_1; Q_3]$ (median and interquartile range). The nonparametric Spearman's rank correlation coefficient (*Rs*) was used to determine the degree of correlation between two variables. The differences were statistically significant at $p < 0.05$.

RESULTS

Of the 47 patients included in the study, 17 were included in group 1 with cold airway hyperresponsiveness, while 30 were in group 2 without a cold trigger response. The patients in both groups were comparable in terms of gender and key physiological parameters: age 37.1 ± 3.5 and 43.2 ± 2.9 years ($p = 0.188$), respectively; height 174.3 ± 2.6 and 170.1 ± 1.5 cm ($p = 0.151$); body mass index 26.0 ± 1.5 and 27.6 ± 1.2 kg / m² ($p = 0.419$), respectively. Smokers comprised 35% of group 1 and 23% of group 2 ($\chi^2 = 0.29$, $p > 0.05$).

The groups differed significantly in several flow parameters recorded during the initial evaluation (Table 1). Median FEV_1 and MMEF values in patients with CAHR were significantly lower, indicating bronchial obstruction. These patients also had lower MEF_{50} (60 [56;87]%) and MEF_{75} (46 [42;54]%) compared to group 2 patients (76 [66;94]%, $p = 0.021$, and 61 [49;83]%, $p = 0.012$, respectively), suggesting the persistence of chronic inflammation in the small airways.

Table 1

Initial lung ventilation parameters and bronchial response to IHCA, $Me\ [Q_1; Q_3]$

Group	FEV_1 % predicted	FEV_1/ VC %	MMEF % predicted	ΔFEV_1 %
Group 1	84.0 [83.0; 93.0]	73.0 [70.0; 76.8]	55.0 [51.0; 67.0]	–16.0 [–19.0; –12.0]
Group 2	99.0 [85.0; 105.0]	78.1 [72.8; 82.4]	76.0 [59.0; 88.0]	–2.2 [–3.5; 0.2]
p between group 1 and group 2	0.012	0.165	0.021	0.0001

When assessing the cytokine content in the blood serum of patients in group 1, higher median values of IL-4 and IFNγ were registered compared to group 2

(Table 2). In the group of patients with CAHR, there was a positive correlation between IL-4 and IFNγ in the blood (Rs = 0.65; p = 0.016). Additionally, IL-4 was inversely correlated with the airway response to the bronchoprovocation test ($\Delta FEV_1/VC$) (Rs = –0.70; p = 0.007).

In the sputum of group 1 patients, a greater number of neutrophils and desquamated epithelial cells was observed (Table 3), and the levels of neutrophils, eosinophils and macrophages directly influenced the severity of the bronchoconstrictor response (ΔFEV_1) during the IHCA test (Rs = –0.50; p = 0.029; Rs = –0.51; p = 0.027; Rs = 0.56; p = 0.013, respectively). It is important to note that in this group there was an inverse correlation between the baseline FEV_1 value, reflecting bronchial patency, and the number of epithelial cells found in sputum (Rs = –0.74; p = 0.00003). Figures 1 and 2 illustrate the various degrees of destructive changes in epithelial cells.

Table 2

IL-4 and IFNγ content in the blood serum of asthma patients, *Me* [Q_1; Q_3], pg / ml

Group	IL-4	IFNγ	IL-4/IFNγ
Group 1	11.48 [10.82; 22.48]	26.98 [17.24;73.51]	0.43[0.31;0.70]
Group 2	1.88 [0.66; 5.96]	7.24 [1.54;26.98]	0.16[0.22;0.40]
p between group 1 and group 2	0.003	0.047	0.049

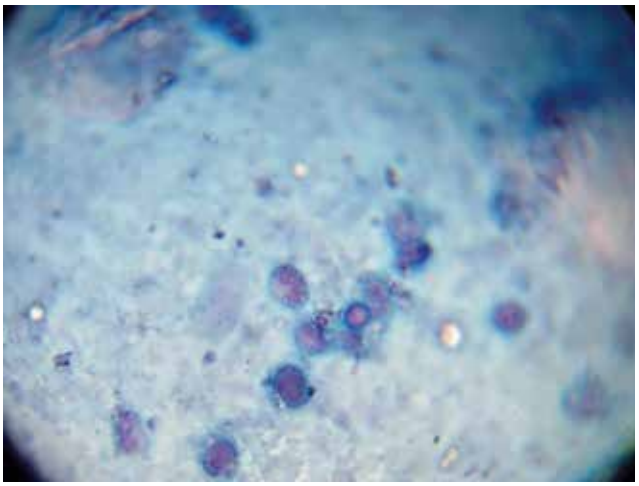

Fig. 1. In the center of the preparation, goblet cells show varying degrees of cytoplasmic and nuclear destruction. Toward the peripheral areas, there are fully destroyed epithelial cells with disintegration of the nucleus and cytoplasm, containing mucins. Here and in Fig.2, induced sputum smear from a patient with BA and CAHR. Stained with Alcian blue. Magnification x 1,250.

Table 3

Cellular composition of induced sputum in asthma patients, *Me* [Q_1; Q_3], %

Group	Neutrophils	Macrophages	Eosinophils	Epithelial cells
Group 1	22.5 [19.5; 26.3]	54.7 [45.6; 66.8]	19.8 [12.6; 21.2]	1.6 [1.2; 2.7]
Group 2	16.9 [15.4; 20.0]	60.4 [56.9; 67.6]	17.0 [3.0; 21.3]	0.2 [0.1; 1.0]
p between group 1 and group 2	0.049	0.119	0.112	0.0013

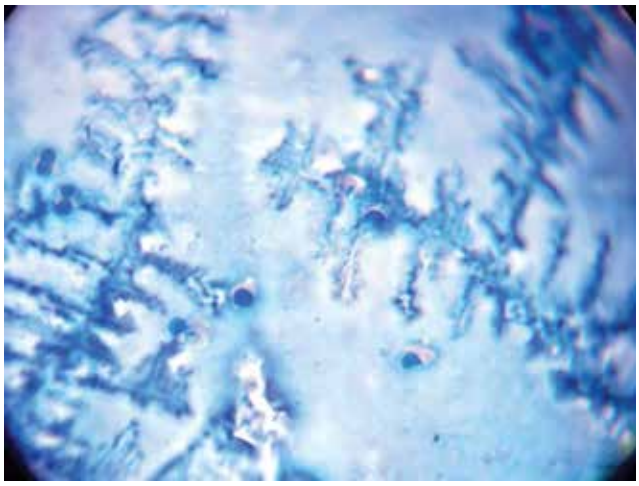

Fig. 2. Goblet cells containing large amounts of mucoproteins are located within an abundance of mucus exhibiting signs of biocrystallization

DISCUSSION

Airway remodeling in BA, affecting all parts of the walls of small bronchi, involves changes in the connective tissue due to fragmentation and homogenization of its fibrous framework by metalloproteinases, hyperproduction and accumulation of proteoglycans, increased fibroblastic synthesis, decreased extracellular matrix protein degradation, fibrillogenesis, and the development of subepithelial fibrosis and diffuse sclerosis. This process affects smooth muscle cells, transforming them from a contractile to a secretory and proliferative phenotype due to hypertrophy and hyperplasia occurring against the background of myofibroblast differentiation and enhanced angiogenesis mediated by vascular endothelial growth factor (VEGF) release. Additionally, epithelial lining disruptions occur in the form of desquamation and destruction of cells, exposing the hyalinized basement membrane, destroying ciliated epithelium, goblet cell hyperplasia, and metaplasia [2, 4].

IL-4 and IFNγ, whose main functions include mutual inhibition, belong to a wide range of cytokines involved in the disruption of the structural integrity of the epithelial barrier and causing the development of epithelial dysfunction of the bronchi [3]. In this study, we observed an increase in IL-4 and IFNγ levels in patients with CAHR compared to those who did not respond to cold air provocation (Table 2). IFNγ activity is linked to the weakening of the atopic phenotype of BA [6, 9] due to significant antagonistic role of the IFNγ/STAT1 signaling pathway (T-bet pathway) against GATA-3 expression, which suppresses Th1 development and activates Th2 proliferation [8–10]. IL-4 induces GATA-3 expression via the STAT6-dependent pathway, which suppresses Th1-specific transcription factors and stimulates Th2 cytokine synthesis, resulting in Th2-associated eosinophilic inflammation, epithelial destruction, secretory hyperplasia, and ciliary dysfunction, accompanying hyperresponsiveness and airway remodeling in BA [2,11,15].

A well-studied effect of Th2 cytokines, induced by IL-4, on the bronchial epithelium in BA is mucus hypersecretion [3, 15]. Increased expression and secretion of mucins MUC5AC, produced by goblet cells, and MUC5B, synthesized by glandular epithelium, intensifies as the disease progresses. This is accompanied by impaired tissue fluid circulation in the bronchi, dehydration of the mucin gel, increased viscosity due to elevated chondroitin sulfate levels, and decreased hyaluronic acid and heparin content in mucins, resulting in firmer adhesion of the gel to the epithelial surface. It has been shown that cold air induces MUC5AC hypersecretion by bronchial epithelium through TRPM8 ion channels [16].

In our previous studies, we demonstrated that BA patients with CAHR have an elevated baseline concentration of glycoproteins and glycosaminoglycans (GAGs) in the bronchial lining. After the IHCA test, simultaneously with the increase in the number of goblet cells and the generation of mucopolysaccharides, disorganization and desquamation of the epithelium, destruction and cytolysis of mucocytes intensify. [17]. During prolonged exposure to cold air *in vitro*, pronounced destructive changes were observed in ciliated epithelial cells, with positive staining for mucins and an abundance of mucus secretions containing high amounts of GAGs and microorganisms on the surface of the epithelial layer [17, 18].

It is suggested that mucous ciliated cells represent a molecular phenotype unique to the respiratory tracts of BA patients, wherein ciliated epithelial cells can express MUC5AC and other goblet cell-specific genes. These metaplastic cells, whose formation is induced by IL-4/IL-13 signaling, express IL-4/IL-13-induced genes and are considered transitional from the ciliated epithelium phenotype to the secretory cell phenotype [2]. In BA, IL-4/IL-13 signaling is linked to the stimulation of the Notch signaling pathway and high levels of Notch signaling, which lead to the activation of differentiation and an increase in the number of goblet cells that produce mucus [15].

Among our patients with CAHR, varying degrees of destructive changes were observed in epithelial cells synthesizing and secreting glycoproteins: from mild, with partial (no more than 1/2) cytoplasmic destruction and preservation of normal nuclear structure, to complete destruction with disintegration of the cytoplasm and nucleus (Fig. 1). In cases where fully destroyed cells containing mucins were found in the induced sputum smears (Fig. 1), it was difficult or impossible to differentiate them as goblet cells or ciliated epithelial cells that had undergone secretory metaplasia. The presence of goblet cell clusters containing large amounts of mucoproteins in abundant, viscous mucus (Fig. 2) in patients from group 1 indicated the development of pronounced mucociliary dysfunction, which exacerbated airway remodeling and obstruction and was associated with increased IL-4 levels in the cytokine profiles of these patients.

Epithelial desquamation in BA patients with CAHR was more intense compared to patients without cold-induced bronchoconstriction: a greater number of desquamated epithelial cells were found in the induced sputum of group 1 patients (Table 3), indicating increased damage to intercellular junctions and heightened epithelial barrier permeability in the bronchi in CAHR. Intercellular junctions in the bronchial epithelium include tight junctions (TJs), located at the apical surface, which contain proteins, such as claudins, occludins, and junctional adhesion molecules (JAMs), forming a multi-protein complex known as the zonula occludens (ZO); adherens junctions (AJs), which contain cadherins and catenins; desmosomes, connecting intermediate filaments of adjacent cells; and hemidesmosomes, anchoring basal cells and other epithelial cells to the basement membrane [6, 15].

The loss of several proteins from intercellular TJs and AJs is considered a key feature of airway hyperreactivity and remodeling in BA [2, 6]. Deficiency of E-cadherin, as the main membrane protein of AJs, is associated with desquamation of ciliated cells, exposure of the basement membrane, induction of proliferation of club cells, and suppression of their differentiation, leading to impaired epithelial repair and the development of proinflammatory and non-regenerative reactions in the airways [2].

It has been shown that IL-4 and IL-13 play a central role in inhibiting the surface expression of ZO-1, occludin, α-catenin, β-catenin, and E-cadherin in bronchial epithelial cells, with decreased levels of E-cadherin in sputum correlating with BA severity [6]. Clinical findings regarding role of IL-4 in disruption of the epithelial barrier in the airways align with *in vitro* studies, which show that the cytokine inhibits the expression of membrane components of AJs: when acting on the apical and basolateral monolayers of cultured epithelial cells, IL-4 increases paracellular permeability and decreases transepithelial resistance [19].

Higher IL-4 concentrations detected in group 1 patients compared to group 2 suggest that IL-4 is a triggering factor for barrier dysfunction and bronchial remodeling in patients with CAHR, associated with Th2-type allergic inflammation. In addition, the bronchial response to cold stimuli is linked to Th1 immune response, and the role of IFNγ in the development and exacerbation of bronchial remodeling and its connection to the neutrophil count, which was higher in the induced sputum of CAHR patients, should not be overlooked (Table 3).

IFNγ marks the Th1 immune response in non-allergic BA phenotypes, which is associated with chronic inflammation persistence, increased neutrophil survival, and activation of the neutrophil inflammatory component, while reducing atopic activity, a factor that contributes to the development of glucocorticoid resistance [20]. Neutrophil pool mobilization in CAHR patients was associated with induction of proinflammatory cytokines and chemokines that recruit neutrophils to the bronchial infiltrate. Neutrophil infiltration stimulated the persistence of chronic inflammation, culminating in diffuse interstitial sclerosis, leading to structural modification of the bronchi and progression of airway obstruction and remodeling.

IFNγ involvement in bronchial epithelial destruction was also linked to the impact of proinflammatory cytokines expressed under its influence, along with oxidative damage caused by reactive oxygen species (ROS) and other toxic metabolites. A critical factor in free-radical epithelial damage is the activation of the respiratory burst in macrophages, stimulated by IFNγ through the induction of cytosolic components of NADPH oxidase [8, 10, 21, 22], associated with IFNγ-regulated phagocyte differentiation. When IFNγ interacts with its receptor on macrophages, the T-bet signaling pathway is activated, which induces STAT1 target genes [22, 23] and polarizes lung interstitial macrophages, which interact with neutrophils in the Th1/Th17 cytokine cascade, into the classic M1 inflammatory phenotype [23, 24].

A possible reason for the lower median macrophage values in the sputum of CAHR patients (Table 3) may have been cytolysis resulting from the intensification of the respiratory burst induced by IFNγ. The escalation of proinflammatory and pro-oxidant functions of IFNγ in these patients indicates a shift in the balance of Th2 cytokine activation, regulated by IL-4, towards the Th1 immune response, which, alongside Th2 immune responses, contributes to airway remodeling in BA patients with CAHR.

CONCLUSION

Patients with BA and CAHR exhibit higher levels of IL-4, associated with increased desquamation, destruction, and marked secretory activity of bronchial epithelial cells, and IFNγ, linked to neutrophil pool mobilization and an increase in neutrophil counts in the inflammatory pattern of the bronchi. Desquamation, destruction, goblet cell hyperplasia and metaplasia, and mucus hypersecretion in the bronchial epithelium, stimulated by IL-4 activation and exacerbating mucociliary and barrier dysfunction, contribute to more pronounced airway obstruction in BA patients with CAHR.

The escalation of the proinflammatory and pro-oxidant functions of IFNγ in BA patients with CAHR indicates a shift from IL-4-regulated Th2 cytokine activation, traditionally responsible for structural reorganization of the bronchial walls in BA, toward a Th1 immune response, which stimulates bronchial remodeling in CAHR.

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Authors' contribution

Pirogov A.B. – conception, analysis of the data, drafting of the manuscript. Prikhodko A.G. – analysis of the manuscript, critical revision of the manuscript for important intellectual content. Pirogova N.A. – analysis of literature data, drafting of the manuscript. Naumov D.E. – analysis of the data, carrying out of biochemical studies. Gassan D.A. – carrying out of biochemical studies. Perelman J.M. – critical revision of the manuscript for important intellectual content, final approval of the manuscript for publication, responsibility for the integrity of all parts of the article.

Authors' information

Pirogov Aleksey B. – Cand. Sci. (Med.), Associate Professor, Senior Researcher, Laboratory of Functional Research of the Respiratory System, Far Eastern Scientific Center of Physiology and Pathology of Respiration, Blagoveshchensk, dncfpd@dncfpd.ru, https://orcid.org/0000-0001-5846-3276

Prikhodko Anna G. – Dr. Sci. (Med.), Chief Researcher, Laboratory of Functional Research of the Respiratory System, Far Eastern Scientific Center of Physiology and Pathology of Respiration, Blagoveshchensk, prih-anya@ya.ru, https://orcid.org/0000-0003-2847-7380

Pirogova Natalia A. – Cand. Sci. (Med.), Researcher, Laboratory of Functional Research of the Respiratory System, Far Eastern Scientific Center of Physiology and Pathology of Respiration, Blagoveshchensk, dncfpd@dncfpd.ru, https://orcid.org/0000-0001-6350-7392

Gassan Dina A. – Cand. Sci. (Med.), Head of the Laboratory for Mechanisms of Virus-Associated Pathologies, Far Eastern Scientific Center of Physiology and Pathology of Respiration, Blagoveshchensk, dani-shi@mail.ru, https://orcid.org/0000-0003-3718-9962

Naumov Denis E. – Cand. Sci. (Med.), Head of the Laboratory for Molecular and Translational Research, Far Eastern Scientific Center of Physiology and Pathology of Respiration, Blagoveshchensk, denn1985@bk.ru, https://orcid.org/0000-0003-3921-8755

Perelman Juliy M. – Corresponding Member of the Russian Academy of Sciences, Dr. Sci. (Med.), Professor, Head of the Laboratory of Functional Research of the Respiratory System, Far Eastern Scientific Center of Physiology and Pathology of Respiration, Blagoveshchensk, jperelman@mail.ru, https://orcid.org/0000-0002-9411-7474

(✉) **Perelman Juliy M.,** jperelman@mail.ru

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The influence of the criterion of abnormal DLco value on the prediction of impaired lung diffusion capacity after SARS-CoV-2 infection

Savushkina O.I.[1,2], Muraveva E.S.[3], Davydov D.V.[1], Kryukov E.V.[4]

[1]*Main Military Clinical Hospital named after academician N. N. Burdenko of the Russian Defense Ministry 3, Gospitalnaya Sq., Moscow, 105229, Russian Federation*

[2]*Pulmonology Scientific Research Institute under Federal Medical and Biological Agency of Russian Federation 28, Orekhovy Blvd., Moscow, 115682, Russian Federation*

[3]*Pirogov Russian National Research Medical University 1, Ostrovityanova Str., Moscow, 117997, Russian Federation*

[4] *S.M. Kirov Military Medical Academy 6, Akademika Lebedeva Str., Saint Petersburg, 194044, Russian Federation*

ABSTRACT

Aim. To predict impaired lung diffusion capacity after SARS-CoV-2 infection depending on the criteria of pathological deviation of DLco value (carbon monoxide transfer factor).

Materials and methods The retrospective study included 341 patients (median age was 48 years, 76.8% of the participants were men) after SARS-CoV-2-associated lung injury. The median volume of lung injury during the acute phase of COVID-19 was 50%. All patients underwent a diffusion test. Descriptive statistics, logistic regression analysis were applied, taking into account the previously obtained model for prognosis of abnormal DLco (<80% of the predicted value (%pred.)) [11]. In the present study on the same sample of patients, the prognosis of abnormal DLco was studied depending on the *criterion 1*: DLco < 80%pred. or *criterion 2*: DLco < predicted – 1.645SD (SD – standard deviation). ROC analysis was used to assess the quality of the binary classifier models.

Results. The coefficients of the logistic regression equations were obtained on the training sample with regard to the chosen criterion of pathological deviation of DLco. The ROC analysis procedure showed that, when applying *criterion 1*, area under curve (AUC) was 0.776, $p < 0.001$ (0.707–0.824 95% confidence interval (CI)), sensitivity and specificity of the training model were 81 and 66%, respectively. When applying *criterion 2*, AUC was 0.759, $p < 0.001$ (0.701–0.817 95% CI), sensitivity and specificity of the training model were 83.4 and 59%, respectively.

Conclusion. The criterion for determining the lower limit of normal DLco (LLN_{DLco}) does not significantly affect the quality of the model for impaired lung diffusion capacity prognosis after SARS-CoV-2-associated lung injury. It is advisable to give preference to a method that is easier to apply in practice.

Keywords: criteria for abnormal DLco, binary classifier model, SARS-CoV-2 infection

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✉ *Savushkina Olga I.,* olga-savushkina@yandex.ru

Влияние критерия патологического отклонения показателя DLco на прогнозирование нарушения диффузионной способности легких после перенесенной инфекции SARS-CoV-2

Савушкина О.И.[1, 2], Муравьева Е.С.[3], Давыдов Д.В.[1], Крюков Е.В.[4]

[1] Главный военный клинический госпиталь (ГВКГ) им. акад. Н.Н. Бурденко
Россия, 105229, г. Москва, Госпитальная пл., 3

[2] Научно-исследовательский институт (НИИ) пульмонологии
Россия,115682, г. Москва, Ореховый бульвар, 28

[3] Российский национальный исследовательский медицинский университет (РНИМУ) им. Н.И. Пирогова
Россия, 117997, г. Москва, ул. Островитянова, 1

[4] Военно-медицинская академия (ВМедА) им. С.М. Кирова
Россия, 194044, г. Санкт-Петербург, ул. Академика Лебедева, 6

РЕЗЮМЕ

Цель. Прогнозирование нарушения диффузионной способности легких после перенесенной инфекции SARS-CoV-2 в зависимости от выбранного критерия патологического отклонения показателя DLco (трансфер-фактора монооксида углерода).

Материалы и методы. В ретроспективное исследование включен 341 пациент (медиана возраста 48 лет, 76,8% мужчин) после перенесенного SARS-CoV-2-ассоциированного поражения легких. Медиана объема поражения легочной ткани в острый период заболевания составила 50%. Всем пациентам был выполнен диффузионный тест. Анализ DLco проведен с помощью описательной статистики и логистического регрессионного анализа с учетом полученной ранее модели прогнозирования снижения DLco [11], в которой за нижнюю границу нормы DLco было принято фиксированное значение 80% от должного значения (%долж.). В настоящем исследовании на той же выборке пациентов проведен сравнительный анализ качества моделей прогнозирования снижения DLco в зависимости от критериев его патологического отклонения (критерий 1: DLco < 80%долж.; критерий 2: DLco < должное – 1,645SD, SD – стандартное квадратичное отклонение от среднего). Для оценки качества моделей бинарного классификатора использовался ROC-анализ.

Результаты. На обучающей выборке получены коэффициенты уравнений логистической регрессии с учетом выбранных критериев патологического отклонения DLco. Процедура ROC-анализа показала, что при применении критерия 1 значение AUC (площадь под кривой) составило 0,776; $p < 0,001$ (95%-й доверительный интервал (ДИ) 0,707–0,824), чувствительность и специфичность обучающей модели – 81 и 66% соответственно, при применении критерия 2 значение AUC составило 0,759; $p < 0,001$ (95%-й ДИ 0,701–0,817), чувствительность и специфичность обучающей модели – 83,4 и 59% соответственно.

Заключение. Выбор критерия определения нижней границы нормы показателя DLco не оказывает существенного влияния на качество модели прогнозирования нарушения диффузионной способности легких после перенесенного SARS-CoV-2-ассоциированного поражения легких. Целесообразно отдавать предпочтение методу, который проще применять на практике.

Ключевые слова: критерии патологического отклонения DLco, модель бинарного классификатора, инфекция SARS-CoV-2

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

Источник финансирования. Исследование выполнено без участия спонсоров.

Соответствие принципам этики. Все пациенты подписали добровольное информированное согласие на участие в исследовании. Исследование одобрено независимым этическим комитетом ФГБУ «ГВКГ им. Н.Н. Бурденко» Минобороны РФ (протокол № 254 от 20.04.2022).

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INTRODUCTION

Pulmonary function tests (PFTs) reflect the physiological properties of the lungs and are used to diagnose lung diseases, determine the cause of shortness of breath, monitor disease progression and response to treatment. The key aspect of interpreting PFT results that are accurate from a technical perspective is the classification of observed values — understanding whether they are within the normal range in relation to the healthy population. It was previously found that values of lung function parameters depend on the patient's age, height, and gender [1]. Currently, it is also considered important to take into account the patient's race [2, 3]. Taking all these factors into consideration, reference equations were created to calculate the predicted values of lung function parameters in a particular patient. Thus, the evaluation criterion for lung function parameters is to compare the actual obtained value with the predicted value.

Additionally, in a population of healthy subjects, there is a range of normal values, the lower limit of which is defined either as a fixed value equal to 80% of predicted (80%pred.) [2, 4] or as the difference between the predicted value and 1.654 SD (SD — standard deviation) [3, 5]. Initially, it was decided to determine the range of normal values of the lung function parameters within the 95% confidence interval (CI). However, A.O. Navakatikyan [6] took into account the unidirectionality of pathological changes in lung function parameters and recommended using a one-sided criterion for assessing the limits of the norm. Thus, values that deviate from the limits of the norm by more than 1.645 SD were proposed to be considered pathology. This concept was later adopted by other Russian scientists [1].

Regarding the recent COVID-19 pandemic (COronaVIrus Disease 2019 — coronavirus infection 2019) caused by the SARS-CoV-2 virus (Severe Acute Respiratory Syndrome-related COronaVirus 2), assessing lung function in patients with SARS-CoV-2-associated lung injury plays an important role in creating individual medical rehabilitation programs for patients after hospital discharge. Additionally, restoring lung function parameters to normal is one of the criteria for recovery.

Impaired lung diffusion capacity is the most common and long-lasting defect in lung function associated with SARS-CoV-2-related lung injury, as shown in various studies [7–9]. The criterion for impaired lung diffusion capacity is a decreased DLco (carbon monoxide transfer factor) [10].

In a previous study, a multifactorial logistic regression analysis was used to determine a decision rule for predicting decreased DLco using a fixed value of the lower limit of normal equal to 80%pred. [11]. However, no convincing evidence of the advantage of any proposed criteria for assessing pathological changes of DLco has been found in the available literature.

The aim of this study is to compare models for predicting impaired lung diffusion capacity after SARS–CoV-2-associated lung injury depending on the selected criterion for pathological changes of DLco.

MATERIALS AND METHODS

A retrospective study was conducted on 341 patients after COVID-19 with virus-associated lung injury. The maximum volume of lung tissue damage in the acute phase of COVID-19 according to high-resolution computed tomography of the chest (CT_{max}) and DLco were analyzed. The median age of the patients was 48 (41.5–57) years, 76.8% (262/341) were men. The median CT_{max} was 50 (31–75)%.

A diffusion test (evaluation of DLco) was conducted according to international standards [12].

Начать с It is worth noting that (221/341) of patients – underwent diffusion test within 90 days, 23.5% (80/341) of patients between 90 and180 days, and 11.7% (40/341) of patients – within more than 180 days from the onset of COVID-19.

Pathological deviation of DLco (the lower limit of the normal – LLN) was assessed using the following criteria:

Criterion 1: LLN_{DLCO} = 80%pred. (the fixed value of LLN) [2, 4];

Criterion 2: LLN_{DLCO} = predicted – 1.645 SD (SD – standard deviation) (the individual value of LLN) [3, 5].

The predicted value of DLco was determined according to the European Community of Coal and Steel prediction equations (ECCS, 1993) [5].

Statistical analysis was performed via SPSS 21 and MS Excel 2016 programs. The results were analyzed using descriptive statistics and multivariate logistic regression analysis.

Quantitative data with a skewed distribution were described using the median and interquartile range *Me* (Q_1–Q_3), where Q_1 is the lower quartile and Q_3 is the upper quartile. To compare three independent samples, the Kruskal – Wallis test and Mann – Whitney test with Bonferroni correction were used. Differences were considered statistically significant at $p < 0.05$, where p is the significance level.

In the previous study [11], multivariate logistic regression analysis was used to create a binary classifier model to predict abnormal DLco.

The decision rule for predicting abnormal DLco was built on a training sample. For this purpose, via a random number generator, the total sample was divided into a training and a test (validation) sample in a 3:1 ratio. The coefficients of the logistic regression equation Z were obtained on the training sample.

Z is the regression equation, which has the following form:

$$Z = \alpha_0 + \alpha_1 x_1 + \ldots\ldots + \alpha_n x_n,$$

where α_0, α_1, $\ldots\alpha_n$ — are model parameters (coefficients), and $x_1 \ldots x_n$ — are predictors.

P — represents the probability of abnormal DLco, where $P = \frac{1}{1 + e^{-z}}$

Logistic regression predicted a decrease in DLco when the Z value was greater than or equal to 0, while DLco was in the normal range if $Z < 0$.

Using the above algorithm, a decision rule was found to predict decreased DLco after SARS-CoV-2-associated lung injury in patients without underlying lung diseases . The logistic regression equation included a single predictor of CT_{max} [11]:

$$Z = \alpha_0 + \alpha_1 \times x_1 \quad (1)$$

where Z is the regression equation, α_0, α_1 – are model parameters (coefficients), and x_1 – is the predictor of CT_{max}.

The decision rule described by equation (1) was used in this study to compare the results of the binary classifier model depending on the selected LLN criterion of DLco.

To assess the quality of the binary classifier model and find the optimal cut-off value for dividing objects into classes, a ROC analysis was performed. The criterion for choosing the cut-off value was the requirement of the maximum sum of sensitivity and specificity. The ability of the created model to recognize the presence or absence of abnormal DLco was assessed by the value of AUC (area under the curve) and the difference between the ROC curve and the diagonal reference line.

RESULTS

The analysis of the Dlco parameter in the study sample is presented in Table 1.

Table 1

DLco parameter at different time intervals (days) from the COVID-19 onset, complicated by virus-associated lung injury, in patients without underlying lung diseases, *Me* (Q_1–Q_3)

Parameter	Total sample n = 341	Sample 1 <90 days (n = 221; 64.8%)	Sample 2 90–180 days (n = 80; 23.5%)	Sample 3 >180 days (n = 40; 11.7%)	p-value: p_{total} / p_{1-2} / p_{1-3} / p_{2-3}
DLco, %pred.	75 (61.7–88.3)	72 (54–84)	81 (67–93.5)	83 (75–95.5)	<0.001[1]/<0.001[2]/ <0.001[2]/0.45[2]

Note. The data are presented as median (lower quartile – upper quartile). p_{total} – significance level between samples 1–3, p_{1-2} – significance level between samples 1 and 2, p_{2-3} – significance level between samples 2 and 3, p_{1-3} – significance level between samples 1 and 3. 1 – Kruskal – Wallis test, 2 – Mann – Whitney test with Bonferroni correction for multiple comparisons.

Table 1 demonstrates that the median DLco in the total sample was decreased. Depending on the time interval between the onset of COVID-19 and the diffusion test, the median DLco tended to increase. Pairwise comparison revealed statistically significant differences between samples 1 and 2, as well as between samples 1 and 3. However, no statistically significant differences in DLco medians were found between samples 2 and 3.

To compare criteria 1 and 2 for determining pathological deviation of DLco, the total sample on which the decision rule was obtained in the previous study [11] was re-divided via a random number generator into a training (n = 262) sample and a validation (n = 79) sample. Further research was conducted in two stages.

Stage 1. Building a binary classifier model if LLN_{DLco} = 80%pred.

Using equation (1), the coefficients of the logistic regression equation were obtained from the training sample:

$$Z = -1.793 + 0.044 \times x_1 \quad (2)$$

The classification results are presented in Table 2.

Table 2

Classification results of DLco in the training sample (CT_{max} is the predictor)

Parameter	$DL_{CO} \geq$ 80%pred. (predicted)	$DL_{CO} <$ 80% pred. (predicted)	Classified correctly, %
$DL_{CO} \geq$ 80%pred., n	66	37	64.1
$DL_{CO} <$ 80%pred., n	27	132	83.0
Overall			75.6

Table 2 demonstrates that the sensitivity, specificity, and accuracy for the training sample using equation 1 were 83, 64.1, and 75.6%, respectively, respectively.

The quality of the model described by equation 2 was verified using the ROC analysis procedure. The ROC curve for the training sample is presented in Fig. 1.

Predicting decreased DLco (<80%pred.), the AUC value was 0.776, $p < 0.001$ (95% CI 0.707–0.824), with sensitivity and specificity (at a cut–off point of 0.165) being 81 and 66%, respectively. Testing the binary classifier model obtained at this stage from a validation sample yielded sensitivity and specificity of 76.6 and 78%, respectively.

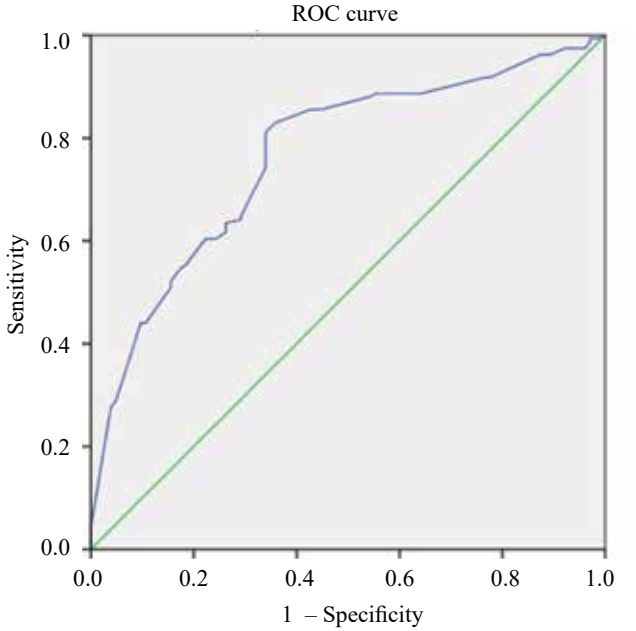

Fig. 1 – the ROC curve of the training sample (CT_{max} is the predictor) to predict abnormal DLco (< 80%pred.), AUC 0.776 (95% CI 0.707–0.824, $p < 0.001$). The cut–off point was 0.165

Stage 2. Building a binary classifier model if LLN_{DLco} = predicted – 1.645 SD.

Similar to *stage 1*, the coefficients of the logistic regression equation were obtained on the training sample using equation (1):

$$Z = -1.997 + 0.043 \times x_1 \quad (3)$$

The classification results are presented in Table 3.

Table 3

Classification results of DLco in the training sample (CT_{max} is the predictor)

Parameter	$DL_{CO} \geq$ LLN, n (predicted)	$DL_{CO} <$ LLN, n (predicted)	Classified correctly, %
$DL_{CO} \geq$ LLN, n	73	44	62.4
$DL_{CO} <$ LLN, n	34	111	76.6
Overall			70.2

Note. LLN is lower limit of normal, equal to predicted – 1.645SD, SD – standard deviation.

Table 3 demonstrates that the sensitivity, specificity, and accuracy for the training sample using equation 1 were 76.6, 62.4, and 70.2%, respectively.

The quality of the model described by equation 3 was verified using the ROC analysis procedure. The ROC curve for the training sample is presented in Fig. 2.

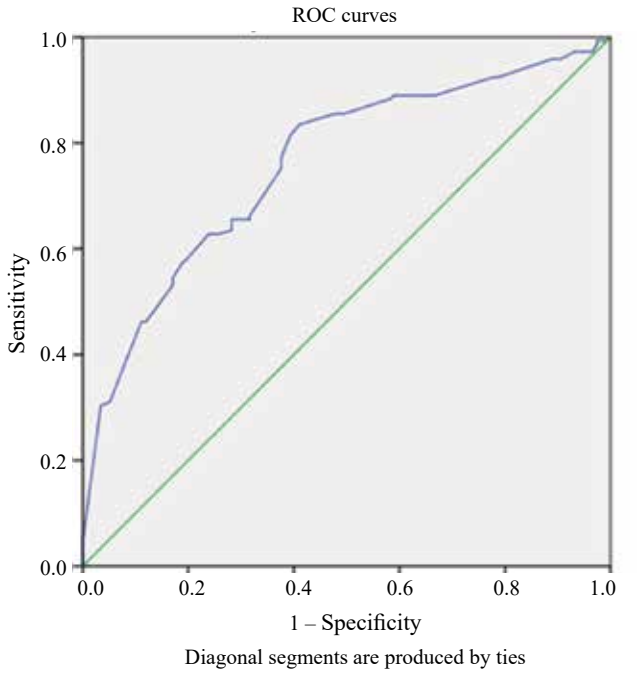

Fig. 2. The ROC curve of the training sample (CT_{max} is the predictor) to predict an abnormal DLco (< predicted – 1.645SD), AUC 0.759 (95% CI 0.701–0.817, $p < 0.001$). The cut–off point was –0.191

Predicting decreased DLco (<predicted – 1.645SD), the AUC value was 0.759, $p < 0.001$ (95% CI 0.701–0.817), with sensitivity and specificity (at a cut–off point of –0.191) being 83.4 and 59%, respectively. Testing the binary classifier model obtained at this stage from a validation sample yielded sensitivity and specificity of 76.2 and 67.6%, respectively.

According to the literature, abnormal lung function can be found in >50% of patients during the follow-up after COVID-19-related hospitalization. Lung diffusion capacity is the most common COVID-19-related complication [13]. M. Bellan et al. revealed that DLco was decreased (<80%pred.) in 51.6% (113/219) of patients and was less than 60%pred. in 15.5% (34/219) of patients after severe COVID-19 [14].

The present study also demonstrated a decrease in DLco within up to 90 days after the onset of COVID-19 and a gradual improvement of lung diffusion capacity as the period of time from the disease onset increases, which is consistent with data obtained in other patient populations [15, 16]. The issue of the lung function parameters dynamics remains important to this day and is being studied both in cases of mild/moderate and severe/extremely severe COVID-19 [17, 18].

In many studies devoted to the lung function after a SARS-CoV-2 infection, LLN of DLco 80%pred. was applied [19–21]. At the same time, in 2022, the American Thoracic Society and European Respiratory Society recommended using the 5th percentile or 1.645SD from the predicted value (Z-score = –1.645) as LLN for all lung function parameters [3]. This is not a new idea, as it was proposed and supported by Russian researchers in the 1960–1980s [1, 6]. However, the lack of appropriate software at that time did not allow this approach to be widely used in clinical practice. In turn, the approach proposed by the American Thoracic Society to use a fixed value of 80%pred. as LLN of the lung function parameters [2] was easy to use and proved itself well in clinical practice.

It should be noted that in a few studies dedicated to the study of the lung function after COVID-19, Z-score = –1.96 was taken as LLN of the lung function parameters [22]. At the same time, no justification was found in the literature for the advantage of any of the proposed criteria for LLN DLco and its effect on the accuracy of diagnosing impaired lung diffusion capacity.

In the present study, via a binary classifier model that includes a single predictor (CTmax), the effect of the pathological DLco deviation criterion on the prediction of lung diffusion capacity was analyzed in an examined group of patients. The study was conducted on a sample of patients without underlying lung diseases who had suffered SARS-CoV-2-associated lung injury. There are no similar studies found in the literature.

In the present study, the analysis of the classification results of the obtained models did not demonstrate significant differences in predicting impaired lung diffusion capacity depending on the criterion for LLN of DLco. Thus, the accuracy of the obtained models was 75.6 and 70.2% for criterion 1 (LLN_{DLco} = 80% pred.) and criterion 2 (LLN_{DLco} = predicted –1.645SD), respectively. The ROC analysis on a

training sample demonstrated that the sensitivity of the model was slightly higher when using criterion 2 in comparison with criterion 1 (83.4 and 81%, respectively).

However, the specificity was higher when using criterion 1 in comparison with criterion 2 (66 and 59%, respectively). In the validation sample, the sensitivity of the models was almost the same (76.6 and 76.2% for criterion 1 and 2, respectively), while the specificity was higher when using criterion 1 (78 and 67.6% for criterion 1 and 2, respectively).

The limitations of this study include the insufficient number of enrolled patients in the period from 6 months to 1 year from COVID-19 onset. Additionally, the ECCS 1993 reference value system was used to determine the predicted value of DLco, while the GLI (Global Lung Function Initiative) system is being widely introduced into clinical practice [3]. However, the effectiveness of the GLI system in clinical practice, its consistency with the ECCS 1993 system, as well as the correspondence of DLco, the predicted value of which is calculated using the GLI system, clinical and X-ray data, has not yet been studied in Russia.

CONCLUSION

For patients without underlying lung diseases, it was shown that the choice of the criterion for assessing LLN of DLco does not significantly affect the sensitivity of the prediction model of DLco decrease after suffering SARS-CoV-2-associated lung injury. However, the specificity of the prediction model was higher when using a fixed value of LLN of DLco (LLN_{DLco} = 80% pred.). In this regard, the authors do not see the advantages of determining LLN of DLco according to any of the criteria considered. In such cases, it is advisable to give preference to a method that is easier to apply in practice.

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Authors' contribution

Savushkina O.I. – development of the concept and design, selection and examination of patients, analysis and interpretation of data, critical revision of the article for important intellectual content, writing the text of the article. Muraveva E.S. – data analysis and statistical processing, graphical representation of data, writing the text of the article. Davydov D.V. – critical revision of the article for important intellectual content. Kryukov E.V. – final approval of the manuscript for publication, critical revision of the article for important intellectual content.

Authors' information

Savushkina Olga I. – Cand. Sci. (Biology), Head of the External Respiration Function Research Department for the Center for Functional Diagnostic Research, Main Military Clinical Hospital named after academician N. N. Burdenko; Senior Researcher, Laboratory of Functional and Ultrasound Research Methods, Research Institute for Pulmonology of the Federal Medical Biological Agency, Moscow, olga-savushkina@yandex.ru, https://orcid.org/0000-0002-7486-4990

Muraveva Elena S. – Cand. Sci. (Biology), Associate Professor, Bioinformatics Department, Faculty of Medicine and Biology, Pirogov Russian National Research Medical University, Moscow, esmuraviova@mail.ru

Davydov Denis V. – Dr. Sci. (Med.), Professor, Head of the Main Military Clinical Hospital named after academician N. N. Burdenko, Moscow, dvdavydov@yandex.ru, https://orcid.org/0000-0001-5449-9394

Kryukov Evgeniy V. – Dr. Sci. (Med.), Professor, Academician of the Russian Academy of Sciences, Head of the Military Medical Academy named after S. M. Kirov, Saint Peterburg, evgeniy.md@mail.ru, https://orcid.org/0000-0002-8396-1936

(✉) **Savushkina Olga I.,** olga-savushkina@yandex.ru

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Association of Toll-like receptor polymorphism and gene expression level with the risk of developing chronic obstructive pulmonary disease (COPD) and its severity

Salamaikina S.A.[1, 2], Korchagin V.I.[1], Mironov K.O.[1], Karnaushkina M.A.[3]

[1] Central Research Institute of Epidemiology of the Russian Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing
3a, Novogireevskaya Str., Moscow, 111123, Russian Federation

[2] Moscow Institute of Physics and Technology (MIPT)
9, Institutsky Lane, Dolgoprudny, Moscow Region, 141701, Russian Federation

[3] Patrice Lumumba Peoples' Friendship University of Russia (RUDN University)
6, Miklukho-Maklaya Str., Moscow, 117198, Russian Federation

ABSTRACT

Aim. To determine how genetic factors of innate immunity influence the risk of development and features of the course of COPD.

Materials and methods. The study included 103 patients diagnosed with chronic obstructive pulmonary disease and 47 apparently healthy people without any chronic bronchopulmonary pathologies. The expression level of TLR genes and alleles of rs5743551 (*TLR1*), rs5743708 (*TLR2*), rs3804100 (*TLR2*), rs3806790 (*TLR4*), rs5743810 (*TLR6*), rs3804880 (*TLR8*) single nucleotide polymorphisms were analyzed via real-time polymerase chain reaction (PCR).

Results. Several trends were observed: an increase in the proportion of GG homozygotes in the rs5743810 (*TLR6*) locus in patients with severe COPD and a negative correlation between *TLR2* and *TLR6* gene expression level and oxygen saturation in blood, dyspnea and COPD severity.

Conclusion. No statistically significant association with rs5743551 (*TLR1*), rs5743708 (*TLR2*), rs3804100 (*TLR2*), rs4986790 (*TLR4*), rs5743810 (*TLR6*), rs3764880 (*TLR8*) single nucleotide polymorphisms was found. The observed trend toward an increase in TLR gene expression may be associated with the remodeling of lung tissues and activation of the immune response that occur during COPD.

Keywords: COPD, TLR, single nucleotide polymorphisms, gene expression

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. The study complies with the ethical principles developed in accordance with the *Ethical Principles for Medical Research Involving Human Participants* in WMA Declaration of Helsinki with amendments of 2000 and the *Rules of Good Clinical Practice of the Russian Federation* approved by Order No. 266 of the Ministry of Health of the Russian Federation dated 19 June 2003. All patients signed an informed consent to participate in the study. The study was approved by the Ethics Committee of the Medical Institute of the Peoples' Friendship University of Russia (Protocol No. 30 of 17.06.2021).

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✉ *Salamaikina Svetlana A.,* salamaykina@cmd.su

Ассоциация полиморфизма и уровня экспрессии генов Toll-подобных рецепторов с риском развития и тяжестью течения хронической обструктивной болезни легких

Саламайкина С.А.[1, 2], Корчагин В.И.[1], Миронов К.О.[1], Карнаушкина М.А.[3]

[1] *Центральный научно-исследовательский институт (НИИ) эпидемиологии*
Россия, 111123, г. Москва, ул. Новогиреевская, 3а

[2] *Московский физико-технический институт (МФТИ)*
Россия, 141701, г. Долгопрудный, Институтский пер., 9

[3] *Российский университет дружбы народов (РУДН) им. Патриса Лумумбы*
Россия, 117198, г. Москва, ул. Миклухо-Маклая, 6

РЕЗЮМЕ

Цель: определение вклада генетических факторов врожденного иммунитета в риск развития и особенности течения хронической обструктивной болезни легких (ХОБЛ).

Материалы и методы. В исследование включены 103 пациента с диагнозом «хроническая обструктивная болезнь легких» и 47 условно здоровых человек без хронической бронхолегочной патологии. Определен уровень экспрессии генов *TLR* и аллели однонуклеотидных полиморфизмов rs5743551 (*TLR1*), rs5743708 (*TLR2*), rs3804100 (*TLR2*), rs3806790 (*TLR4*), rs5743810 (*TLR6*), rs3804880 (*TLR8*) методом полимеразной цепной реакции в режиме реального времени.

Результаты. Выявлена тенденция к увеличению доли гомозигот GG в локусе rs5743810 (*TLR6*) у пациентов с тяжелым течением ХОБЛ и обратная корреляция уровня экспрессии генов *TLR2* и *TLR6* с сатурацией кислорода в крови, выраженностью одышки и тяжестью течения заболевания.

Заключение. Для однонуклеотидных полиморфизмов rs5743551 (*TLR1*), rs5743708 (*TLR2*), rs3804100 (*TLR2*), rs4986790 (*TLR4*), rs5743810 (*TLR6*), rs3764880 (*TLR8*) не обнаружено статистически значимой ассоциации. Наблюдаемые тенденции повышения уровня экспрессии генов *TLR* могут быть связаны с возникающим в процессе течения ХОБЛ ремоделированием легочных тканей и активацией пути иммунного ответа.

Ключевые слова: ХОБЛ, TLR, однонуклеотидные полиморфизмы, экспрессия генов

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Соответствие принципам этики. Все пациенты подписали информированное согласие на участие в исследовании. Исследование одобрено этическим комитетом Медицинского института ФГАОУ ВО «Российский университет дружбы народов» (протокол № 30 от 17.06.2021).

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INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a heterogeneous lung disease characterized by progressive airflow limitation due to the development of obliterative bronchiolitis, chronic bronchitis, emphysema, and lung tissue remodeling in response to inhaled particles and gasses [1]. These processes have a progressive course associated with the development of inflammation.

COPD exacerbations lead to a progressive decrease in lung capacity, increasing respiratory insufficiency, disease progression and are one of the most frequent reasons for patients to seek emergency medical care, which is associated with significant economic costs [1].

Smoking is a major risk factor for COPD, although not all smokers develop clinically significant lung tissue damage, which indicates that apart from pollutants there are also other factors (including genetics) that influence the development of COPD. According to the literature, Toll-like receptors (TLRs) mediate many cellular immunity responses, including the cytokine response [2]. Activation of these receptors occurs due to the binding of pathogen molecular structures (pathogen-associated molecular patterns, PAMPs) and tissue damage products (damage-associated molecular patterns, DAMPs). Innate immunity activation caused by chronic inflammatory respiratory diseases could be associated with the immune system gene polymorphisms. Specifically, with single nucleotide polymorphisms (SNPs) in TLR genes.

TLRs are mediators of smoking-induced inflammation. Smokers have an increased *TLR2* expression in monocyte cells [3]. Increased expression levels of *TLR4* and *TLR9* are associated with inflammatory processes of the lower airway tissues in patients with COPD [4]. Cigarette smoke-induced oxidative stress and DAMP-induced inflammation are important mechanisms of the immune response[5].

Organic dust-stimulated IL-6 production may be associated with one or more synonymous SNP variants in the *TLR1* gene [6]. rs1898830 (*TLR2*) and rs11938228 (*TLR2*) are associated with accelerated FEV-1 decline and higher inflammatory cell counts in sputum [7]. A population-based study reported an association between rs4986790 (*TLR4*), rs4986791 (*TLR4*), and rs5743708 (*TLR2*) polymorphisms and the development of COPD [8]. A slight association was found between the rs5743810 (*TLR6*) polymorphism and the risk of developing chronic lower respiratory tract diseases [9]. Further studies are needed to clarify the role of TLR gene polymorphisms in COPD.

Thus, the aim of this study was to determine how genetic factors of innate immunity influence the risk of development and features of the course of COPD..

MATERIALS AND METHODS

A case-control study was conducted using biological material obtained during the period from January 2022 to February 2024 at the V.V. Vinogradov City Clinical Hospital of the Moscow Health Department. The study complies with the ethical principles developed in accordance with the *Ethical Principles for Medical Research Involving Human Participants* in WMA Declaration of Helsinki (with amendments of 2000) and the *Rules of Good Clinical Practice of the Russian Federation* approved by Order No. 266 of the Ministry of Health of the Russian Federation dated 19 June 2003.

The COPD group included patients aged 40 to 70 with a clinically confirmed diagnosis established by a pulmonologist 12 or more months prior to inclusion in the study, smokers at the time of inclusion in the study (smoking index of more than 10 pack-years), hospitalized patients with a COPD exacerbation. All of the participants signed an informed consent.

The study did not include those patients with COPD who also suffered from severe concomitant pathologies, oncological or mental diseases, patients taking medications that may cause lung tissue damage as a side effect, patients who were vaccinated against pneumococcus, as well as patients with other chronic diseases of the bronchopulmonary system, including bronchial asthma.

Exclusion criteria were clinical, laboratory or instrumental signs discovered during medical examination that indicated the absence of COPD or were indicative of other causes for lung tissue damage.

All patients with COPD underwent physical examination and participated in spirometry with a bronchodilator reversibility test (in accordance with ATS requirements) [10]. The severity of COPD was assessed using the BODE index (B – body mass index, O – obstruction, D – dyspnea, E – exercise tolerance) and the scale proposed by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) [1]. To exclude other bronchopulmonary pathologies and to clarify the nature of changes in the lung tissue, multispiral computed tomography of the chest organs (MSCT CO) was performed.

The control group included patients with a smoking history of at least 10 pack-years with $SatO_2 \geq 95\%$ saturation who showed no evidence of any respiratory diseases during spirometry with a bronchodilator test and MSCT CO, as well as showed no signs of the right ventricular dysfunction or elevation of mean pulmonary arterial pressure during echocardiography (ECHO CG).

Whole blood samples were collected anonymously in vacutainers with K_3EDTA as anticoagulant for genetic studies. Biological material was stored for no more than 4 hours before the nucleic acid mixture was isolated. The study used reagents and kits manufactured at the Central Research Institute of Epidemiology (AmpliSens, Moscow, Russia). SNP alleles and TLR gene expression levels were determined via real-time polymerase chain reaction (PCR). Allele detection of rs5743551 (*TLR1*), rs5743708 (*TLR2*), rs3804100 (*TLR2*), rs4986790 (*TLR4*), rs5743810 (*TLR6*), rs3764880 (*TLR8*) polymorphisms was performed in accordance with the previously described methodology [11].

Reverse transcription reaction was performed using the REVERTA-L reagent kit. Method of normalization of genes with relatively stable expression (housekeeping genes) – *HPRT1*, *SDHA*, *GAPDH* and *TBP* – was used

to calculate the expression level of target genes (*TLR1*, *TLR2*, *TLR4*, *TLR6*, *TLR8*). Normalization index was formed using the *BestKeeper* algorithm [12] in order to select genes with the smallest spread of cycle threshold (Ct) values. Information about the development and optimization of the method for determining expression level was described in earlier studies [13].

Statistical analysis was performed using the R environment (version 4.4.0), including standard contingency table analysis functions: Pearson's $\chi 2$ test and Fisher's exact test. Association analysis and odds ratio (OR) estimation were performed using SNPassoc [14] and epitools [15] packages. The Holm – Bonferroni method was used to correct multiple comparisons. Results were considered statistically significant at $p < 0.05$. All graphical results were obtained using ggplot2 [16] and ggstatsplot [17] packages.

RESULTS

The COPD group (n = 103) included patients with a diagnosis confirmed by a pulmonologist. There was an irregular distribution among the study participants by sex (80 men and 23 women), the mean age of the participants was 67.5 ± 11.6 years, and the mean smoking history was 71 (43; 88) pack-days. In the control group of apparently healthy smokers (n = 47), the mean age was 61.6 ± 9.4 years and the mean smoking history was 65 (32; 79) pack-days. A comparison of clinical and demographic characteristics revealed that the groups differed significantly in age (p = 0.0025). Clinical and demographic characteristics of the study groups are presented in Table 1.

Table 1

Characteristics of the studied groups

Parameter	COPD (n = 103)	Control (n = 47)
Sex, n (%)		
male, 117 (72.2%)	80 (77.7%)	36 (76.6%)
female, 45 (27.8%)	23 (22.3%)	11 (23.4%)
Age, years	69 (60.5–74)	61 (54–68)
Dyspnea, mMRC score	3 (2–3)	–
Smoking index, pack-years	71 (43; 88)	65 (32; 79)
FEV1, % of predicted (after bronchodilator test)	47 (45; 59)	92 (86; 98)
FVC, % of predicted (after bronchodilator test)	73 (68; 82)	88 (81; 97)
FEV1/ FVC (after bronchodilator test)	0.52 (0.41; 0.64)	0.83 (0,76; 0.91)
Saturation	93 (90–95)	97 (95; 98)
Obstruction severity on the GOLD scale	3 (2–3)	0
Severity on the BODE scale	2 (1–3)	1 (0; 1)

Note. FEV1 – forced expiratory volume in 1 second; FVC – forced vital capacity.

rs5743551 (*TLR1*), rs3804100 (*TLR2*), rs4986790 (*TLR4*), rs5743810 (*TLR6*), and rs3764880 (*TLR8*) polymorphic loci were analyzed in order to determine whether there was an association between them and the risk of development and severity of COPD. The studied groups were not significantly different in the frequency of alleles of the studied SNPs (Table 2). rs5743708 (*TLR2*) locus was the exception. For this locus, frequency of a rare A allele was significantly lower (0.5%) in the COPD group than in the control group (9%). Thus, a rare allele may be associated with a protective effect — the risk of COPD in its carriers is lower than in carriers of a wild-type allele (odds ratio (OR) = 0.05; 95% confidence interval (CI) = 0.01–0.43; p = 0.0005).

The COPD group was stratified into four subgroups by disease severity according to the spirometric classification of the severity of bronchial obstruction and recommendations [1]. An analysis of the allele frequency distribution in the studied subgroups was carried out. There were no statistically significant differences between the groups in allele frequency for the studied polymorphic loci. For the rs5743810 (*TLR6*) polymorphism, the number of GG homozygotes tended to increase from the group with moderate disease severity to the group with severe COPD, while the number of rare AA homozygotes decreased and the frequency of heterozygotes remained the same. Table 3 shows the distribution of genotypes in the rs5743810 (*TLR6*) locus and the statistical significance of differences between groups 1 and 3.

During the next stage of the study, the expression levels of TLR genes in the control group (n = 47) and the COPD group (n = 33) were analyzed. Based on the stability analysis of housekeeping genes in the analyzed samples, a complex *BestKeeper* index was formed. The index was based on the geometric mean Ct value of the two most stable genes — *HPRT1* and *SDHA*. Normalization allows, to a certain extent, to exclude the influence of the quality and quantity differences of the source material on the obtained values. In order to determine the expression level differences between patients with different disease severity, they were divided into subgroups for each analyzed parameter. In the group of patients with a saturation level ($SatO_2$) below normal (<95%), there was an increased expression of the *TLR2* and *TLR6* genes. The results are shown in Figure 1. There was a similar trend for the other *TLR* genes but the comparison results were not statistically significant ($p > 0.05$).

Analysis of the TLR gene expression level between subgroups of patients using the modified Medical Research Council Dyspnea Scale (mMRC) [18] showed an increase in the expression of *TLR2* and *TLR6* genes with increasing severity of dyspnea. The results of the analysis are shown in Figure 2.

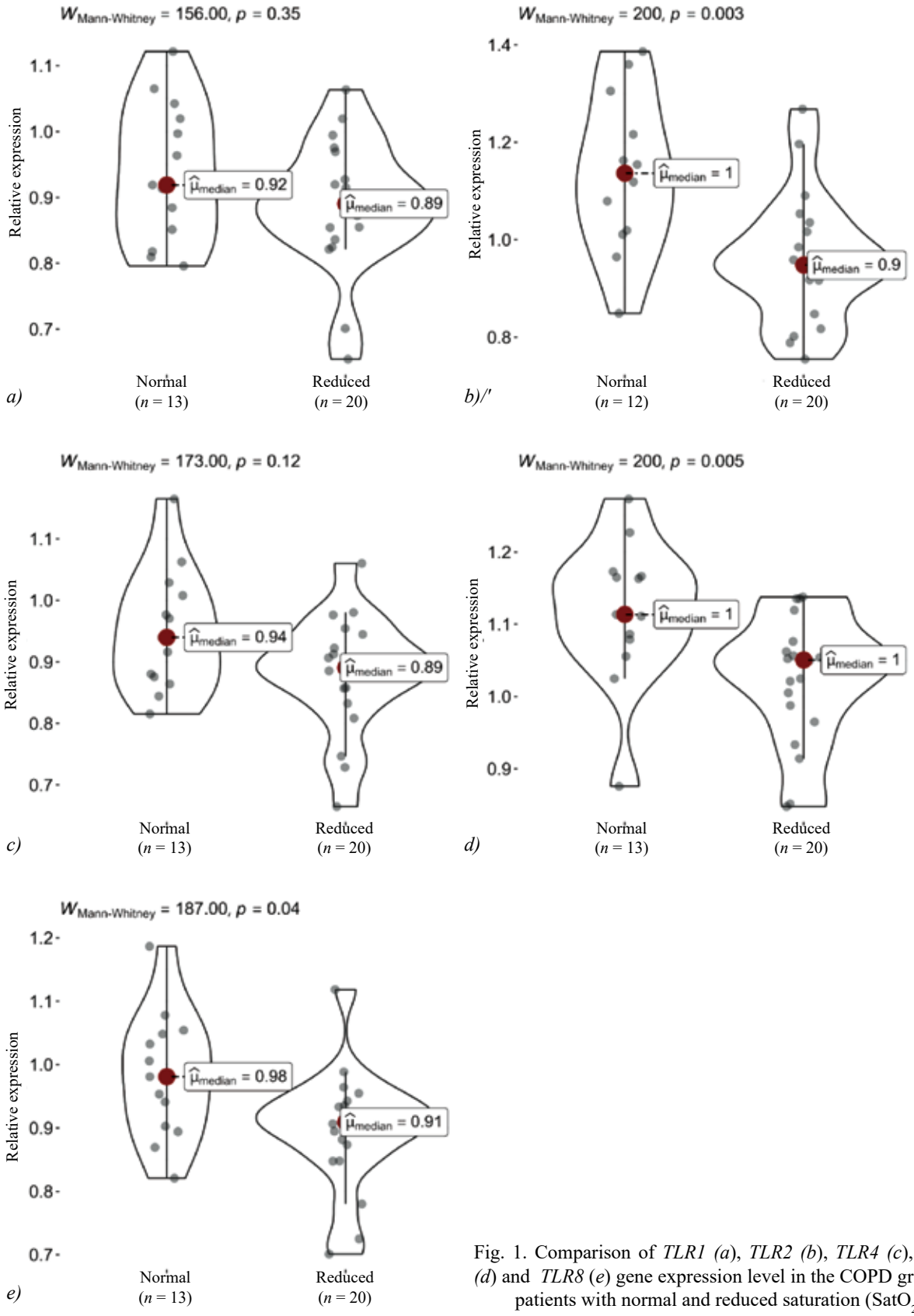

Fig. 1. Comparison of *TLR1 (a)*, *TLR2 (b)*, *TLR4 (c)*, *TLR6 (d)* and *TLR8* (*e*) gene expression level in the COPD group of patients with normal and reduced saturation ($SatO_2$)

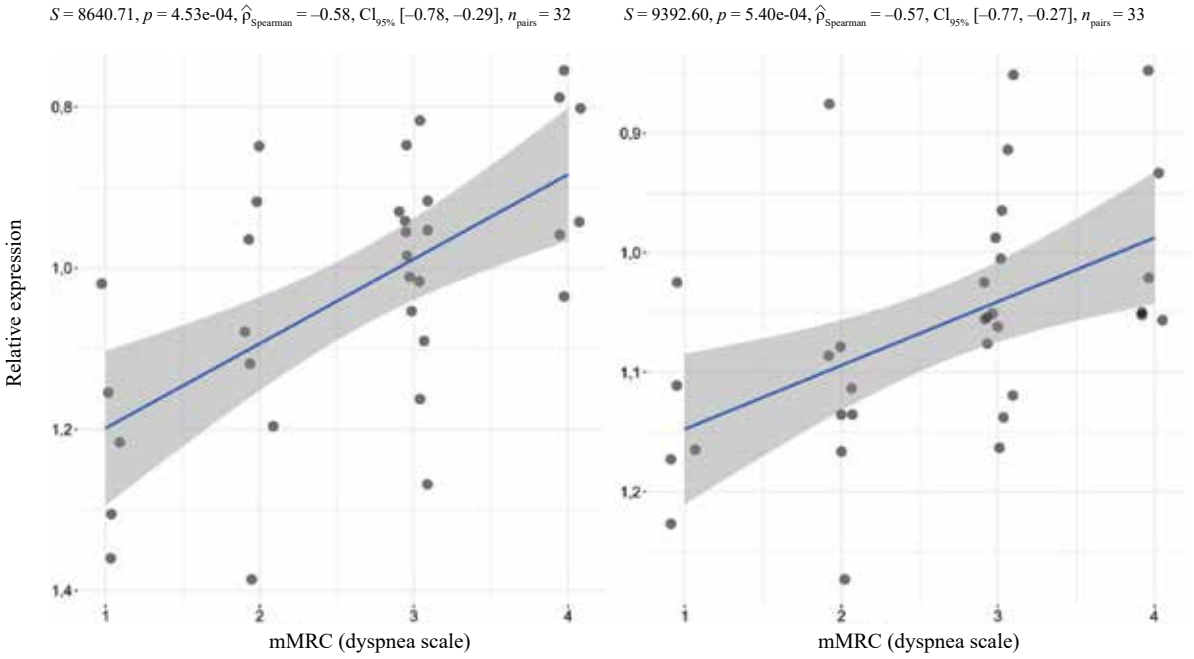

Fig. 2. Correlation between (*a*) TLR2 and (*b*) TLR6 gene expression level and the mMRC dyspnea scale

Table 2

Allele frequencies of polymorphic TLR loci associated with the risk of development and severity of COPD

Locus	Allele	Groups		Fisher's exact test, p ($p_{Bonferroni}$)
		COPD (n = 103)	Control (n = 47)	
rs5743551 (*TLR1*)	A	167 (81%)	73 (78%)	0.5346 (1)
	G	39 (19%)	21 (22%)	
rs5743708 (*TLR2*)	A	1 (0.5%)	8 (9%)	**0.0005 (0.003)**
	G	205 (99.5%)	86 (91%)	
rs3804100 (*TLR2*)	C	9 (4%)	7 (7%)	0.2781 (1)
	T	197 (96%)	87 (93%)	
rs4986790 (*TLR4*)	A	187 (91%)	88 (94%)	0.5033 (1)
	G	19 (9%)	6 (6%)	
rs5743810 (*TLR6*)	A	77 (37%)	35 (37%)	1 (1)
	G	129 (63%)	59 (63%)	
rs3764880 (*TLR8*)	A	160 (78%)	73 (78%)	1 (1)
	G	46 (22%)	21 (22%)	

Table 3

Distribution of genotype frequencies in the COPD group by severity of the disease (according to FEV1)

Locus	Genotype	Severity of COPD according to FEV1				Fisher's exact test, p ($p_{Holm-Bonferroni}$)
		1	2	3	4	
rs5743810 (*TLR6*)	A/A	0 (0%)	7 (20%)	6 (15%)	1 (8%)	0.034 (0.20*)
	A/G	12 (75%)	16 (46%)	15 (39%)	6 (46%)	
	G/G	4 (25%)	12 (34%)	18 (46%)	6 (46%)	

* with correction for multiple comparisons (Holm-Bonferroni)

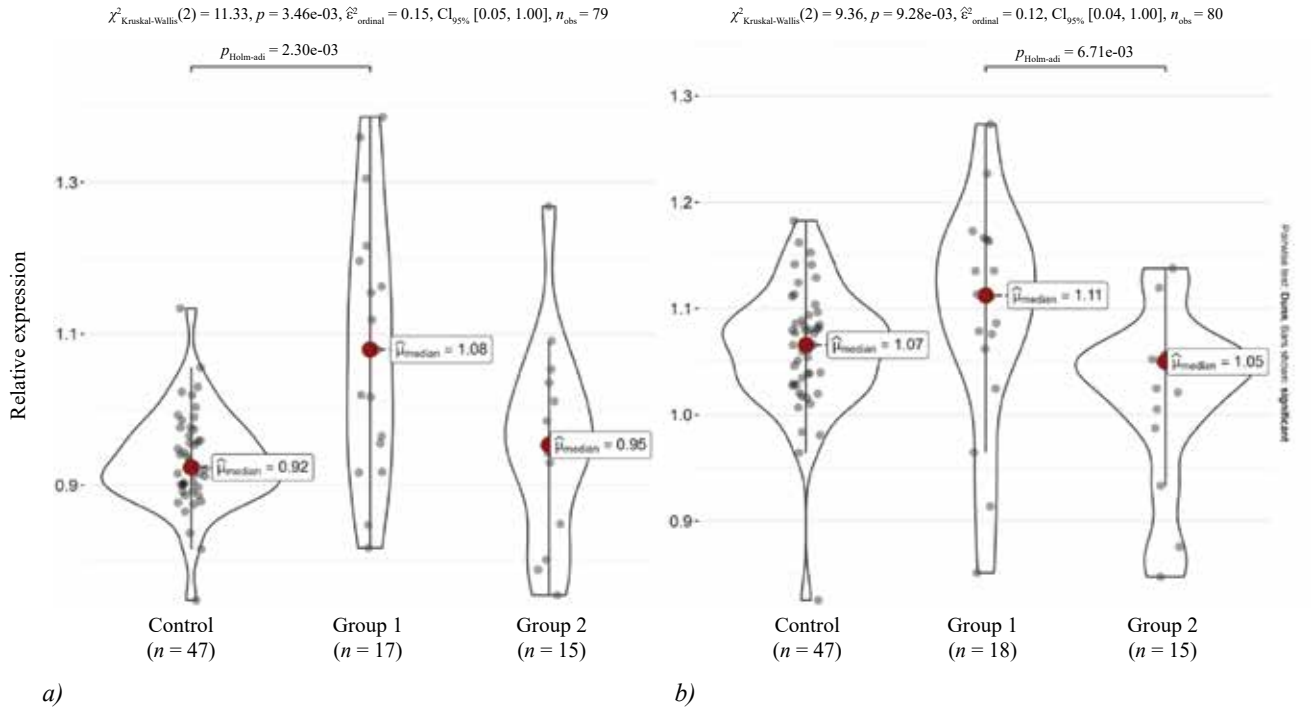

Fig. 3. Expression of TLR2 (*a*) and TLR6 (*b*) genes in the control group and in subgroups with mild and severe COPD according to the severity scale (GOLD) [1]

Considering that the studied indicators were part of the COPD severity assessment according to the severity scale (GOLD) [1], the COPD group was divided into subgroups according to COPD severity. Subgroups 1 and 2 were combined into a subgroup with mild/ moderate COPD, and subgroups 3 and 4 were combined into a subgroup with a more severe COPD. The results of the analysis are shown in Figure 3. There was an increased expression of *TLR2* and *TLR6* genes in patients with severe COPD and in the control group.

When the patients were divided according to the severity of COPD using the A, B, E scale (assessment of the FEV1 level, mMRC, and the frequency of exacerbations), only subgroups with mild and severe COPD significantly differed in the level of *TLR2* and *TLR6* gene expression.

DISCUSSION

The absence of a statistically significant association between alleles and genotypes of the studied polymorphisms is not consistent with the previously obtained data on the associations of the studied alleles with other pathologies of the lower respiratory tract. In previous studies, the rs5743551-G (*TLR1*) and rs4986790-G (*TLR4*) alleles were identified to have an association with the risk of developing NETosis [19] – a marker of the inflammatory process severity. There was also an association discovered between the rs4986790-G (*TLR4*) allele and the risk of developing tuberculosis [20]. Although COPD exacerbations may be associated with the development of inflammation in the lungs caused by bacteria, earlier findings on the connection between the rs5743551-G (*TLR1*) allele and the development of acute bacterial infection in patients with pneumonia [11] were not confirmed in this study. Such a result may be explained by the predominance of non-bacterial reasons for COPD exacerbations in the study group. This is tied to the specific features of the disease: the main pathogenetic mechanism of COPD is a combination of modifiable environmental factors (smoking, ecology), that are a lot more influential than genetics. Viral infections are also a common factor for COPD exacerbations and the immunologic defense mechanisms to them are different from bacterial infections.

The findings in this study are consistent with those previously published; however, most studies have focused on the *TLR2* and *TLR4* genes. In the study published by S.E. Budulac et al., an association of FEV1 with several SNPs in the *TLR2* gene was identified, while the results for the rs5743708 and rs3804100 loci were not statistically significant [7]. Another study analyzed the contribution of the rs5743836 (*TLR9*) allele to the development of alveolar macrophage dysfunction and the progression of COPD [21].

Studying the connection between gene polymorphic variants and expression level allows not only to better

understand how genetic factors influence the emergence and the development of multifactorial diseases, but also how polymorphic variants affect gene functions. This study did not find an association between SNPs in TLR genes and changes in expression levels, but the observed trends towards increased expression levels may be explained by other factors. TLR molecular patterns activate not only in response to pathogens but also in response to lung tissue damage [22]. Since COPD development is usually associated with long-term exposure to cigarette smoke, COPD patients tend to have non-infectious damage to the lung tissue.

According to the literature, exposure to cigarette smoke can activate signaling cascades of DAMPs and oxidative stress [23]. Similar to TLR activation, oxidative stress is closely linked to interleukin production [24, 25]. Increased *TLR2* and *TLR6* expression levels, low blood oxygen saturation, and severe dyspnea may be associated with activation of oxidative stress and production of interleukins in response to smoking-induced lung tissue damage. High levels of interleukins may also contribute to decreased *TLR2* and *TLR6* gene expression in patients with COPD, which requires further studying.

CONCLUSION

This study investigated the connection between polymorphic variants of TLR genes and the likelihood of COPD development and its course. No statistically significant association with rs5743551 (*TLR1*), rs5743708 (*TLR2*), rs3804100 (*TLR2*), rs4986790 (*TLR4*), rs5743810 (*TLR6*), rs3764880 (*TLR8*) single nucleotide polymorphisms was found. The observed trend toward an increase in TLR gene expression may be associated with the remodeling of lung tissues and activation of the immune response by DAMPs that occur during COPD.

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Authors' information

Salamaikina Svetlana A. — Researcher, Laboratory of Molecular Methods for Analysis of Genetic Polymorphisms, Central Research Institute of Epidemiology, PhD student, Molecular and Cellular Biology Department, Moscow Institute of Physics and Technology, Moscow, Russian Federation, salamaykina@cmd.su, https://orcid.org/0000-0002-2517-5048

Korchagin Vitaly I. — Cand. Sci. (Biology), Senior Researcher, Laboratory of Molecular Methods for Analysis of Genetic Polymorphisms, Central Research Institute of Epidemiology, Moscow, Russian Federation, Korchagin@cmd.su, https://orcid.org/0000-0003-2264-6294

Mironov Konstantin O. — Dr. Sci. (Med.), Head of Laboratory of Molecular Methods for Analysis of Genetic Polymorphisms, Central Research Institute of Epidemiology, Moscow, Russian Federation, mironov@cmd.su, https://orcid.org/0000-0001-8207-9215

Karnaushkina Maria A. — Dr. Sci. (Med.), Associate Professor, Professor of the Department of Internal Diseases with Cardiology and Functional Diagnostics Course named after Academician V.S. Moiseev, Patrice Lumumba Peoples' Friendship University of Russia (RUDN University), Moscow, Russian Federation, kar3745@yandex.ru, https://orcid.org/0000-0002-8791-2920

(✉) **Salamaikina Svetlana A.,** salamaykina@cmd.su

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Osteogenic potential of mesenchymal stem cells of epicardial adipose tissue in patients with coronary heart disease

Uchasova E.G.[1], Dyleva Yu.A.[1], Slesareva T.A.[1, 2], Belik E.V.[1], Ponasenko A.V.[1], Velikanova E.A.[1], Matveeva V.G.[1], Dvadtsatov I.V.[1], Tarasova O.L. [2], Gruzdeva O. V. [1, 2]

[1] *Research Institute for Complex Issues of Cardiovascular Diseases Federal State Budgetary Institution 6, Barbarasha Blvd. Kemerovo, 650000, Russian Federation*

[2] *Kemerovo State Medical University 22a, Voroshilova Str., Kemerovo, 650000, Russian Federation*

ABSTRACT

Aim. To assess the osteogenic potential of mesenchymal stem cells (MSCs) of epicardial adipose tissue (EAT) in patients with stable coronary heart disease based on obtaining gene profiles (osteogenesis markers).

Materials and methods. In EAT MSCs, the expression levels of the *RUNX2* (RUNX transcription factor encoding gene), BGLAP (osteocalcin encoding gene), SPP1 (osteopontin encoding gene), *SP7* (Osterix encoding gene) genes were determined using real-time polymerase chain reaction (PCR). Using immunofluorescence staining, the amount of RUNX2, osteocalcin, osteopontin, and Osterix proteins was determined in the supernatant of cultured MSCs.

Results. It was found that the expression of *RUNX2* in cells cultured in a medium with osteoinducers was 1.88 times higher than in undifferentiated MSCs ($p = 0.012$). The level of *RUNX2* protein was also higher in a differentiated cell culture ($p < 0.05$). Similar results were obtained regarding the level of *SPP1* mRNA expression ($p = 0.012$). *BGLAP* expression did not differ between differentiated and undifferentiated MSC cultures. The level of *SP7* gene expression did not differ in cells either with or without an osteoblastic medium. It is worth noting that using immunofluorescence staining, there were no differences detected in the expression of Osterix and OCN between cultures of differentiated and undifferentiated cells.

Conclusion. It was found that EAT MSCs have osteogenic potential, which was manifested by the expression of osteogenesis genes in both differentiated and undifferentiated MSCs. The increase in the expression level of *SSP1* and *RUNX2* mRNA on day 15 of cultivation with osteoblastic medium indicates that the studied cells are preosteoblasts and are at the stage of extracellular matrix synthesis.

Keywords: mesenchymal stem cells, adipose tissue, coronary heart disease, osteogenesis genes, calcification

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. This study was approved by the local Ethics Committee of the Research

✉ *Uchasova Evgenia G.,* evg.uchasova@yandex.ru

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Остеогенный потенциал мезенхимальных стволовых клеток эпикардиальной жировой ткани у пациентов с ишемической болезнью сердца

Учасова Е.Г.[1], Дылева Ю.А.[1], Слесарева Т.А.[1, 2], Белик Е.В.[1], Понасенко А.В.[1], Великанова Е.А.[1], Матвеева В.Г.[1], Двадцатов И.В.[1], Тарасова О.Л.[2], Груздева О.В.[1, 2]

[1] Научно-исследовательский институт комплексных проблем сердечно-сосудистых заболеваний (НИИ КПССЗ)
Россия, 650002, г. Кемерово, бульвар имени академика Л.С. Барбараша, 6

[2] Кемеровский государственный медицинский университет (КемГМУ)
Россия, 650000, г. Кемерово, Ворошилова, 22а

РЕЗЮМЕ

Цель. На основе получения профиля генов – маркеров остеогенеза – оценить остеогенный потенциал мезенхимальных стволовых клеток (МСК) эпикардиальной жировой ткани (ЭЖТ) у пациентов со стабильной ишемической болезнью сердца.

Материалы и методы. В МСК ЭЖТ методом полимеразной цепной реакции (ПЦР) в реальном времени определяли уровни экспрессии генов *RUNX2* (ген, кодирующий транскрипционный фактор RUNX), *BGLAP* (ген, кодирующий остеокальцин OCN), *SPP1* (ген, кодирующий остеопонтин OPN), *SP7* (ген, кодирующий Osterix). С помощью иммунофлуоресцентного окрашивания в супернатанте культивируемых МСК определяли количество белка RUNX2, OCN, OPN и Osterix.

Результаты. Установлено, что экспрессия RUNX2 в клетках, культивированных в среде с остеоиндукторами, была в 1,88 раза выше, чем в недифференцированных МСК ($p = 0{,}012$). Уровень белка *RUNX* также был выше в дифференцированной культуре клеток ($p < 0{,}05$). Аналогичные результаты были получены в отношении уровня экспрессии мРНК *SPP1* ($p = 0{,}012$). Экспрессия *BGLAP* не отличалась в дифференцированных и недифференцированных культурах МСК так же, как уровень экспрессии гена *SP7* в клетках с остеобластной средой и без нее. Стоит отметить, что методом иммунофлуоресцентной окраски нами не выявлено различий в экспрессии Osterix и OCN между культурами дифференцированных и недифференцированных клеток.

Заключение. МСК ЭЖТ имеют остеогенный потенциал, что проявилось экспрессией генов остеогенеза как дифференцированных, так и недифференцированных МСК. Увеличение уровня экспрессии мРНК *SSP1* и *RUNX2* на 15-е сут культивирования с остеобластной средой свидетельствует о том, что изучаемые нами клетки являются преостеобластами и находятся на стадии синтеза внеклеточного матрикса.

Ключевые слова: мезенхимальные стволовые клетки, жировая ткань, ишемическая болезнь сердца, гены остеогенеза, кальцификация

Конфликт интересов. Авторы заявляют об отсутствии явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Работа выполнена в рамках фундаментальной темы НИИ КПССЗ № 0419-2022-0002 (период выполнения 2022–2026 гг.) «Разработка инновационных моделей управления риском

развития болезней системы кровообращения с учетом коморбидности на основе изучения фундаментальных, клинических, эпидемиологических механизмов и организационных технологий медицинской помощи в условиях промышленного региона Сибири».

Соответствие принципам этики. Все пациенты подписали информированное согласие на использование биологического материала в исследовании. Исследование одобрено локальным этическим комитетом НИИ КПССЗ (протокол № 12 от 20.03.2023).

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INTRODUCTION

Atherosclerosis and its associated pathology – coronary heart disease (CHD) are leading causes of mortality in economically developed countries, despite significant advancements in treatment. A common complication accompanying CHD is calcification of the coronary arteries [1]. Although vascular calcification was recognized as a form of extra-skeletal ossification over a century ago, it is now understood to be a strictly regulated process. Its stages are similar to bone morphogenesis, including the expression of major pro-osteogenic factors such as osteocalcin, osteoprotegerin, osteopontin, and other markers [2]. The exact mechanisms underlying the pathogenesis of coronary artery calcification remain unclear.

For a long time, it was believed that at least 4 types of cells can lead to vascular calcification: 1) pericytes in microvessels; 2) pericyte-like calcifying vascular cells in the intima of the aorta; 3) smooth muscle cells in the media; and 4) myofibroblasts in adventitia. Along with resident cells, circulating cells, including bone marrow-derived mesenchymal stem cellss, can migrate into the vessel wall and contribute to calcification [3, 4]. However, adipose tissue (AT) surrounding the heart and blood vessels may also be the source of mesenchymal stem cells (MSCs). [5]. It is assumed that the main function of AT MSCs is to regenerate damaged areas surrounding the organ, as well as to produce biologically active substances, including anti-apoptotic, growth, and immunomodulatory factors. At the same time, AT MSCs are multipotent cells, possessing the ability to differentiate into osteogenic, chondrogenic, and adipogenic lines [6]. Information regarding the function of AT MSCs surrounding the heart and blood vessels is limited. Existing literature on this topic indicates that the AT surrounding the heart and blood vessels is a source of MSCs that are capable of differentiating into various cell types [7, 8]. It is also known that the paracrine activity of AT MSCs, which involves the production of growth factors and cytokines, has an effect on inducing angiogenesis and increasing the survival of cardiomyocytes [5].

The ability of AT MSCs to differentiate into osteoblasts secreting calcium salts into the extracellular space may be a link in the pathogenesis of vascular wall calcification. Currently, the role of AT MSCs in the formation of vascular calcification remains understudied. However, this area holds significant promise from both fundamental and clinical perspectives, since MSCs may not only be one of the key participants in the development of pathological calcification but also serve as a therapeutic target for regulating osteoblastic potential. Existing literature presents contradictory information regarding the osteogenic ability of AT MSCs. For example, A.B. Malashicheva et al.compared subcutaneous fat MSCs from healthy donors and patients with aortic valve calcification, revealing a reduced osteogenic potential in the MSCs of patients with aortic valve calcification [9]. Conversely, there is evidence that the ability of AT MSCs to undergo osteogenic differentiation depends on the tissue localization [10].

The aim of this study is to evaluate the osteogenic potential of MSCs derived from epicardial adipose tissue by assessing the expression levels of osteoblastic differentiation genes and their corresponding proteins in patients with stable coronary heart disease.

MATERIALS AND METHODS

The study included 5 patients with CHD, all under the age of 75,who signed a voluntary informed consent to participate. All patients had indications for open-heart surgery, specifically direct myocardial revascularization via coronary artery bypass grafting (CABG). The study did not include patients over the age of 75 and those with clinically

significant concomitant pathologies (such as type 1 and type 2 diabetes mellitus, myocardial infarction (MI), anemia, renal and liver failure, oncological and infectious-inflammatory diseases during exacerbation, autoimmune diseases). Epicardial adipose tissue (EAT) stem cells were isolated from biopsies of epicardial localization, with each biopsy weighing from 3 to 5 grams.The source of EAT was the right parts of the heart, particularly the regions with the highest presence, namely the right atrium and right ventricle. The obtained EAT samples were thoroughly washed with a sterile phosphate-buffered saline (PBS) (Gibco, USA) to remove erythrocytes, blood clots, and local anesthetics from the surface. Subsequently, the EAT was cut into small, irregularly shaped pieces (1–3 mm^2) using scissors, with an average weight of about 4 g per piece. The tissue pieces were then placed in 20 ml of PBS supplemented with penicillin (600 U/ml) (Gibco, USA) and streptomycin (300 mg/ml, Gibco, USA) in a 50 ml test tube for 5–10 minutes at room temperature to remove remnants of connective tissue and/or dermis, blood vessels. Following this, the small pieces of adipose tissue were pipetted into 25 cm^2 culture vials (Biologix, Germany). The cells were incubated in a CO_2 incubator (5% CO_2, 95% air, 37 °C), in a medium supporting the growth of MSCs (MesenCult Proliferation Kit, STEMCELL Technologies, Canada) with the addition of 100 U/ml penicillin and 100 U/ml streptomycin (Gibco, USA). When the primary cells reached 80–90% confluence, they were treated with 0.25% trypsin solution (Trypsin, PanEco, Russia) and transferred into 75 cm^2 culture vials (Biologix, Germany). The cells were then cultured to 80–90% cell fusion.

IMMUNOPHENOTYPING OF CELLS (FLOW CYTOMETRY)

To confirm that the resulting cell culture consisted of mesenchymal stem cells, their phenotype was assessed using a combination of conjugated monoclonal antibodies: CD90 FITC (BC, IM1839U), CD 34 APC (BC, PN IM2472U), CD73 APCCy7 (Biolegend, 344022), CD 105 PE (Biolegend, 323206). The phenotype CD34-, CD73+, CD 90+, CD 105+ was regarded as corresponding to the AT MSC according to the definition of the International Society for Cell Therapy (ISCT). The results are presented as a percentage of the number of cells exhibiting the corresponding antigens.

The gating strategy included the removal of duplicates on the FSC-H/FSC-A histogram, followed by the isolation of the studied population based on FSC/SSC parameters. According to the expression level of CD90 and CD73 receptors, the main cell population was isolated: CD90+CD73+ (79.47%). This population was subsequently examined for the presence of CD105 and CD34 membrane antigens. CD105 and CD90 were detected in 79.71% of the cells, while CD105 antigenic marker was present in 17.54% of the cells, and CD34 was found in only 3.76% of the cells. Thus, the phenotype of the cell culture obtained from the EAT was determined to be CD73+CD 90+CD105+CD34-/+

OSTEOGENIC DIFFERENTIATION OF MSCS OF EPICARDIAL ADIPOSE TISSUE

To perform osteogenic differentiation, cells from the 3rd passage of EAT were transferred into two T-75 vials. At the preparatory stage, human fibronectin (PanEco, Russia) was diluted to a concentration of 20 μg/ml in a single-use PBS and introduced into a 6-well tablet (for RNA isolation) and an 8-well Ibidi chamber (Germany) to ensure that the protein covered the culture plastic. Following this, the fibronectin was removed, and the PBS wells were washed (Gibco, China), cells were added in an appropriate volume. To start osteogenic cell differentiation, the MesenCult™ Osteogenic Differentiation Kit (Human, STEMCELL Technologies, Canada) was used. Cell differentiation process was conducted over a period of 15 days, with the osteogenic medium being replaced every 2–3 days. Cells that did not undergo osteogenic differentiation served as control samples.

DETERMINATION OF OSTEOGENIC POTENTIAL OF AT MSCS BY GENE EXPRESSION LEVEL VIA PCR

Total ribonucleic acid (RNA) extraction from MSCs was performed on day 15 of osteogenic differentiation to assess expression levels of a screening panel of osteogenic differentiation genes (quantitative polymerase chain reaction (PCR) after reverse transcription), including *RUNX2* (which encodes the transcription factor of the same name), *SP7* (which encodes Osterix transcription factor), *BGLAP* (which encodes osteocalcin), and *SPP1* (which encodes osteopontin). To do this, the medium was removed from the 6-well tablet, and each well was washed with 1 ml of single-use PBS.

Subsequently, 1 ml of Trizol was added to each well for 5 minutes and the MSC lysate was collected using a scraper. In total, there were three wells designated for osteogenically differentiated MSCs, while two wells were allocated for undifferentiated MSCs.

To isolate the RNA, the samples were placed in a cooled Trizol (*Extract RNA Reagent*, Eurogene, Russia) for 5 minutes. RNA was then extracted using guanidine-thiocyanate-chloroform extraction with Trizol (*Extract RNA Reagent*, Eurogene, Russia) according to the manufacturer's instructions. The amount and purity of the isolated RNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), while its quality was determined on a Qubit 4 fluorimeter (Invitrogen, USA) by evaluating the RIQ index (RNA Integrity and Quality) using a set of reagents Qubit RNA IQ Assay Kit (Invitrogen, USA). Reverse transcription and synthesis of complementary DNA (cDNA) from the isolated RNA were conducted using the OT-M-MuLV-RH kit (Biolabmix, Russia). The amount of synthesized cDNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). All samples were diluted in RNase and DNase-free water (*Sterile water treated with diethylpyrocarbonate (DEPC, DEPC), without RNase and DNase*, Biolabmix, Russia) to a volume of 1.5 ml, achieving a cDNA concentration of 20 ng / l.

The results of the analysis are presented as a relative expression values. To calculate these values, the ΔCt method (a variant of the Livak method) was used. This method is based on determining the difference between the Ct values of the reference genes and the target Ct values for each sample. Normalization of PCR results was performed relative to the geometric mean of the Ct values of three reference genes: *ACTB* (β-actin), *GAPDH* (glyceraldehyde-3-phosphatedehydrogenase), and *B2M* (beta-2-microglobulin).

Table 1

Primers used to evaluate the expression of osteogenic differentiation genes

Gene name	Primer	
	Forward	Reverse
Genes used for normalization in calculating expression levels		
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC
B2M	TCCATCCGACATTGAAGTTG	CGGCAGGCATACTCATCTT
ACTB	CATCGAGCACGGCATCGTCA	TAGCACAGCCTGGACAGCAAC
Genes of interest		
RUNX2	AGATGGACCTCGGGAACCCA	TGAGGCGGGACACCTACTCT
SP7	TGCTTGAGGAGGAAGTTCAC	AGGTCACTGCCCACAGAGTA
BGLAP	TCACACTCCTCGCCCTATTG	TAGCGCCTGGGTCTCTTCAC
SPP1	CATCACCTGTGCCATACCAGTT	TTGGAAGGGTCTGTGGGGCTA

Note: ACTB – β-actin, GAPDH-glyceraldehyde-3-phosphatedehydrogenase, B2M-beta-2-microglobulin, *RUNX2* (encodes the transcription factor of the same name), *SP7* (encodes Osterix transcription factor), BGLAP (encodes osteocalcin), SPP1 (encodes osteopontin).

IMMUNOFLUORESCENCE STAINING OF AT MSCS AND OSTEOBLASTS

For immunofluorescence staining, rabbit and mouse antibodies were selected as primary antibodies, including RUNX2, osteopontin, osteocalcin, Osterix, (Abcam, UK). Cells were stained with a blue fluorescent dye for nucleic acids (DAPI) (dilution 1:100), the detection of the results of the study was performed using a confocal microscope. Fluorescence intensity was analyzed using the ImageJ software. Ten visual fields were analyzed from each sample, and the results are presented as conventional units of fluorescence intensity.

Statistical analysis. Statistical data processing and graphical representation of the results were conducted using the standard package of statistical methods of IBM SPSS Statistics 27. The data are presented as median values along with the 25th and 75th percentiles: *Me* [25%; 75%]. The nonparametric Mann – Whitney U-test was used to evaluate differences in quantitative characteristics when comparing two independent groups with distributions that deviate from normality. A critical significance level of p is <0.05.

RESULTS

Evaluation of the immunophenotype of the EAT cell culture

The analysis revealed that CD105 and CD90 were expressed in 79.71% of the cells in the EAT of a patient with CHD. Notably, the surface marker CD105 was present in 17.54% of the cells (Fig. 1). Antigenic markers CD73 and CD90 were found in 79.47% of the cells, with CD73 being expressed on the surface of 18.26% of the cells. In contrast, CD34 was present only in 3.76% of the cells. Thus, the phenotype of the main cell culture derived from the EAT was CD34-/+, CD73+, CD 90+, CD 105+, which corresponds to one of the criteria of the AT MSC [11]. In addition to the main cell population, two minor populations were identified within the EAT culture: 1. CD90+,CD34+,CD73+,CD105 – presumably representing an endothelial population, 2. CD90+ CD105-CD34-CD73 – the smallest cell population.

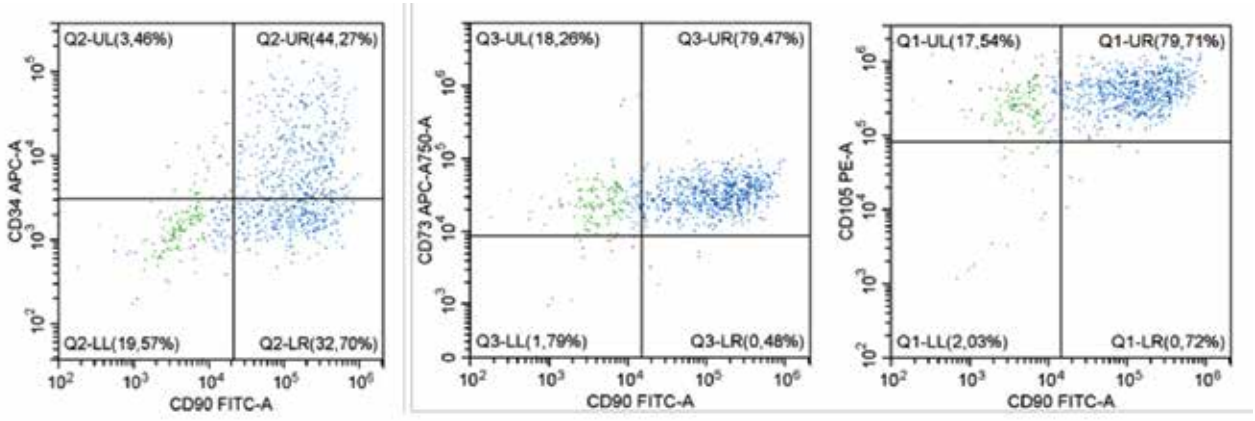

Fig. 1. Flow cytometry of cells derived from epicardial adipose tissue of a patient with coronary heart disease.
Note: Blue color in the figure indicates the largest cell population according to the immunophenotype belonging to the MSC, green and pink are two minor cell populations.

Expression of osteoblastic differentiation genes

The activation of transcription of genes involved in osteogenic induction was assessed on day 15 of culture, since during this period MSCs acquire specific properties of preosteoblasts and actively synthesize bone matrix proteins. Real-time PCR analysis revealed that the expression of the key osteogenic factor, the *RUNX2* gene, in cells cultured in a medium with osteoinducers was 1.88 times higher than in undifferentiated MSCs (Fig. 2).

Similar results were obtained for the expression level of *SPP1* mRNA (OPN, osteopontin), which, like *RUNX2*, is expressed at the early stages of mesenchymal cells differentiation into osteoblasts. Thus, the expression of the *SPP1* gene was found to be 1.35 times higher in MSCs cultured in the presence of an osteogenic medium compared to the control sample.

The expression of *BGLAP*, which encodes osteocalcin (OSN) and is responsible for the formation of mature osteoblasts, did not differ between differentiated and undifferentiated MSC cultures. The expression of the *SP7* (Osterix) gene, responsible for the differentiation of cells into mature osteoblasts and finally into osteocytes during bone formation, did not differ between cells cultured in osteoblastic medium and those without it.

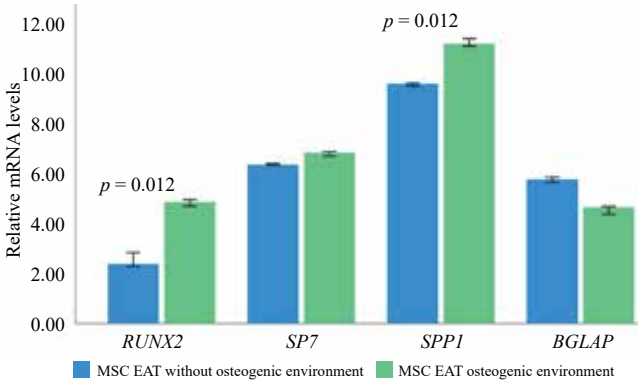

Fig. 2. Expression levels of osteogenic genes in differentiated and undifferentiated mesenchymal stem cells of epicardial adipose tissue of patients with coronary heart disease on day 15 of culture. Note: MSC – Mesenchymal stem cells, EAT – epicardial adipose tissue, *RUNX2* – encodes the transcription factor of the same name, *SP7* – encodes the transcription factor Osterix, *BGLAP* – encodes osteocalcin, *SPP1* – encodes osteopontin

Immunofluorescence staining

Based on the results of staining cells with specific antibodies, an analysis was conducted to evaluate the effect of directed osteogenic differentiation on the culture of MSCs derived from EAT. It was shown that a significant ($p < 0.05$) increase in half of the studied markers was observed in the culture of MSCs of EAT obtained on day 15 after incubation with an osteoblastic medium compared to control MSCs (Fig. 3, 4). Thus, the key regulator and marker of osteogenic differentiation – RUNX2 – was 1.6 times higher in osteogenic-induced MSCs than in intact (control) cell cultures. Another marker of early osteogenic differentiation – OPN – was also increased by 1.6 times in differentiated MSCs. It is worth noting that by the method of immunofluorescence staining, there were no differences detected in the expression of Osterix and OCN between cultures of differentiated and undifferentiated cells (Fig. 3, 4).

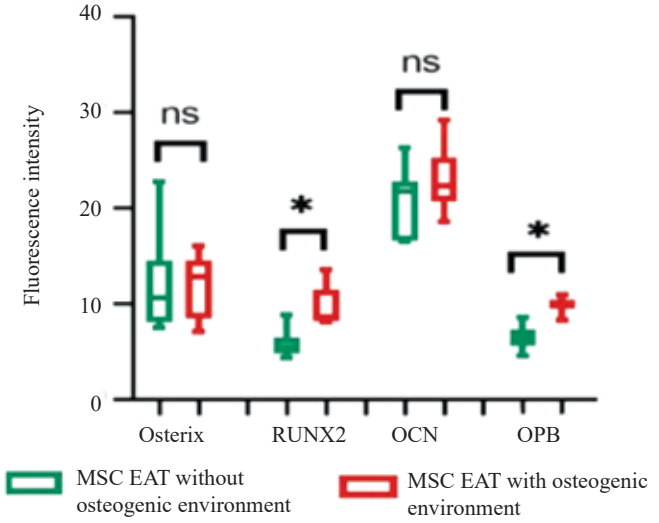

Fig. 3. Quantitative analysis of osteogenic differentiation markers of MSCs of EAT in the 3rd passage (day 15) by immunofluorescence staining: MSC – Mesenchymal stem cells, EAT – epicardial adipose tissue, OCN – osteocalcin, OPN – osteopontin

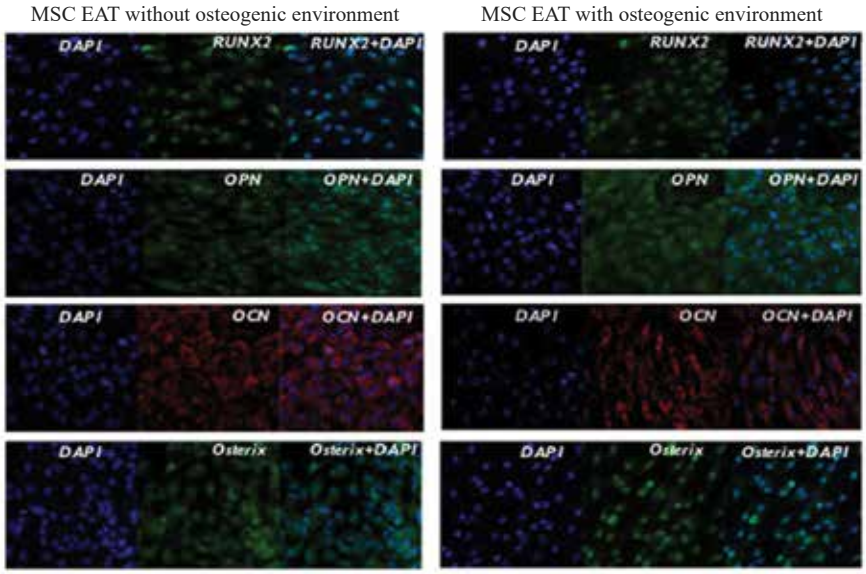

Fig. 4. Immunofluorescence staining of the studied osteoblastic differentiation markers. Note: DAPI – nuclear dye (4',6-diamidino-2-phenylindole), OCN – osteocalcin, OPN – osteopontin.

DISCUSSION

The study of the osteo-cardiovascular continuum represents a relatively new and promising area of contemporary scientific research. Recent studies have shown that during vascular calcification, various signaling pathways associated with bone formation and repair are activated in cells. For example, G. Fadini et al. have shown that cells derived from bone marrow can migrate from blood circulation to blood vessels, transform into osteogenic cells, and then contribute to the development of vascular calcification [12]. With the potential for osteogenic differentiation and recruitment of damaged vessels,

MSCs play a crucial role in the "circulating calcifying cell theory," suggesting that they may serve as a source of osteoblast-like cells. However, the role of MSC in the process of vascular calcification remains unclear and controversial. The question of whether MSCs contribute to or inhibit the development of vascular calcification is still to be determined.

In this study, we focused on the activation of transcription genes involved in osteogenic induction on day 15 of culturing MSCs derived from EAT, since it is known that multipotent cells acquire specific properties of preosteoblasts and begin to actively synthesize bone matrix proteins during this period [13, 14].

It is known that the maturation and function of osteoblasts are directly related to the expression of two main transcription factors of osteogenesis: *RUNX2* and *SP7* transcription factor (Osterix). The *RUNX2* transcription factor is an important regulator of bone formation and the osteogenic differentiation of MSCs; it initiates differentiation of MSCs into preosteoblasts and suppresses adipogenic and chondrogenic differentiation [15]. During osteoblast differentiation, *RUNX2* expression increases in preosteoblasts, reaches a maximum level in progenitor cells, and decreases in mature osteoblasts [16]. *RUNX2* activates the expression of calcification-related proteins such as osteopontin, bone sialoprotein II, and osteocalcin, thus inducing extracellular bone matrix synthesis and mineralization.

According to the literature, *RUNX2* is slightly expressed in undifferentiated MSCs and increases during the proliferation of preosteoblasts, which corresponds to day 7 of culture in an osteogenic medium. Its expression level is maintained at a relatively low level throughout the entire period of osteocyte differentiation. RT-PCR analysis has shown that the expression of the *RUNX2* gene increases after the day 7 of cultivation and reaches peak values on day 21 [17]. In this study, it was shown that the level of *RUNX2* mRNA in cells cultured in an osteogenic medium was 66% higher than in undifferentiated MSCs. According to the results of the immunofluorescence study, it was also shown that RUNX2 protein levels were higher in differentiated cell culture.

The second most important factor (after RUNX2) inducing osteoblast differentiation and the synthesis of bone-specific proteins is Osterix [18]. Both of these factors regulate the activation cascade of genes encoding bone-specific proteins that form bone tissue. [19]. The expression of *SP7* is necessary for the differentiation of preosteoblasts into mature and functional osteoblasts. However, one of the most important functions of this protein is its ability to inhibit the differentiation of chondrocytes in RUNX2-expressing osteoblast precursors [20]. In this study, the expression level of the *SP7* gene did not differ between differentiated and undifferentiated MSCs. Immunofluorescence staining of this osteogenesis marker also revealed no significant difference in Osterix levels between cultures of differentiated and undifferentiated cells. At the same time, the expression levels of *SP7* mRNA and Osterix protein were relatively high in MSCs incubated both with and without osteoblastic medium. It is possible that the absence of a significant difference between differentiated and undifferentiated MSCs may be attributed to the fact that, according to the literature, an increase in *SP7* gene expression is noted at a later stage, approximately on day 16-21 of osteoblast formation (the phase of extracellular matrix synthesis) [21].Osteopontin (OPN) is one of the main non-collagenous bone proteins and plays an important role in bone remodeling. OPN not only mediates the early differentiation of osteoblasts but also activates the function of osteoclasts during resorption. A high level of expression of the *SPP1* gene encoding OPN synthesis indicates an active process of bone extracellular matrix formation, since OPN is the main non-collagenous bone protein. The maximum activity of OPN corresponds to the stage of mineralization in the process of osteogenesis [22]. The results of this study are consistent with existing data indicating that the peak of *SPP1* expression during osteoblast differentiation is achieved twice: during proliferation and mineralization, which corresponds to days 3 and 14 of differentiation. Using confocal microscopy on day 15 of osteoblastic differentiation, it was also found that OPN levels were higher in differentiated cells. In addition, the *SSP1* gene expression and the OPN level obtained by immunofluorescence test were higher than other studied osteogenic markers. These data may indicate that the process of osteogenesis in cell cultures is in the initial stage of extracellular matrix synthesis.

The next step was to evaluate the expression of the *BGLAP* gene encoding OCN. Its level was lower than that of other studied markers, which is consistent with the literature. OCN performs a mechanical

function in the bone matrix due to its ability to firmly bind hydroxyapatite and form a complex with collagen through the osteopontin matrix protein [23]. OCN is used as a late marker of bone formation, as it is expressed at the later stages of extracellular matrix mineralization by mature osteoblasts, which corresponds to day 16-21 of differentiation. This may explain why, in this study, we did not observe an increase in the levels of the *BGLAP* gene and OCN protein in cultures of differentiated MSCs.

CONCLUSION

It was found that MSCs derived from EAT have osteogenic potential, which is manifested by the expression of osteogenic differentiation genes in both differentiated and undifferentiated MSC cultures. These data can be the basis for further study of EAT-derived MSCs from the perspective of their role in the formation of vascular calcification in patients with coronary heart disease. The high level of *SPP1* expression, along with relatively low levels of *RUNX2* and *BGLAP* in differentiated cultures, may indicate that on day 15 of incubation, EAT-derived MSCs are preosteoblasts and are at the initial stage of extracellular matrix synthesis.

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Authors' contribution

Uchasova E.G., Gruzdeva O.V., Dyleva Yu.A. – development of the concept and design of the study. Uchasova E.G., Slesareva T.A., Ponasenko A.V., Velikanova E.A., Matveeva V.G., Dvadtsatov I.V. – data analysis and interpretation. Uchasova E.G, Dyleva Yu.A., Belik E.V., Gruzdeva O.V. – substantiation of the manuscript or critical revision of the manuscript for important intellectual content. Uchasova E.G., Slesareva T.A., Tarasova O.L., Gruzdeva O.V. – final approval of the manuscript for publication.

Authors' information

Uchasova Evgenia G. – Cand. Sci. (Med.), Senior Researcher, Laboratory for Homeostasis Studies, Research Institute for Complex Issues of Cardiovascular Diseases, Kemerovo, Russia, evg.uchasova@yandex.ru, https://orcid.org/0000-0003-4321-8977

Dyleva Yulia A. – Cand. Sci. (Med.), Senior Researcher, Laboratory for Homeostasis Studies, Research Institute for Complex Issues of Cardiovascular Diseases, Kemerovo, Russia, dyleva87@yandex.ru, https://orcid.org/0000-0002-6890-3287

Slesareva Tamara A. – Doctor of Clinical Laboratory Diagnostics, Research Institute of Complex Problems of Cardiovascular Diseases. Postgraduate student, Department of Pathological Physiology, Kemerovo State Medical University, Kemerovo, Russia, soloveva081296@mail.ru, https://orcid.org/0000-0003-0749-4093

Belik Ekaterina V. – Cand. Sci. (Med.), Researcher, Laboratory for Homeostasis Studies, Research Institute for Complex Issues of Cardiovascular Diseases, Kemerovo, Russia, sionina.ev@mail.ru, https://orcid.org/ 0000-0003-3996-3325

Panasenko Anastasia V. – Cand. Sci. (Med.), Head of the Laboratory of Genomic Medicine, Research Institute for Complex Issues of Cardiovascular Diseases, Kemerovo, Russia, PonaAV@kemcardio.ru, https://orcid.org/ 0000-0002-3002-2863

Velikanova Elena A. – Cand. Sci. (Med.), Researcher, Laboratory of Cell Technologies, Research Institute for Complex Issues of Cardiovascular Diseases Federal State Budgetary Institution, Kemerovo, Russia, veliea@kemcardio.ru, https://orcid.org/0000-0002-1079-1956

Matveeva Vera G. – Cand. Sci. (Med.), Senior Researcher, Laboratory of Cell Technologies, Research Institute for Complex Issues of Cardiovascular Diseases, Kemerovo, Russia, matveeva_vg@mail.ru, https://orcid.org/0000-0002-4146-3373

Dvadtsatov Ivan V. – Cand. Sci. (Med.), Cardiovascular Surgeon, Research Institute for Complex Issues of Cardiovascular Diseases, Kemerovo, Russia, dvadiv@kemcardio.ru, https://orcid.org/0000-0003-2243-1621

Tarasova Olga L. – Cand. Sci. (Med), Associate Professor, Head of the Department of Pathological Physiology, Kemerovo State Medical University, Kemerovo, Russia, pathophysiology_kaf@mail.ru, https://orcid.org/0000-0002-7992-645X

Gruzdeva Olga V. – Dr. Sci. (Med.), Professor of the Russian Academy of Sciences, Head of the Laboratory for Homeostasis Studies, Research Institute for Complex Issues of Cardiovascular Diseases, Head of the Department of Medical Biochemistry, Kemerovo State Medical University, Kemerovo, Russia, o_gruzdeva@mail.ru, https://orcid.org/0000-0002-7780-829X

(✉) **Uchasova Evgenia G.,** evg.uchasova@yandex.ru

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Analysis of the relationship between low-grade inflammation markers and the severity of atherosclerotic coronary bed lesions

Ushakov A.V.[1], Zakharyan E.A.[1], Grigoriev P.E.[2,3], Malyi K.D.[1]

[1] The Order of the Red Banner of Labour Medical Institute named after S.I. Georgievsky of the V.I. Vernadsky Crimean Federal University (MI CFU)
5/7, Lenina Blvd., Simferopol, 295051, Russian Federation

[2] Sevastopol State University (SevSU)
33 Universitetskaya Str., Sevastopol, 299053, Russian Federation

[3]Academic Scientific Research Institute of Physical Methods of Treatment, Medical Climatology and Rehabilitation named after I.M.Sechenov (Sechenov ASRI)
10/3 Mukhina Str., Yalta, 298603, Russian Federation

ABSTRACT

Aim. To study serum concentrations of low-grade inflammation markers and the severity of atherosclerotic processes in the coronary artery in patients with coronary heart disease (CHD) in the context of their clinical and instrumental characteristics.

Materials and methods. The study included 264 participants (161 men and 103 women), with 220 of them being diagnosed with CHD. Subgroups were identified among the participants, including those with a history of myocardial infarction (110 patients) and angina pectoris (152 patients). A control group consisted of healthy volunteers (44 persons). The patients underwent coronary angiography, echocardiography, duplex ultrasound scanning of the extracranial segments of the brachiocephalic arteries. The level of C-reactive protein (CRP (mg / l)), tumor necrosis factor alpha (TNFα (pg/ml)), growth differentiation factor 15 (GDF-15 (pg/ml)), and endothelial cell specific molecule-1 (ESM-1 (ng/ml)) in the blood serum were measured. Statistical significance was considered at $p < 0.05$.

Results. A significantly higher concentration of all laboratory markers of low-grade inflammation in the CHD group of patients compared to the control group, as well as a significant increase in their values with enhanced severity of coronary atherosclerosis ($p < 0.0001$) was found. Significant differences in marker levels were also found between patients with angina pectoris and a history of myocardial infarction compared to those without these conditions. A correlation was revealed between the value of markers and various clinical and instrumental characteristics of the patients. Multivariate linear regression analysis revealed a statistically significant association of SYNTAX score with the concentration of GDF-15 and ESM-1, but not with CRP and TNFα.

Conclusion. The simultaneous measurement of multiple laboratory parameters may be a more effective method for assessing the risk of CHD progression. The study also showed that endocan and GDF-15 have high prognostic significance in evaluating the severity of atherosclerotic processes in the coronary arteries.

Keywords: inflammation, C-reactive protein, tumor necrosis factor alpha, growth differentiation factor 15, specific molecule of endothelial cells-1, endocan, atherosclerosis, coronary heart disease

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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✉ *Zakharyan Elena A.,* locren@yandex.ru

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Анализ взаимосвязи маркеров низкоинтенсивного воспаления с выраженностью атеросклеротического поражения коронарного русла

Ушаков А.В.[1], Захарьян Е.А.[1], Григорьев П.Е.[2, 3], Малый К.Д.[1]

[1] Ордена Трудового Красного Знамени Медицинский институт им. С.И. Георгиевского, Крымский федеральный университет им. В.И. Вернадского» (МИ КФУ)
Россия, 295051, г. Симферополь, бульвар Ленина, 5/7

[2] Севастопольский государственный университет (СевГУ)
Россия, 299053, г. Севастополь, ул. Университетская, 33

[3] Академический научно-исследовательский институт физических методов лечения, медицинской климатологии и реабилитации им. И.М. Сеченова (АНИИ им. И.М.Сеченова)
Россия, 298603, г. Ялта, ул. Мухина, 10/3,

РЕЗЮМЕ

Цель. Изучение сывороточных концентраций маркеров низкоинтенсивного воспаления у пациентов с ишемической болезнью сердца (ИБС) в контексте их клинико-инструментальных характеристик, а также оценка их предиктивной ценности в выраженности атеросклеротических процессов коронарного русла.

Материалы и методы. В исследование включены 264 человека (161 мужчина и 103 женщины), из них 220 – пациенты с диагнозом ИБС. Среди пациентов были выделены подгруппы с наличием инфаркта миокарда в анамнезе (110 человек) и стенокардией (152 человека). Группа контроля представлена здоровыми добровольцами (44 человека). Пациентам выполнены коронароангиография; эхокардиографическое исследование; дуплексное ультразвуковое сканирование внечерепных отделов брахиоцефальных артерий. Проведено исследование уровня С-реактивного белка (СРБ, мг/л), фактора некроза опухоли альфа (ФНО-α, пг/мл), фактора дифференцировки роста 15 (GDF-15, пг/мл) и специфической молекулы эндотелиальных клеток-1 (ESM-1, нг/мл) в сыворотке крови. Статистически значимыми считали различия при $p < 0,05$.

Результаты. Выявлена значимо большая концентрация всех лабораторных маркеров субклинического воспаления в группе пациентов с ИБС в сравнении с контролем, а также значимое повышение их значений по мере увеличения выраженности коронарного атеросклероза ($p < 0,0001$). Показана статистическая значимость различий уровня маркеров между группами пациентов с наличием стенокардии и инфаркта миокарда в анамнезе в сравнении с пациентами без данных признаков. Выявлена корреляционная связь разной силы и значимости между значением маркеров и рядом клинико-инструментальных характеристик пациентов. При проведении линейного многофакторного регрессионного анализа выявлена статистически значимая связь баллов по шкале SYNTAX с концентрацией GDF-15 и ESM-1 при отсутствии таковой с СРБ и ФНО-α.

Заключение. Одновременное количественное определение нескольких лабораторных показателей может быть более мощным инструментом для оценки риска прогрессирования ИБС. Показано, что эндокан и GDF-15 имеют высокую предиктивную значимость в оценке выраженности атеросклеротических процессов в коронарных артериях.

Ключевые слова: воспаление, С-реактивный белок, фактор некроза опухоли альфа, фактор дифференцировки роста 15, специфическая молекула эндотелиальных клеток-1, эндокан, атеросклероз, ишемическая болезнь сердца

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 дали добровольное информированное согласие на участие в исследовании. Исследование одобрено этическим комитетом ФГАОУ ВО «КФУ им. В.И. Вернадского» (протокол № 5 от 19.05.2022).

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INTRODUCTION

In recent years, inflammation has been recognized as a critical component in the pathogenesis of cardiovascular diseases. Numerous studies have explained the complex relationship between inflammation and coronary heart disease (CHD) [1]. Chronic low-grade inflammation, in particular, has been recognized as a factor contributing to the onset and progression of various cardiovascular diseases, including atherosclerosis, acute coronary syndrome, and heart failure [1]. Moreover, arterial hypertension, dyslipidemia, diabetes, and obesity are associated with low-grade inflammatory processes [2].

Numerous studies have demonstrated the role of C-reactive protein (CRP) and tumor necrosis factor-alpha (TNFα) as markers of low-grade inflammation in diseases of the circulatory system [1–7]. There are also isolated studies devoted to the study of a similar role of such indicators as growth differentiation factor 15 (GDF-15) and endothelial cell specific molecule-1, or endocan (endothelial cell specific molecule-1, ESM-1) [8–11].

GDF-15, a member of the transforming growth factor beta superfamily, is a marker of inflammation and apoptosis of cells, primarily atypical ones. Its expression is induced in macrophages by interleukin-1 and TNFα, leading to inhibition of both their activation and the inflammatory reaction itself [8]. In turn, a number of studies have shown that ESM-1, being a surrogate marker of inflammation and endothelial dysfunction, plays a crucial role in the processes of angiogenesis, inflammation, and vascular permeability [11].

As evidenced by the foregoing, it seems relevant to study the relationship between serum concentrations of the listed laboratory markers in patients with CHD and to assess their predictive ability in the progression of coronary atherosclerosis.

The aim of the study was to investigate the relationship between serum concentrations of low-grade inflammation markers and the severity of atherosclerotic processes in the coronary bed in patients with CHD in the context of their clinical and instrumental characteristics.

MATERIALS AND METHODS

The inclusion criterion for the patients into the study was the presence of clinically and instrumentally verified CHD. *The exclusion criteria* were: myocardial infarction (MI) or stroke occurred within the past 6 weeks; any acute and chronic inflammatory diseases that can affect serum concentrations of CRP, TNFα, GDF-15 and ESM-1; chronic kidney disease ≥ stage III (glomerular filtration rate <60 ml/min/1.73 m^2); primary and secondary cardiomyopathy, inflammatory heart diseases; oncological diseases, blood diseases and immune system diseases; pregnancy or lactation; mental disorders that hinder the contact with the patient during the cancer; and violation of the protocol or patient's refusal to participate in the study.

A total of 264 people (161 men and 103 women) were enrolled in this study, including 220 patients with an established diagnosis of CHD and 44 healthy volunteers (the control group).

All patients underwent coronary angiography using the General Electric Optima IGS 330 angiographic system. The SYNTAX score, an online calculator (https://officialsyntaxscore.com), was used for an objective quantitative assessment of the severity of atherosclerotic lesions in coronary arteries (CA). Considering that this score is a reliable tool for determining the severity of CA atherosclerosis, all patients were divided into the following groups: Group 1 – with moderate atherosclerotic CA lesions, having a SYNTAX score of 22 or less (124 patients); Group 2 – with severe CA atherosclerosis, having a score of 23–32 (53 patients); Group 3 – with extremely severe CA lesions, having a score of 33 or more (43 patients). Among the participants with CHD, several subgroups were identified: patients who underwent percutaneous coronary intervention (stenting) within the past 4 months to 6 years – 45 persons, patients with multifocal atherosclerosis (MFA) – 46 persons, patients with a history of MI –110 persons, and patients with angina pectoris – 152 persons. The control group (Group 4) consisted of 44 healthy volunteers, in whom cardiovascular pathology was excluded due

to the absence of any clinical, anamnestic, or electrocardiographic signs of heart disease. All groups were comparable in terms of sex and age.

Echocardiographic examination (EchoCG) was performed using the Samsung Accuvix A30 ultrasound scanner (Samsung-Medison, South Korea), using two-dimensional EchoCG, Doppler EchoCG in pulsed and continuous wave modes, and color Doppler scanning. Standard structural parameters of the ventricles and atria, contractile and diastolic function of the left ventricle (LV), and valvular apparatus competency were evaluated. The intima-media thickness (IMT) was measured using duplex ultrasound scanning of the extracranial sections of the brachiocephalic arteries with the Samsung UGEO H60 ultrasound scanner (Samsung-Medison, South Korea).

The study of the concentrations of CRP, TNFα, GDF-15, and ESM-1 in the blood serum was also performed. For this purpose, venous blood was collected on an empty stomach before coronary angiography. Commercial test systems manufactured by Cloud Clone, USA (TNFα, pg/ml; GDF-15, pg/ml), Biomerica, USA (CRP, mg/l), and Aviscera, USA (ESM-1, ng/ml) were used.

The statistical processing of the study results was carried out using the STATISTICA 12.0 and MedStat programs. The data were presented as a median (Me) and interquartile interval (Q – 25th and 75th percentiles). The Mann-Whitney U-test was used to test statistical hypotheses when comparing two independent groups. The Kruskal – Wallis test was used for multiple comparisons in independent samples for quantitative or ordinal data. The Mann – Whitney test with the Bonferroni correction was used as a posteriori criterion for pairwise comparisons. The multiple comparisons of the proportions of nominal features in independent samples were performed using the Pearson's chi-square test. The Marascuilo procedure was used as a posteriori criterion for pairwise comparisons. The statistical relationship between two features was measured using Spearman's rank correlation. The multivariate linear regression analysis was performed to estimate the dependence of atherosclerotic lesions in the CAs according to the SYNTAX score on laboratory parameters of low-grade inflammation. The critical significance level p for all used analysis procedures was set at 0.05.

RESULTS

The clinical, anamnestic, and laboratory-instrumental characteristics of the patients enrolled in the study are presented in Table 1.

Table 1

Clinical, anamnestic, and laboratory-instrumental characteristics of the patients

Parameter	Group 1, $n = 124$	Group 2, $n = 53$	Group 3, $n = 43$	p	p_{1-2}	p_{1-3}	p_{2-3}
Age (years), *Me* (Q_{25}; Q_{75})	64.0 [58.0; 69.0]	66.0 [60.0; 70.0]	66.0 [60.0; 70.0]	0.882	0.961	0.778	1.000
SYNTAX, (score), *Me* (Q_{25}; Q_{75})	12.25 [5.0; 17.0]	27.5 [24.0; 29.5]	36.25 [34.0; 40.5]	<0.001	<0.001	<0.001	<0.001
LVEF, %, *Me* (Q_{25}; Q_{75})	57.0 [49.0; 62.0]	54.0 [47.0; 59.0]	52.0 [44.0; 59.0]	0.063	0.419	0.091	1.000
Angina, *n* (%)	73 (58.9)	42 (79.2)	37 (86.0)	0.004	0.063	0.026	0.973
Angina class 2, *n* (%)	25 (20.2)	12 (22.6)	6 (14)	0.547	–	–	–
Angina class, 3 *n* (%)	49 (39.5)	29 (54.7)	27 (62.8)	0.016	0.237	0.047	0.840
Angina class 4, *n* (%)	–	–	4 (9.3)	<0.001	1.000	0.039	0.174
NYHA class II, *n* (%)	44 (35.5)	14 (26.4)	6 (14.0)	0.110	–	–	–
NYHA class III, *n* (%)	80 (64.5)	39 (73.6)	31 (72.0)	0.793	–	–	–
NYHA class IV, *n* (%)	–	–	6 (14.0)	<0.001	1.000	0.003	0.033
History of MI, *n* (%)	45 (36.3)	41 (77.4)	25 (58.1)	<0.001	<0.001	0.069	0.198
IMT, cm, *Me* (Q_{25}; Q_{75})	0.8 [0.8; 0.9]	1.05 [0.9; 1.1]	0.9 [0.8; 1.0]	0.024	0.030	0.641	0.972

Note. IMT – intima-media thickness; LVEF – left ventricular ejection fraction; MI – myocardial infarction.

The study revealed a significantly higher concentration of all laboratory markers of low-grade inflammation in patients with CHD compared to the control group (Table 2).

When examining the levels of laboratory markers in the blood serum of patients in three groups according to the SYNTAX score, a statistically significant enhance in the concentration of CRP, TNFα, GDF-15, and ESM-1 was found as the severity of coronary atherosclerosis increased (Table 3).

It is necessary to note the statistical significance of the differences in the concentration of low-grade inflammation markers in the blood serum depending on the presence of certain clinical signs (Tables 4, 5).

Also noteworthy are the discovered correlations between the concentration of low-grade inflammation markers in the blood serum and several clinical and instrumental indicators (Table 6).

Table 2

Laboratory values in patients with coronary heart disease and the control group

Parameter	Patients with CHD (n = 220)	Control group (n = 44)	p
CRP, mg/l	7.73 [6.29; 9.21]	3.22 [2.15; 3.76]	<0.001
TNFα, pg/ml	4.6 [3.6; 5.8]	1.4 [1.1; 2.7]	<0.001
GDF-15, pg/ml	723 [579; 912]	405 [291; 591]	<0.001
ESM-1, ng/ml	18.95 [11.51; 26.13]	5.97 [4.38; 8.25]	<0.001

Table 3

Laboratory values in patients of three groups

Parameter	Group 1 (n = 124)	Group 2 (n = 53)	Group 3 (n = 43)	p	p_{1-2}	p_{1-3}	p_{2-3}
CRP, mg/l	7.23 [5.64; 7.86]	8.49 [7.99; 9.15]	9.99 [9.32; 11.63]	<0.001	<0.001	<0.001	0.023
TNFα, pg/ml	4.0 [3.2; 4.8]	5.5 [4.7; 5.9]	7.05 [5.2; 7.8]	<0.001	<0.001	<0.001	0.102
GDF-15, pg/ml	613.0 [422.5; 695.5]	891.0 [800; 944]	1245.0 [1100; 1400]	<0.001	<0.001	<0.001	<0.001
ESM-1, ng/ml	14.40 [10.19; 19.91]	20.31 [12.75; 24.12]	32.10 [22.12; 38.21]	<0.001	0.039	<0.001	<0.001

Note. The Kruskal–Wallis test was used for comparisons of the quantitative or ordinal data.

Table 4

Laboratory values in patients depending on the presence of angina

Parameter	Patients with angina (n = 152)	Patients without angina (n = 68)	p
CRP, mg/l	8.15 [6.84; 9.56]	7.44 [5.24; 8.12]	0.003
TNFα, pg/ml	5.0 [3.8; 6.1]	4.2 [3.4; 5.4]	0.006
GDF-15, pg/ml	789 [632; 979]	656.5 [500; 842]	<0.001
ESM-1, ng/ml	20.05 [13.38; 29.57]	13.29 [9.23; 20.05]	<0.001

Table 5

Laboratory values in patients depending on the presence of a history of myocardial infarction

Parameter	Patients with history of MI (n = 110)	Patients without history of MI (n = 110)	p
CRP, mg/l	8.33 [6.89; 9.32]	7.44 [6.21; 8.45]	0.039
TNFα, pg/ml	5.1 [3.9; 6.1]	4.3 [3.3; 5.4]	0.004
GDF-15, pg/ml	866 [690; 980]	633 [497; 800]	<0.001
ESM-1, ng/ml	20.605 [14.78; 30.10]	12.105 [6.78; 19.21]	<0.001

Table 6

Evaluation of statistical relationships between clinical, instrumental, and laboratory parameters using the Spearman's *R* rank correlation coefficient

Parameter	R (p-value)			
	CRP	TNFα	GDF-15	ESM-1
SYNTAX score	+0.487 (<0.001)	+0.573 (<0.001)	–0.830 (<0.001)	+0.474 (<0.001)
IMT	+0.178 (0.184)	+0.288 (0.030)	–0.499 (<0.001)	+0.436 (<0.001)
LVEF	–0.092 (0.174)	–0.125 (0.064)	–0.210 (0.002)	–0.197 (0.003)
FC of angina	+0.236 (<0.001)	+0.233 (<0.001)	+0.434 (<0.001)	+0.443 (<0.001)
FCHF by NYHA	+0.153 (0.023)	+0.220 (<0.001)	+0.307 (<0.001)	+0.110 (0.106)
Mounts passed after MI	+0.222 (0.625)	+0.198 (0.102)	+0.367 (0.683)	+0.261 (0.270)

Note. HF – heart failure.

The multivariate linear regression analysis was performed to estimate the dependence of the atherosclerotic lesions of the CAs according to SYNTAX score on laboratory indices of low-grade inflammation. This model was shown to be acceptable for prediction. This is evidenced by the highly significant value of the Fisher's criterion: $F = 76.138$ ($p < 0.00001$). The multiple correlation coefficient was 0.7686, while the adjusted determination coefficient was 0.5830. The Durbin – Watson coefficient was 1.9168, which is close to 2 and indicates the absence of autocorrelation in the residuals and the adequacy of the constructed model.

While analyzing the regression results, it should be noted that among the measured laboratory markers, a statistically significant relationship between the SYNTAX score was found only for GDF-15 and, to a lesser extent, for ESM-1 (Table 7), in contrast to CRP and TNFα.

Subsequently, after recalculating everything with the inclusion of only two indicators (GDF-15 and ESM-1), the model still appears acceptable for forecasting, The Fisher's criterion value was $F = 126.30$, ($p < 0.00001$); the multiple correlation coefficient was 0.7358; the adjusted coefficient of determination was 0.5371; and the Durbin – Watson coefficient was 1.9605.

Table 7

Results of the multivariate linear regression analysis of the relationship between SYNTAX score and laboratory parameters

Parameter	β	Standard error β	*b*	Standard error *b*	*p*
Intercept	–	–	–6.00057	1.694136	0.000489
GDF-15, pg/ml	0.650666	0.051819	0.02600	0.002070	<0.000001
ESM-1, ng/ml	0.136217	0.049111	0.15113	0.054489	0.006040
CRP, mg/l	0.075386	0.045461	0.20774	0.125274	0.098748
TNFα, pg/ml	0.071769	0.046442	0.23795	0.153976	0.123760

Table 8

Results of the multivariate linear regression analysis of the relationship between SYNTAX score and GDF-15 and endocan values

Parameter	β	Standard error β	*b*	Standard error *b*	*p*
Intercept	–	–	–2.35452	1.546611	0.129391
GDF-15, pg/ml	0.652809	0.051894	0.02430	0.001932	0.000000
ESM-1, ng/ml	0.154705	0.051894	0.17061	0.057228	0.003204

DISCUSSION

When studying cardiovascular risk factors, the relationship between atherosclerotic and inflammatory processes becomes obvious. A persistent increase in inflammation markers is closely associated with the development of adverse cardiovascular events caused by the rupture of atherosclerotic plaques [2].

It is necessary to note a significant number of studies examining the role of CRP and TNFα as representative laboratory markers for predicting major cardiovascular events [3]. Thus, more than twenty years ago, P.M. Ridker et al. showed that the inclusion of CRP and lipids is more effective in predicting the risk of MI compared to models using lipids only. Additionally, initial CRP levels predicted the risk of MI even in individuals with low total cholesterol or a high total cholesterol/high-density lipoproteins ratio [12]. In particular, the Reynolds scales were developed to estimate the risk of adverse cardiovascular events during a 10-year period in both women (Reynolds Risk Score) and men (Reynolds Risk Score for men), which included, among other things, the CRP level [13]. Of particular interest is a 2018 study involving 7,382 persons to confirm a new risk scoring system that incorporated factors such as CRP levels and quantification of calcium in the coronary arteries. This model, known as Astronaut Cardiovascular Health and Risk Modification (Astro-CHARM), surpassed traditional scales, making it a potentially valuable tool for making risk-based decisions for the prevention of cardiovascular diseases [14].

In turn, it is well known that TNFα activates endothelial cells and induces the expression of cytokines and chemokines by monocytes/

macrophages, which lead to the progression of atherosclerotic processes. Apoptosis of endothelial cells plays an important role in the development of atherosclerosis [15]. TNFα induces apoptosis of endothelial cells by enhancing autophagy and promotes their premature aging [16]. It has been extensively studied that TNFα suppresses the regulation of the eNOS gene, leading to a decrease in the production of nitric oxide (NO) and, as a result, endothelial proliferation and inhibition of endothelium-dependent vasodilation [17].

A number of studies have demonstrated that TNFα causes endothelial dysfunction, promotes the formation of foam cells, angiogenesis, proliferation of smooth muscle cells, and thrombosis [18, 19].

However, there is growing interest in studying new laboratory markers of low-grade inflammation, such as GDF-15 and endocan. It is the ability of cardiomyocytes to produce GDF-15 in response to stress that underlies the diagnostic value of this marker. L. Lind et al. [20] studied the intima-media thickness and the plaque height in the carotid arteries using ultrasound and found that the proportion of thickened atherosclerotic plaques increased with the higher levels of GDF-15. A. Rohatgi et al. [21] demonstrated a positive correlation between GDF-15 and signs of subclinical coronary atherosclerosis and mortality. The data obtained in eight studies from an examination of 4,126 patients with heart failure demonstrated an association between excessive expression of GDF-15 and an increased risk of death [22]. Moreover, GDF-15 meets the criteria of R.S. Vasan (2006) as a biological marker of increased cardiovascular risk [23].

In turn, endocan expression in endothelial cells increases in response to inflammatory triggers such as lipopolysaccharides and cytokines (TNFα, transforming growth factor β1, fibroblast growth factor 2, interleukin-1β, hypoxia-inducible factor 1α), and decreases with interferon γ [24, 25]. Endocan also enhances the production of proinflammatory cytokines by endothelial cells, the expression of adhesion molecules, and the adhesion between monocytes and endothelial cells. In addition, endocan-activated adhesion molecules can secrete potent chemokines such as IL-8 and monocyte chemoattractant protein-1, which are necessary for the inflammatory response and contribute to the progression of atherosclerosis [26]. Several studies have investigated the role of endocan as a biomarker for predicting the severity of CHD using the Gensini and SYNTAX score, which take into consideration the anatomy, morphology, and severity of coronary artery stenosis and are widely used in clinical practice to choose the optimal type of treatment and predict overall cardiovascular risk. However, there have been conflicting results regarding the correlation of endocan with both scales, with some studies reporting significant, independent, and positive correlations [27, 28], while others did not find any significant associations [29, 30].

The present study has revealed higher concentrations of all low-grade inflammation markers in patients with CHD compared to the control group ($p < 0.0001$). There was also an increase in their values with the progression of the CA lesions ($p < 0.0001$). Statistically significant differences were found depending on the presence of such clinical signs as angina pectoris and a history of MI, as well as a number of correlations between their values and a number of clinical and instrumental characteristics. Subsequently, using the multivariate linear regression analysis, an attempt was made to assess the predictive significance of serum concentrations of CRP, TNFα, GDF-15, and ESM-1. The last two indicators demonstrated an independent relationship with the severity of atherosclerotic processes in the coronary bed. It should be noted, that GDF-15 turned out to be the most significant in this model ($\beta = 0.651$; $b = 0.026$; $p < 0.0001$).

CONCLUSION

The study demonstrated that simultaneous quantitative measurement of several laboratory parameters may be a more powerful tool for estimating the risk of coronary heart disease progression. This approach allows for a more accurate assessment of multiple aspects of pathogenesis. Furthermore, it was shown that endocan and, to a greater extent, GDF-15 are associated with the severity of atherosclerotic processes in the coronary arteries.

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Authors' contribution

Ushakov A.V. – literature analysis, data interpretation, study coordination, and final approval of the manuscript for publication. Zakharyan E.A. – conception and design, study coordination, obtaining and interpreting clinical data, drafting of the manuscript, and final approval of the manuscript for publication. Grigoriev P.E. – database compilation, statistical processing of study results, critical revision of the manuscript for important intellectual content, and final approval of the manuscript for publication. Malyi K.D. – literature analysis, obtaining and interpreting clinical data, and database compilation.

Authors' information

Ushakov Alexey V. – Dr. Sci. (Med.), Professor, Head of the Internal Medicine Department, MI CFU, Simferopol, ushakovav88@mail.ru, http://orcid.org/0000-0002-7020-4442.

Zakharyan Elena A. – Cand. Sci. (Med.), Assistant Professor of the Internal Medicine Department No.1, MI CFU, Simferopol, http://orcid.org/0000-0002-7384-9705.

Grigoriev Pavel E. – Dr. Sci. (Biol.), Associate Professor, Professor of the Psychology Department, SevSU, Sevastopol; Leading Researcher of Sechenov ASRI, Yalta, http://orcid.org/0000-0001-7390-9109.

Malyi Konstantin D. – Cand. Sci. (Med.), Assistant Professor of the Biochemistry Department, MI CFU, Simferopol, http://orcid.org/0000-0002-6591-2719.

(✉) **Zakharyan Elena A.,** locren@yandex.ru, +7 (978)-787-93-53.

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Study of gut microbiota in cholangiocarcinoma patients

Fedorova O.S., Kovshirina A.E., Sokolova T.S., Kulenich V.V., Ogorodova L.M.

Siberian State Medical University
2, Moscow Trakt, Tomsk, 634050, Russian Federation

ABSTRACT

Aim. To analyze the taxonomic composition of the intestinal microbiota in patients with cholangiocarcinoma (CCA) and compare it to individuals without oncopathology.

Materials and methods. The study included patients with histologically verified cholangiocarcinoma (n = 30) and a control group (n = 27). An integrated approach was used, including clinical and anamnestic, laboratory, and instrumental methods. The intestinal microbiota was studied through amplicon sequencing of the bacterial 16S rRNA gene.

Results. The assessment of alpha- and beta-diversity of the microbiota in patients with CCA did not show any significant differences compared to the control group. However, a comparative analysis revealed changes in the representation of a number of microorganisms at different taxonomic levels, including a higher content of *Bacteroides* and *Lachnospiraceae_NK4A136_group* in patients with CCA. Additionally, bacteria that influence the change in the global balance of microorganisms were identified in both groups, such as *[Ruminococcus]_torques_group, Subdoligranulum, Parasutterella, unclassified Firmicutes* in samples of patients with CCA and *Oscillospiraceae* and *Erysipelotrichaceae UCG-006* in the control group.

Conclusion. The study found a number of significant differences in bacterial representation between patients with cholangiocarcinoma and control group participants. Further research on the intestinal microbiota has the potential to develop non-invasive tools for early diagnosis of CCA.

Keywords: cholangiocarcinoma, gut microbiota, amplicon sequencing of bacterial 16S rRNA, liver cancer

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 local Ethics Committee at Siberian State Medical University (Protocol No. 9389 of 27.02.2023).

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Исследование микробиоты кишечника у больных холангиокарциномой

Федорова О.С., Ковширина А.Е., Соколова Т.С., Куленич В.В., Огородова Л.М.

Сибирский государственный медицинский университет (СибГМУ)
Россия, 634050, г. Томск, Московский тракт, 2

РЕЗЮМЕ

Цель исследования: проанализировать таксономический состав кишечной микробиоты у пациентов с холангиокарциномой (ХК) по сравнению с индивидуумами без онкопатологии.

✉ *Fedorova Olga S.,* olga.se rgeevna.fedorova@gmail.com

Материалы и методы. В исследование включены пациенты с гистологически верифицированной холангиокарциномой (n = 30) и контрольная группа (n = 27). Для решения задач данного проекта использован комплексный подход, включающий клинико-анамнестические, лабораторные и инструментальные методы. Исследование микробиоты кишечника выполнено методом ампликонного секвенирования гена бактериальной 16S рРНК.

Результаты. При оценке альфа- и бета-разнообразия микробиоты у пациентов с ХК в сравнении с контрольной группой значимых различий не выявлено. Сравнительный анализ показал изменения в представленности ряда микроорганизмов на разных таксономических уровнях, в том числе более высокое содержание *Bacteroides* и *Lachnospiraceae_NK4A136_group* у пациентов с ХК. Также определены бактерии, оказывающие влияние на изменение глобального баланса микроорганизмов в образцах для пациентов с ХК *([Ruminococcus]_torques_group, Subdoligranulum, Parasutterella,* неклассифицированные *Firmicutes*), и контроля (*Oscillospiraceae и Erysipelotrichaceae UCG-006*).

З**аключение.** В результате исследования выявлен ряд значимых различий в представленности бактерий у пациентов с холангиокарциномой в сравнении с участниками контрольной группы. Дальнейшие исследования кишечной микробиоты представляют перспективу для разработки неинвазивных инструментов ранней диагностики ХК.

Ключевые слова: холангиокарцинома, кишечная микробиота, ампликонное секвенирование бактериальной 16S рРНК, рак печени

Конфликт интересов: авторы заявляют об отсутствии конфликта интересов.

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Соответствие принципам этики. Все пациенты подписали информированное согласие на участие в исследовании. Исследование одобрено локальным этическим комитетом СибГМУ (протокол № 9389 от 27.02.2023).

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INTRODUCTION

According to world statistics, liver cancer holds the sixth position among the most common localizations of malignant neoplasms. The incidence of hepatobiliary cancer is increasing worldwide, accounting for 15% of all primary liver cancers and 3% of malignant neoplasms of the digestive tract [1]. Cholangiocellular carcinoma (cholangiocarcinoma, CCA) is one of the leading causes of mortality in oncology due to its aggressive nature, lack of specific symptoms, prolonged asymptomatic course and methods of preclinical diagnosis, and resistance to therapy [2].

The highest incidence of CCA in the world is registered in the countries of Southeast Asia and North Africa, with more than 20 cases per 100 thousand people annually [2]. In the Russian Federation, the highest incidence of CCA is observed in the regions of Western Siberia, where it is more than twice the world average (more than 9 cases per 100 thousand people annually) and poses a significant socioeconomic burden [2, 3].

CCA belongs to multifactorial diseases, in the development of which genetic, infectious, environmental, and epidemiological risk factors are involved. The most significant risk factors include primary sclerosing cholangitis/ulcerative colitis, chronic viral hepatitis C and B, hepatic trematodoses, Epstein-Barr virus carrier, non-alcoholic fatty liver disease, cholelithiasis, and (or) malformations of the biliary system, food carcinogens (N-dinitrosodimethylamine), deposits of X-ray contrast agents (thorotrast) in bile ducts [4–6]. Immune mechanisms play a crucial role in carcinogenesis: chronic inflammation leads to increased exposure of cholangiocytes to proinflammatory mediators – interleukin-6, tumor necrosis factor, cyclooxygenase-2 and activation of Wnt signaling pathway with progression of mutations

in tumor suppressor genes, proto-oncogenes and DNA repair genes, and increased risk of carcinogenesis [7].

According to clinical and experimental studies, the intestinal microbiota is the most important factor in the development of liver diseases along the gut – liver axis [8, 9]. Inflammatory reactions resulting from changes in the gut microbiome are associated with the development of a number of chronic non-communicable diseases and are also considered as a potential carcinogenic mechanism [10–13]. However, the fundamental mechanisms underlying this relationship are still unclear. Thus, it is essential to study the taxonomic composition of the intestinal microbiota in patients with CCA and individuals without cancer.

MATERIALS AND METHODS

To solve the set tasks, the study was conducted in a case-control design in parallel groups. The study protocol was approved by the Local Ethics Committee of Siberian State Medical University (Protocol No. 9389 of 27.02.2023).

The study included the following groups: patients with histologically confirmed CCA (n = 30) and participants without cancer and/or clinically significant changes in the hepatobiliary system (control group, n = 27). Among the patients with CCA, 16.6% received chemotherapy, 13.3% received a short course of antibiotic therapy (up to 7 days), and 33.3% underwent surgical intervention on the organs of the hepatobiliary system within 2-4 weeks prior to inclusion in the study. The control group did not receive any of these treatments during the specified periods.

CCA was diagnosed through the histologic and/or immunohistochemical examination of biopsy or postoperative liver material, according to the following ICD-10 codes: C22.1 – cancer of the intrahepatic bile duct; C24 – malignant neoplasm of other and unspecified parts of the biliary tract (n = 29); C24.0 – malignant neoplasm of the extrahepatic bile duct (n = 1). The inclusion criterion for all participants was a signed informed consent to participate in the study.

During the study, the medical histories and physical examination data of the participants were analyzed, including vital signs, anthropometric data, and system and organ examination data.

All participants underwent ultrasound examination of the hepatobiliary system using a high-resolution mobile ultrasound scanner (Mindray M7, Shenzhen Mindray Bio-Medical Electronics Co, Ltd, PRC) in accordance with the protocol proposed within the framework of epidemiologic studies of opisthorchiasis previously conducted in Southeast Asia [14]. We evaluated the liver size and structural features of the liver parenchyma, including echo signs of CCA and periductal fibrosis (defined as an increase in periportal echogenicity of more than 3 mm around the intrahepatic bile ducts of the second order), as well as dilation and thickening of the bile duct walls. The presence of liver masses was verified by computed tomography (CT) and/or magnetic resonance imaging (MRI) in patients with CCA.

To assess the composition of the intestinal microbiota, stool samples were collected in special sterile containers and stored at minus 80 degrees Celsius until analysis. Additionally, stool samples were evaluated for the presence of O. felineus infection through microscopy of two stool samples using Parasep concentrators (DiaSysLtd, UK).

To isolate DNA from the stool samples, we used the Nobias DNA Extraction Kit (Nobias Technologies LLC, Russia) with the extraction protocol, including the stage of stool sample homogenization with the help of solid particles (bead beating) and precipitation of inhibitors.

The microbiota of stool samples from patients with CCA and the control group was studied through amplicon sequencing of the V3-V4 fragment of the 16S rRNA gene. As a result of sample preparation and sequencing, 6 samples were excluded due to inadequate quality for analysis, and data from 51 patients' samples were included in the microbiota study data analysis. Sequencing of the V4 region of the bacterial 16S rRNA gene was performed on an Illumina MiSeq instrument.

Bioinformatic analysis of the obtained reads was performed using Qlime 2 software. Data were aggregated into ASVs (amplicon sequence variants) using the DADA2 plugin, and taxonomic analysis was performed using the RDP classifier plugin and the Silva taxonomy database. Statistical analysis was performed using R 4.3.3. Alpha diversity was assessed using the total number of operational taxonomic units (OTUs), Shannon, Chao1, and Simpson diversity indices (rbiom 1.0.3). Alpha diversity between groups was compared using the non-parametric Mann – Whitney test. Beta diversity was analyzed through nonparametric permutation analysis of variance

(PERMANOVA) using distance matrices based on the Eitchison distance and Jaccard's measure. Beta diversity differences were analyzed using the adonis test (vegan 2.6-4). Microbial representation between groups was compared using the nonparametric Mann – Whitney test and compositional analysis through the balance selection method (selbal 0.1.0) for two groups, and the Kraskell – Wallis criterion followed by Dunn's test (dunn.test 1.3.6) for three or more groups. Correlation analysis was performed using Spearman rank correlation (Hmisc 5.1-2). Benjamini – Hochberg multiple comparisons correction was applied to all statistical tests. The level of statistical significance was chosen at 0.05.

RESULTS

During the study, two clinical groups of patients were formed: patients with histologically verified CCA (n = 30) and a control group (n = 27). In the CCA group, 96.7% of cases were diagnosed with intrahepatic localization of cholangiocarcinoma, and only 1 patient had an extrahepatic Klatskin tumor; 46.7% of patients were diagnosed at stage I-II and 53.3% – at stage III-IV according to the TNM classification. Chemotherapy was given to 16.7% of patients and surgical treatment – to 30% of participants, while 53.3% received only palliative care.

The most frequent symptoms in patients with CCA were abdominal pain, bloating, positive bladder symptoms of Ker and Ortner, liver pain on palpation, as well as significantly higher levels of bilirubin, aspartate aminotransferase, alanine aminotransferase and detection of hepatomegaly, periductal fibrosis, and bile duct dilatation on ultrasound.

Neoplasms were detected by ultrasound and confirmed by abdominal MRI / CT.. All clinical data for the studied groups are presented in Table 1.

Table 1.

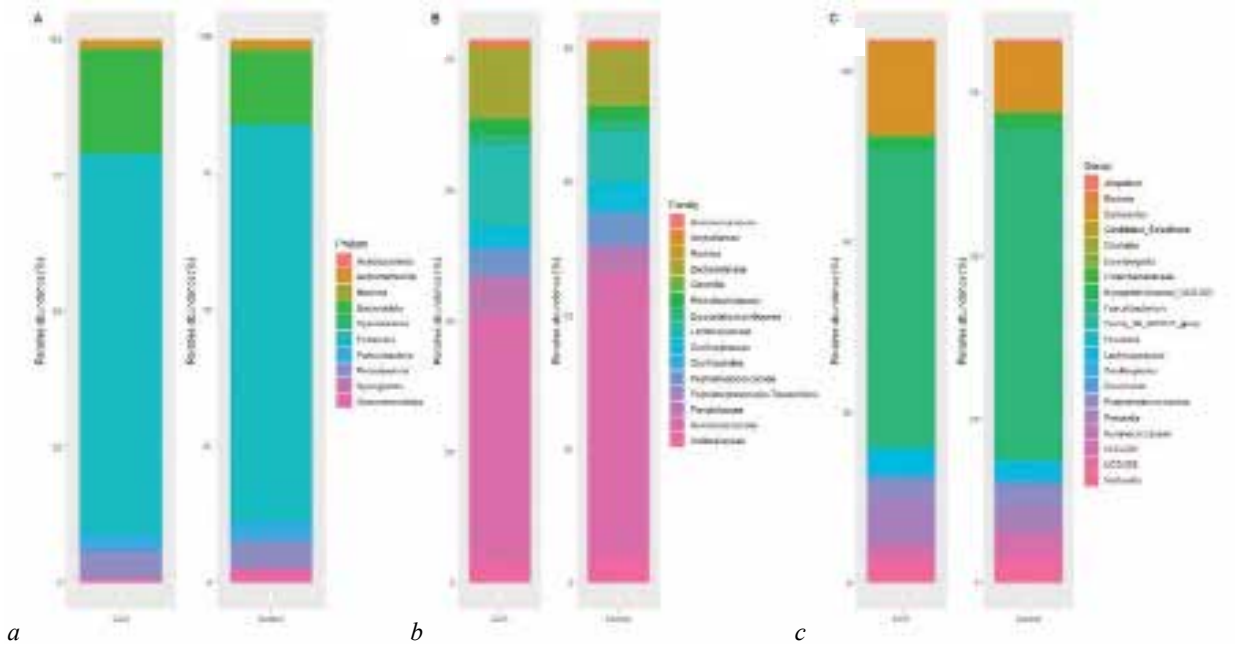
Clinical characteristics of the studied groups

Parameter	Patients with CCA (n = 30)	Control group (n = 27)	p
Demographic and anamnestic characteristics			
Gender, n (%) male female	 18 (60.0) 12 (40.0)	 14 (51.8) 13 (48.2)	$p > 0.05$
Age, years (N (Q_1; Q_2))	59.96 (54.0 ; 67.0)	62,8 (58.0 ; 68.0)	$p > 0.05$
Smoking, n (%)	21 (70.0)	11 (36.7)	$p < 0.05$
Average history of smoking (years)	12.2 (0.0 ; 20.0)	10.8 (0.0 ; 20.0)	$p > 0.05$
Alcohol consumption, n (%)	15 (50,0)	11 (36.7)	$p > 0.05$
Gastric and/or duodenal ulcer disease, n (%)	8 (26.7)	4 (14.8)	$p < 0.05$
Hypertension, n (%)	15 (50.0)	19 (70.4)	$p > 0.05$
Chronic heart failure, n (%)	4 (13.3)	9 (33.3)	$p < 0.05$
Type 2 diabetes mellitus, n (%)	3 (10.0)	4 (14.8)	$p > 0.05$
Cholelithiasis, n (%)	8 (26.7)	2 (7.4)	$p < 0.05$
Clinical characteristics			
Body mass index (kg/cm2)	26.6 (21.0 ; 28.4)	29.1 (23.0 ; 33.5)	$p > 0.05$
Fever, n (%)	4 (6.7)	–	
Jaundice of the skin and visible mucosae, n (%)	11 (36.7)	–	
Hepatomegaly on the liver palpation, n (%)	14 (43.3)	4 (15.4)	$p < 0.05$
Positive gall bladder symptoms, n (%)	10 (33.3)	–	
Biochemical markers			
Total protein, g/l	67.2 (62.0 ; 73.0)	68.7 (63.0 ; 75.0)	$p > 0.05$
Total bilirubin, mcmol/l	54.2 (8.9 ; 41.0)	11.7 (6.0 ; 14.2)	$p < 0.05$
Conjugated bilirubin, mcmol/l	28.4 (2.3 ; 32.0)	1.8 (0.0 ; 2.3)	$p < 0.05$
ALT, IU/I	61.8 (25.0 ; 73.0)	30.2 (14.0 ; 33.0)	$p < 0.05$
AST, IU/I	58.2 (32.0 ; 73.0)	30.5 (17.0 ; 42.0)	$p < 0.05$
Ultrasound examination of the hepatobiliary system			
Hepatomegaly, n (%)	8 (26.7)	2 (7.4)	$p < 0.05$
Dilation of the bile ducts, n (%)	14 (46.7)	–	
Thickening, irregular walls of bile ducts, n (%)	7 (23.3)	1 (3.7)	$p < 0.05$
Periductal fibrosis, n (%)	12 (40.0)	–	

BIOINFORMATICS AND STATISTICAL ANALYSIS OF GUT MICROBIOTA COMPOSITION DATA

The most represented types in the microbiota of stool samples from patients with CCA were *Firmicutes* (70.1%), *Bacteroidota* (18.9%), *Proteobacteria* (5.1%), *Patescibacteria* (2.9%), and *Actinobacteriota* (1.6%). The control group had a higher proportion of *Firmicutes* (72.6%), *Bacteroidota* (13.4%), *Proteobacteria* (4.7%), *Patescibacteria* (3.6%), and *Verrucomicrobiota* (2.5%, Fig. 1, *a*). At the family level, patients with CCA were dominated by *Ruminococcaceae* (38.0%), *Lachnospiraceae* (12.5%), *Bacteroidaceae* (10.7%), *Prevotellaceae* (5.9%), and *Peptostreptococcaceae* (4.1%). In the control group, the most represented families of microorganisms were the following: *Ruminococcaceae* (42.9%), *Bacteroidaceae* (8.3%), *Lachnospiraceae* (7.3%), *Peptostreptococcaceae* (4.7%), and *Oscillospiraceae* (4.5%, Fig. 1, *b*).

At the genus level, patients with CCA had a predominant rate of *Faecalibacterium* (34.7%), *Bacteroides* (10.7%), *Prevotella* (5.2%), unclassified genera of the families *Lachnospiraceae* (3.1%) and *Peptostreptococcaceae* (2.3%). In the control group, the most represented genera of microorganisms were *Faecalibacterium* (40.5%), *Bacteroides* (8.3%), *Prevotella* (3.0%), UCG-002 (2.7%) and unclassified genera of the family *Peptostreptococcaceae* (2.7%, Fig. 1, *c*).



a *b* *c*

Fig. 1. The bar graph shows the most represented microbial taxa in stool samples from patients with cholangiocarcinoma (CCA) and control groups (Control): *a* – at the order level, *b* – at the family level, *c* – at the genus level

The comparative analysis of alpha diversity of the microbiota of stool samples showed no statistically significant differences between the group of patients with CCA and the control group. (Fig. 2). Similarly, no differences were found between the groups when beta diversity was assessed.

The Mann – Whitney test was used to identify bacteria (28 taxa) whose abundance was statistically significantly different in the group of patients with CCA compared to the control group. The results showed that CCA is associated with an increase in the abundance of bacteria from the following orders: *Lachnospirales*, *Clostridiales*, *Rhodospirillales*, семейств *Lachnospiraceae*, *Tannerellaceae*, *Clostridiaceae*, unclassified *Rhodospirillales,* and *Bacteroidales,* compared to the control group. The intestinal microbiota of the control group participants was characterized by a higher content of bacteria from the *Staphylococcaceae* family and the genera of *Staphylococcus*, and *Finegoldia*. At the genus level, the microbiota samples of CCA patients were characterized by a higher content of *[Ruminococcus] torques group, Dorea, unclassified Lachnospiraceae, CAG-56, Agathobacter, Clostridium sensu stricto*

1, uncultured Rhodospirillales, Subdoligranulum, CAG-352, Anaerostipes, Parabacteroides, uncultured Bacteroidales, Anaerovoracaceae Family XIII AD3011 group, Oscillospiraceae NK4A214 group, and *Lachnospiraceae NK4A136 group.*

We also used the method of selecting balances with sex as a covariate to reduce the detection of false positives. Only bacteria that were in global balance (R^2 = 0.726, p = 0.006, Fig. 3) were considered significant.

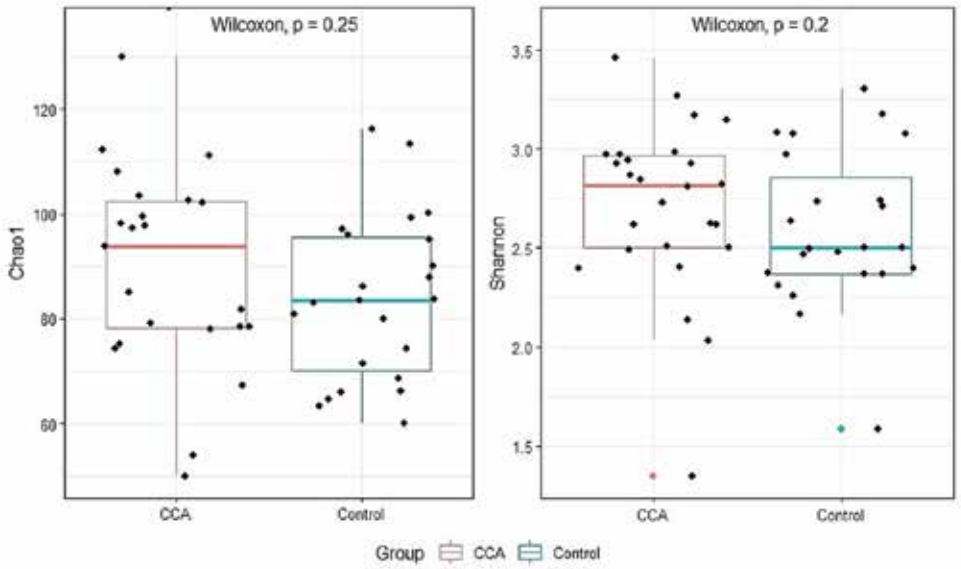


Fig. 2. The graph shows comparison of alpha diversity between cholangiocarcinoma (CCA) and the control group (Control) of patients in terms of number of operative taxonomic units (OTUs), Shannon, Chao1 and Simpson diversity indices. The *p*-value was calculated using the nonparametric Mann – Whitney test

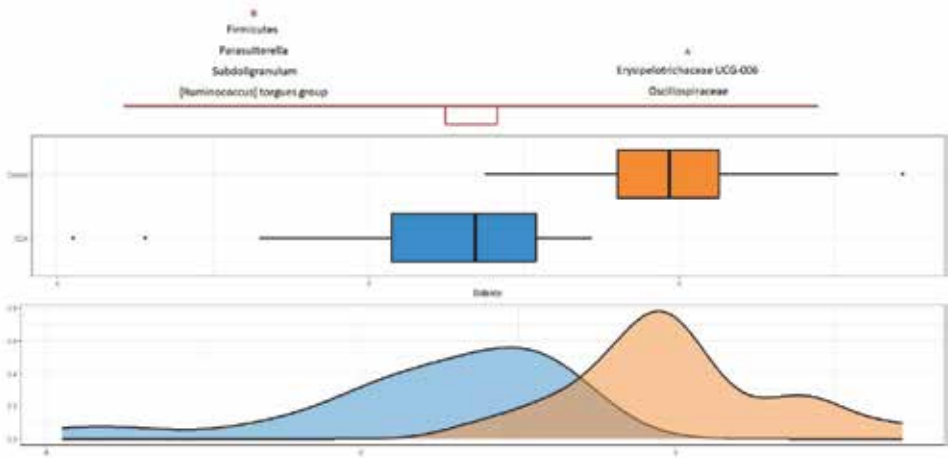


Fig. 3. The selection plot of microbial balances in samples at the genus level. The bacteria presented in the upper part of the graph influence the variation of the global balance of microorganisms in the samples. The middle and bottom parts show box-and-whisker type plots and density distribution curves of the balance for the groups of CCA patients (blue) and the control groups (orange)

Additionally, we performed a correlation analysis between bacteria in the global balance, taking into account the joint influence of several bacterial genera. We found a statistically significant mean correlation between bacteria from the genera *[Ruminococcus] torques group* and *Subdoligranulum* (r = 0.56, $p < 0.0001$), which changed the balance of microorganisms downward.

Within the group of patients with CCA, we investigated the differences in the relative bacterial representation based on clinical features such as bad habits, concomitant diseases, ongoing treatment,

tumor localization and histological characteristics, and macroscopic characteristics of the liver and bile ducts. Thus, in patients with intrahepatic tumor localization, there was a significant increase in the representation of bacteria from the genus *Clostridia UCG-014* (p = 0.023), while in patients with stage 3–4 of the disease, the content of bacteria of the genus *Odoribacter* significantly decreased (p = 0.012). Patients with anaplastic cancer confirmed by histologic examination had a significantly increased content of the genus *Saccharimonadaceae* (p = 0.029), unclassified genera from the family *Rhizobiaceae* (p = 0.036), and the genus *Faecalibacterium* (p = 0.043) compared to patients with other types of cancer. We also observed a significant increase in the abundance of bacteria from the genus *Agathobacter* in patients with dilated hepatic ducts (p = 0.022).

DISCUSSION

This study is the first to investigate the composition of the intestinal microbiota using 16S rRNA sequencing in a Russian population of patients with cholangiocarcinoma. In our study sample, intrahepatic localization of CCA was diagnosed in the majority of cases, more than half of the patients were diagnosed at TNM stages III-IV. The clinical symptoms corresponded to the course of the disease.

The study revealed that the composition of intestinal microbiota in patients with CCA in comparison with the control group is characterized by changes in the quantitative representation of individual microbial communities without significant differences in alpha- and beta-diversity.

The results of foreign studies demonstrated differences in the composition of the intestinal microbiota between patients with biliary tract cancer and healthy participants. However, there is currently no clear trend in changes in specific groups of microorganisms or a characteristic microbial profile in CCA, possibly due to differences in methodological approaches. It should be noted that our study revealed some modifications of microorganism representation comparable to the results of similar previous studies. Thus, in two studies, a change in the representation of the genus Bacteroides was noted in patients with CCA and hepatocellular carcinoma (HCC) [15, 16]. Bacteria of the genus *Bacteroides* are dominant representatives of the normal intestinal microbiota and perform various functions aimed at maintaining intestinal homeostasis [17]. However, certain species of the genus *Bacteroides*, such as *Bacteroides fragilis*, may play a role in the pathogenesis of various diseases and carcinogenesis [18, 19]. A study by Tuo Deng et al, 2022, also found an association with increased representation of the *unclassified Lachnospiraceae group NK4A136* [15].

Another study also noted an increase in *Parabacteroides* in intestinal microbiota samples from patients with hepatocellular carcinoma [20]. The gut microbiota of patients at early stages of HCC was characterized by a decrease in butyrate-producing bacteria and an increase in lipopolysaccharide-producing microorganisms compared to control group samples [20]. High levels of lipopolysaccharides have been shown to activate the NF-κB pathway, produce pro-inflammatory cytokines (TNFα, IL-6 and IL-1) and lead to inflammatory and oxidative damage to the liver, contributing to the development of hepatobiliary cancers [21–23].

The results of gut microbiota diversity indices assessment according to different studies are heterogeneous. A systematic review of studies investigating intestinal microbiota in patients with CCA showed that in most publications, the alpha-diversity index did not differ significantly from the controls, while two studies reported a decrease and in one study an increase in taxonomic diversity [24]. In a study of liver biopsy specimens, it was shown that samples from peritumor sites and HCC tissue had greater bacterial diversity compared to unaffected liver sites [25].

As a result of gut microbiota assessment using the balance selection method, we identified a number of bacteria that influence the change in the global balance of microorganisms in the samples. A microbiota profile with increased representation of *[Ruminococcus]_torques_group*, *Subdoligranulum*, *Parasutterella*, *unclassified Firmicutes* was characteristic of patients with CCA. In contrast, previous studies have noted a decrease in the representation of bacteria of the genus *Subdoligranulum* in patients with liver disease compared to healthy participants [26–28]. Bacteria of the genus *Parasutterella* are involved in bile acid metabolism [29].

The study of the gut microbiota is a challenging task due to the many internal and external factors affecting the composition of microbial communities, and it requires careful study design. The limitations

of our study include a small sample size and a single assessment of the microbiota in a stool sample.

CONCLUSION

This study revealed a number of significant differences in bacterial representation in patients with cholangiocarcinoma compared to control group participants. Considering the results of previous studies, microorganisms such as *Bacteroides* and *Lachnospiraceae_NK4A136_group* may be potential microbial markers of CCA development. Thus, further studies of the gut microbiota hold promise for the development of non-invasive tools for the early diagnosis of CCA.

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Authors' contribution

Fedorova O.S. – final approval of the manuscript for publication,. Kovshirina A.E. – substantiation of the manuscript, conception and design, analysis and interpretation of the data. Sokolova T.S. – conception and design,. Kulenich V.V. – analysis and interpretation of the data. Ogorodova L.M. – critical revision of the manuscript for important intellectual content.

Authors' information

Fedorova Olga S. – Dr. Sci. (Med.), Vice-Rector for Postgraduate Education and Research, Head of the Intermediate-Level Pediatrics Division with a Pediatric Diseases Course, General Medicine Department, Siberian State Medical University, Tomsk, olga.sergeevna.fedorova@gmail.com, https://orcid.org//0000-0002-7130-9609; +7 (3822)901-101(1506).

Kovshirina Anna E. – Teaching Assistant of the Propaedeutics of Internal Diseases Division with a Therapy Course, Department of Pediatrics, Siberian State Medical University, Tomsk, anna.evgenjevna.kovshirina@gmail.com, https://orcid.org/0000-0001-6116-8323.

Sokolova Tatiana S. – PhD, Associate Professor of the Intermediate-Level Pediatrics Division with a Pediatric Diseases Course, General Medicine Department, Siberian State Medical University, Tomsk, sokolova.ts@ssmu.ru, https://orcid.org/0000-0002-1085-0733.

Kulenich Victoria V. – Research Assistant of the Research and Educational Laboratory "Live Laboratory of Population-Based Studies", Siberian State Medical University, kulenich.vv@ssmu.ru, https://orcid.org/0009-0000-7416-5017.

Ogorodova Ludmila M. – Dr. Sci. (Med.), RAS Corresponding Member, Professor of the Intermediate-Level Pediatrics Division with a Pediatric Diseases Course, General Medicine Department, Siberian State Medical University, Tomsk, ogorodova.lm@ssmu.ru, https://orcid.org/0000-0002-2962-1076.

(✉) **Fedorova Olga S.,** olga.sergeevna.fedorova@gmail.

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Perinatal and social predictors of early childhood health in preterm infants: multicenter cohort study results

Khodkevich P.E.[1,2], Fedorova O.S.[1], Kulikova K.V.[3], Deev I.A. [4]

[1] *Siberian State Medical University*
2, Moscow Trakt, Tomsk, 634050, Russian Federation

[2] *Children's Hospital No. 1*
4, Moscow Trakt, Tomsk, 634050, Russian Federation

[3] *I.D. Evtushenko Regional Perinatal Center*
96/1, I. Chernykh Str., Tomsk, 634063, Russian Federation

[4] *Pirogov Russian National Research Medical University*
1, Ostrovityanova Str., Moscow, 117997, Russian Federation

ABSTRACT

Aim. To identify perinatal and social predictors that determine the health of premature infants in early childhood, based on their birth weight.

Materials and methods. This publication is part of a cohort prospective observational study of premature infants that was initiated in Tomsk in 2014 (Deev I.A., Kulikova K.V., Kobyakova O.S. et al., 2016). The main group consisted of 226 premature infants: 78 infants with low birth weight (LBW), 76 – with very low birth weight (VLBW), and 72 – with extremely low birth weight (ELBW), while a control group included 76 term infants. The follow-up period was 3 years, with examinations conducted every 12 months.

Results. The study found that 57.1% (n = 36) of ELBW infants, 34.9% (n = 23) of VLBW infants, and 32.9% (n = 23) of LBW infants showed an "improvement" in their health during early childhood (transition from health groups IV and V to III, as well as transition from health group III to II at subsequent visits). The presence of siblings (for the main group OR = 2.6 [95% CI 1.3–5.3], p = 0.006, for children with ELBW OR = 8.4 [95% CI 1.0–69.6], p = 0.045) and the mother's higher education (for children with VLBW OR = 3.9 [95% CI 1.2–12.2], p = 0.018 and with LBW OR = 3.4 [95% CI 1.2–9.9], p = 0.025) were identified as predictors of a favorable clinical prognosis. Perinatal and social predictors associated with the development of pathological abnormalities included intrauterine growth retardation, intraventricular hemorrhage, severe anemia in the neonatal period, maternal obesity, maternal smoking, parental age over 35 years, and lack of higher education for the mother.

Conclusion. To implement a health-preserving strategy for the group of premature infants, especially those with ELBW, health improvement can be achieved by addressing controllable social factors.

Keywords: health, premature infants, social predictors

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. An informed consent for the child's participation in the study was signed by legal representatives. The study was approved by the local Ethics Committee at Siberian State Medical University (Protocol No.7937 of 28.10.2019).

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✉ *Khodkevich Polina E.,* pkhodkevich@mail.ru

Перинатальные и социальные предикторы, определяющие состояние здоровья недоношенных детей в раннем детском возрасте: результаты когортного многоцентрового исследования

Ходкевич П.Е.[1, 2], Федорова О.С.[1], Куликова К.В.[3], Деев И.А.[4]

[1] *Сибирский государственный медицинский университет (СибГМУ)*
Россия, 634050, г. Томск, Московский тракт, 2

[2] *Детская больница № 1*
Россия, 634050, г. Томск, Московский тракт, 4

[3] *Областной перинатальный центр (ОПЦ) им. И.Д. Евтушенко*
Россия, 634063, г. Томск, ул. Ивана Черных, 96/1

[4] *Российский национальный исследовательский медицинский университет (РНИМУ) им. Н.И. Пирогова*
Россия, 117997, г. Москва, ул. Островитянова, 1

РЕЗЮМЕ

Цель. Установить перинатальные и социальные предикторы, определяющие состояние здоровья недоношенных детей в раннем детском возрасте в зависимости от массы тела при рождении.

Материалы и методы. Публикация является частью когортного проспективного наблюдательного исследования недоношенных новорожденных, инициированного в г. Томске в 2014 г. (Деев И.А., Куликова К.В., Кобякова О.С. и др., 2016). Основную группу составили 226 недоношенных новорожденных (с низкой массой тела (НМТ) – 78, с очень низкой массой тела (ОНМТ) – 76, с экстремально низкой массой тела (ЭНМТ) – 72 ребенка), в группу контроля включены 76 здоровых младенцев. Период катамнестического наблюдения – 3 года, периодичность обследования – 12 мес.

Результаты. Установлено, что 57,1% (n = 36) детей с ЭНМТ, 34,9% (n = 23) детей с ОНМТ и 32,9% (n = 23) детей с НМТ при рождении имели «улучшение» состояния здоровья (переход из IV и V групп здоровья в III, а также переход из III группы здоровья во II на последующих визитах) в раннем детском возрасте. Предикторами благоприятного клинического прогноза являлись наличие сибсов (для основной группы отношение шансов (ОШ) 2,6 [95%-й доверительный интервал (ДИ) 1,3–5,3], p = 0,006, для детей с ЭНМТ при рождении ОШ = 8,4 [95%-й ДИ 1,0–69,6], p = 0,045) и наличие высшего образования у матери (для детей с ОНМТ при рождении ОШ = 3,9 [95%-й ДИ 1,2–12,2], p = 0,018 и с НМТ при рождении ОШ = 3,4 [95%-й ДИ 1,2–9,9], p = 0,025). Также установлены перинатальные и социальные предикторы развития патологических отклонений по разным функциональным системам в зависимости от массы тела при рождении в раннем детском возрасте: задержка внутриутробного развития, внутрижелудочковое кровоизлияние, тяжелая анемия в неонатальном периоде, ожирение у матери, курение матери, возраст родителей более 35 лет, отсутствие высшего образования у матери.

Заключение. Таким образом, в целях реализации стратегии здоровьесбережения в группе недоношенных детей, особенно с ЭНМТ при рождении, улучшение состояния здоровья может быть достигнуто воздействием на управляемые социальные факторы.

Ключевые слова: здоровье, недоношенные дети, социальные предикторы

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. Информированное согласие на участие ребенка в исследовании подписано законными представителями. Исследование одобрено локальным этическим комитетом СибГМУ (протокол № 7937 от 28.10.2019).

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INTRODUCTION

Health preservation strategy is a priority area of medicine. This approach promotes the need for pregravid preparation, since human health is largely determined by the course of the ante-, intra- and neonatal periods of life, and according to the theory of "fetal programming", is the result of a combination of the genetic program under the influence of perinatal factors [1–3]. Premature infants, especially those with very low birth weight (VLBW) and extremely low birth weight (ELBW), are particularly vulnerable to developing various diseases both in the neonatal period and later in life [4–7]. Several studies have identified laboratory, instrumental, and clinical predictors of an unfavorable clinical prognosis for premature infants in the neonatal period have been established [8, 9]. Additionally, some studies have examined perinatal risk factors for chronic health issues in children born with VLBW and ELBW. However, these studies are limited in number and often only focus on one aspect of the body's functioning [5]. At the same time, identifying predictors of a favorable clinical prognosis associated with a decrease in morbidity and the absence of chronic disease exacerbations can be an effective tool in strengthening the health of the population of premature infants.

Thus, we planned and conducted a study to determine perinatal and social predictors that influence the health of premature infants in early childhood, based on their birth weight.

MATERIALS AND METHODS

This study is part of a cohort prospective observational study of premature infants initiated in Tomsk in 2014 [10]. The study included data from 302 children born in 2014–2020 at the I.D. Evtushenko Regional Perinatal Center and the Tomsk Maternity Hospital No. 4. Once the legal representatives signed the informed consent for their child to participate in the study, the child was included in one of the observation groups. The study methodology, inclusion and exclusion criteria, results of the ethical review, study design, and clinical characteristics of the children during the neonatal period were previously published [7, 11]. Four in-person visits were conducted: visit 0 (during the neonatal period), visit 1 (at one year of age), visit 2 (at two years of age), and visit 3 (at three years of age).

This article presents an analysis of the anamnestic data of parents (socioeconomic status, education, health status, anthropometric data, presence of bad habits, and medication intake) and the health groups of the children according to the Order of the Ministry of Health of the Russian Federation dated December 30, 2003 No. 621 "On a comprehensive assessment of the health of children". Additionally, conclusions from narrow medical specialists and medical commissions based on medical documentation (forms 112/u) were used to established medically verified diagnoses (such as malnutrition, obesity, transient hypothyroidism, bronchopulmonary dysplasia, myopia, astigmatism, patent ductus arteriosus, psychomotor retardation, hearing loss, etc.). When assessing the health dynamics of the observed children, a comparison was made between the current visit and the previous visit. A favorable clinical outcome was recorded when a child was assigned to a more "favorable" health group (for example, transitioning from IV to III health group or from III to II health group) or maintained their I, II, or III health group without exacerbation or decompensation of chronic diseases. An unfavorable clinical outcome was recorded when a child was assigned to a less favorable health group (from I and II health groups to III group) or experienced decompensation or exacerbation of chronic diseases.

Statistical analysis was performed using the Statistica for Windows, 13.0 software package. Quantitative data were described using the arithmetic mean and standard deviation (M±SD). For qualitative data, the absolute value and percentage were determined. The Mann-Whitney U-test was used to assess the differences in quantitative data. The Pearson $\chi 2$ test (with Yates' correction for values less than 10) was used to compare the frequencies of qualitative features. The difference in values was considered statistically significant at $p < 0.05$.

RESULTS

The study included the main group of 226 premature infants (low birth weight (LBW) – 78 children, VLBW – 76 children, and ELBW – 72 children), and a control group of 76 healthy infants. At the beginning of the study, the main group consisted of 123 boys (54%) and 103 girls (46%), while the control group had 57 boys (75%) and 19 girls (25%). There were statistically significant differences in anthropometric parameters and gender between the main and control groups, but the groups of premature infants were comparable in terms of gender [7].

When the children were divided into health groups based on their birth weight, it was found that at the visit 0, health group II was predominant among premature infants with LBW (n = 46, 58.9%) than among those with VLBW (n = 24, 31.6%, p < 0.001). In the ELBW group, children with health groups III–IV were more common than in the other groups. Throughout the observation period, children with a birth weight of less than 1,000 grams were less likely to be in health group II than children in other observation groups, and more often belonged to regular health check-up groups III–IV compared to children with LBW and VLBW (Table 1).

Table 1

Distribution of children by health groups based on their birth weight and age, % (n)

Age	Health group	LBW	VLBW	ELBW	Control group
Visit 0	I	0 (0)	0 (0)	0 (0)	67.1 (51)
	II	58.9 (46)	31.6* (24)	0 (0)	32.9 (25)
	III	37.1 (29)	61.8* (47)	45.8 (33)	0 (0)
	IV	1.3 (1)	5.3 (4)	37.5** (27)	0 (0)
	V	2.6 (2)	1.3 (1)	16.7** (12)	0 (0)
Visit 1	I	7.1 (5)	1.5 (1)	0 (0)	57.1** (40)
	II	58.6 (41)	33.3 (22)	7.9$^{\#}$ (5)	31.8 (20)
	III	28.6 (20)	53.0$^{\#\#}$ (35)	55.6$^{\#\#}$ (35)	4.8 (3)
	IV	5.7 (4)	12.2 (8)	33.3$^{\#}$ (21)	0 (0)
	V	0 (0)	0 (0)	3.2 (2)	0 (0)
Visit 2	I	9.8 (4)	5.6 (2)	0 (0)	8.3 (1)
	II	53.7 (22)	41.7 (15)	20.5$^{\#\#}$ (9)	83.3 (10)
	III	26.8 (11)	30.6 (11)	43.2 (19)	8.3 (1)
	IV	9.8 (4)	19.4 (7)	36.4* (16)	0 (0)
	V	0 (0)	2.8 (1)	0 (0)	0 (0)
Visit 3	I	10.5 (4)	8.6 (3)	0 (0)	8.3 (1)
	II	60.5 (23)	45.7 (16)	27.3$^{\#\#}$ (12)	75.0 (9)
	III	21.1 (8)	31.4 (11)	47.7* (21)	16.7 (2)
	IV	5.3 (2)	14.3 (5)	22.7 (10)	0 (0)
	V	0 (0)	0 (0)	2.3 (1)	0 (0)

* p < 0.05 when compared with the LBW group (Pearson χ2 test); ** p < 0.05 when compared with the LBW and VLBW groups (Pearson χ2 test); $^{\#}$ – p < 0.05 when compared with the LBW, VLBW, and control groups (Pearson χ2 test); $^{\#\#}$ p < 0.05 when compared with the LBW and control groups (Pearson χ2 test).

The study also analyzed the prevalence of pathological deviations in the main functional systems of the body. It was found that at the age of one year, children with VLBW (n = 9, 13.6%, p = 0.017) and ELBW (n = 18, 28.6%, p < 0.001) were more likely to have malnutrition than children in the control group (n = 1, 1.6%). At the age of two and three years, statistically significant differences in the presence of malnutrition between premature infants based on birth weight were not found. This deviation was not recorded at visits 2 and 3 (Table 2) among children in the control group.

The study also found that children with ELBW were more likely to have abnormalities in the nervous system (such as delayed psychomotor development, movement disorder syndrome, hypertension syndrome, and cerebral palsy) than children in other groups at the age of one and two years. At the age of three, there were no differences in the prevalence of neurological pathology between the groups.

Children with ELBW were also more likely to have endocrine pathology (mainly transient hypothyroidism) throughout the observation period compared to children in other groups (Table 2).

When analyzing data on the presence of visual organ deviations, children with ELBW (n = 31, 49.2%) were more likely to have ophthalmic pathology (such as retinopathy of prematurity, severe myopia, astigmatism, and others) at the first visit compared to children with LBW (n = 9, 12.9%, p < 0.001) and VLBW (n = 7, 10.6%, p < 0.001). At the age of two, no differences were found between the groups. At visit 3, ophthalmic pathology was statistically significantly more common among

children with ELBW (n = 12, 27.3%) than among children with LBW at birth (n = 2, 5.3%, p = 0.019).

The main lung pathology among premature infants included in the study throughout the entire observation period was bronchopulmonary dysplasia (BPD). Children with ELBW were more likely to have this diagnosis than premature infants of other groups throughout early childhood (Table 2). At the age of one year, according to the results of the study, 26 children (37.1%) with LBW, 29 children (43.9%) with VLBW, and 21 children (33.3%) with ELBW had anemia of varying severity, which was more common than that in the control group – 4 (6.4%, $p < 0.001$).

When studying the prevalence of cardiovascular diseases (congenital heart defects, except for patent ductus arteriosus, arrhythmia), gastrointestinal tract (chronic constipation, biliary tract diseases, functional bowel disorders, etc.), and genitourinary system (hypospadias, congenital kidney defects, spermatic cord cysts, labia minora adhesions, etc.), no statistically significant differences were found between the groups in early childhood [7].

At the end of the follow-up, 27.3% (n = 12) of children with ELBW had a disabling disease, which was significantly more common than among children with LBW (n = 2, 5.3%, p = 0.019) and VLBW (n = 1, 2.9%, p = 0.010). According to our study, having a birth weight of less than 1,000 grams increased the odds of having a disability at the age of three by 9 times compared to premature infants with birth weights between 1,000 and 2,500 grams (OR = 8.8 [95% CI 2.3–3.2], $p < 0.001$). No child in the control group had a disabling disease.

Table 2

Structure of pathological abnormalities in children based on their birth weight in early childhood

Pathological deviations	Main group		LBW		VLBW		ELBW		Control group	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%
1 year										
Malnutrition	34#	17.1	7	10	9#	13.6	18#	28.6	1	1.6
Pathology of the cardiovascular system	42	21.1	15	21.4	12	18.2	15	23.8	0	0
Pathology of the nervous system	48	24.1	15#	21.4	12	18.2	21#	33.3	4	6.4
Endocrine pathology	104	52.3	15	21.4	37*	56.1	52**	82.5	3	4.8
Ophthalmic pathology	47#	23.6	9	12.9	7	10.6	31**	49.2	6	9.5
Lung pathology	38	19.1	1	1.4	4	6.1	33***	52.4	0	0
Pathology of the genitourinary system	27	13.6	10	14.3	12	18.2	5	7.9	0	0
Gastrointestinal tract pathology	10	5.0	3	4.3	3	4.6	4	6.4	0	0
Anemia	76#	38.2	26#	37.1	29#	43.9	21#	33.3	4	6.4
2 years										
Malnutrition	20	16.5	5	12.2	3	8.3	12	27.3	0	0
Pathology of the cardiovascular system	16	13.2	5	12.2	2	5.6	9	20.5	0	0
Pathology of the nervous system	48	39.7	11	26.8	12	33.3	25***	56.8	0	0
Endocrine pathology	51	42.2	7	17.1	13	36.1	31**	70.5	2	16.7
Ophthalmic pathology	21	17.4	4	9.8	3	8.3	14	31.8	1	8.3
Lung pathology	16	13.2	0	0	4	11.1	12	27.3	0	0
Pathology of the genitourinary system	9	7.4	2	4.9	4	11.1	3	6.8	1	8.3
Gastrointestinal tract pathology	22	18.2	7	17.1	7	19.4	8	18.2	1	8.3
3 years										
Malnutrition	8	6.8	0	0	4	11.4	4	9.1	0	0
Pathology of the cardiovascular system	11	9.4	2	5.3	3	8.6	6	13.6	0	0
Pathology of the nervous system	40	34.2	10	26.3	10	28.6	20	45.5	3	25.0
Endocrine pathology	40	34.2	7	18.4	10	28.7	24**	54.6	2	16.7
Ophthalmic pathology	18	15.4	2	5.3	4	11.4	12*	27.3	0	0
Lung pathology	5	4.3	0	0	1	2.9	4	9.1	0	0
Pathology of the genitourinary system	10	8.6	3	7.9	4	11.4	3	6.8	3	25.0
Gastrointestinal tract pathology	17	14.5	6	15.8	4	11.4	7	15.9	1	8.3

# $p < 0.05$ relative to the control group (Pearson $\chi 2$ test); * $p < 0.05$ relative to the LBW group and the control group (Pearson $\chi 2$ test); ** $p < 0.05$ relative to the control group, LBW and VLBW groups (Pearson $\chi 2$ test); *** $p < 0.05$ relative to the LBW and VLBW groups at birth (Pearson $\chi 2$ test).

Social predictors of a favorable clinical prognosis for premature infants in early childhood

During the follow-up, some children were a posteriori assigned to a group of infants with improved health. This group consisted of children who moved to a more favorable health group during subsequent visits, for example, from IV to III health group, from III to II health group. It was found that 57.1% (n = 36) of children with ELBW, 34.9% (n = 23) of children with VLBW, and 32.9% (n = 23) of children with LBW at birth had improved health in early childhood.

An analysis of possible social predictors (such as age, anthropometric data, education and social status of parents, income, presence of bad habits, and chronic diseases) of a favorable clinical prognosis (assignment to a more favorable health group at subsequent visits or maintenance of I, II or III health group in the absence of exacerbation and decompensation of chronic diseases) for premature infants included in the study was conducted during the follow-up period (Fig).

A statistically significant predictor associated with improved health status of premature infants in early childhood, according to the results of our study, was the presence of siblings. The probability of a positive clinical prognosis increased 3-fold among children in the main group (OR = 2.6 [95% CI 1.3–5.3], p = 0.006) and 8-fold among children with ELBW at birth (OR = 8.4 [95% CI 1.0–69.6], p = 0.045). At the same time, according to the logistic regression model, with each subsequent child born in the family, the probability of a favorable clinical prognosis for premature infants increased (OR = 14.2 [95% CI 1.7–118.8], p = 0.007).

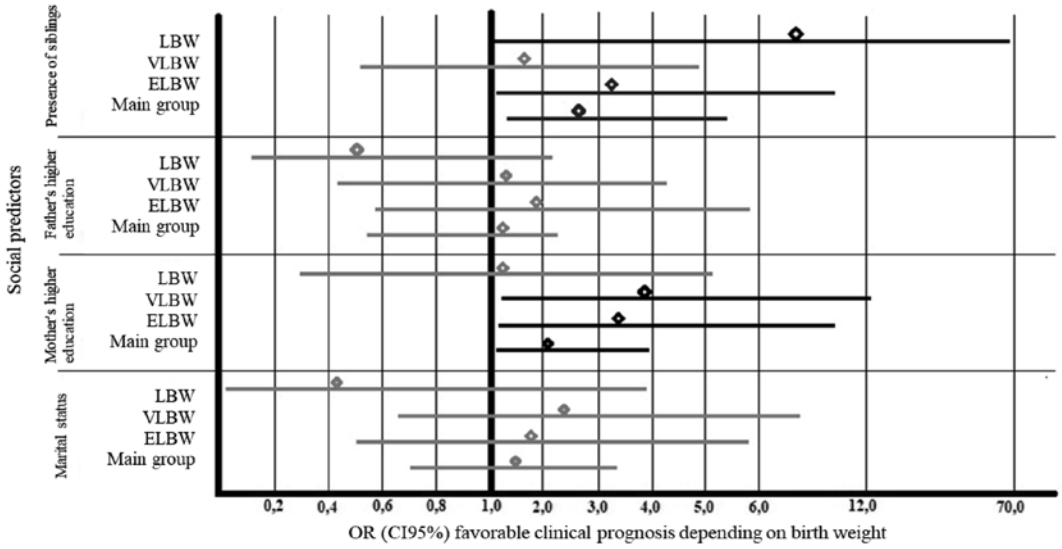

Fig. Social predictors of a favorable clinical prognosis: ** for premature infants in early childhood based on their birth weight. * higher education; ** assignment to a more favorable health group (e.g. transition from IV to III health group, from III to II health group) at subsequent visits or maintenance of I, II or III health group in the absence of exacerbation and decompensation of chronic diseases.

According to the results of the study, a positive predictor of the health status of children in the main group was the presence of higher education for the mother. This factor statistically significantly increased the chance of a favorable clinical prognosis in premature infants with VLBW at birth by 4 times (OR = 3.9 [95% CI 1.2–12.2], p = 0.018) and with LBW at birth by 3 times (OR = 3.4 [95% CI 1.2–9.9], p = 0.025).

The data analysis using the logistic regression method revealed that a decrease in the number of cigarettes consumed per day by the father was associated with a favorable clinical prognosis of premature infants in the groups with LBW (OR = 0.1 [95% CI 0.0–0.6], p = 0.012) and ELBW (OR = 0.001 [95% CI 0.0–0.9], p = 0.009).

Perinatal predictors determining the health status for premature infants in early childhood

The study analyzed the contribution of several perinatal factors (such as anthropometric data at birth, gestational age, presence of intrauterine growth

retardation, intraventricular hemorrhage, severe asphyxia and seizures in the neonatal period, presence of lateral ventricular dilation, invasive ventilation, and infectious diseases in the neonatal period). A number of predictors associated with an increased likelihood of developing pathological abnormalities in individual functional systems and individual nosological units depending on birth weight at the age of one, two, and three years were identified.

According to the results of the study, one of the significant perinatal factors worsening the prognosis for several systems among premature infants of all groups was severe anemia requiring blood transfusion in the early neonatal period. The presence of this deviation increased the probability of nervous system pathology (OR = 6.2 [95% CI 1.4–25.5], p = 0.008) and ophthalmic pathology (OR = 5.5 [95% CI 1.1–28.9], p = 0.029) by 6 times at the age of one year among children with LBW at birth. It also increased the probability of having 4 or more pathological deviations in different functional systems of the body among children with VLBW at birth by 4 times at the age of one year (OR = 4.3 [95% CI 1.3–13.9], p = 0.012) and the probability of having neurological pathology by 5 times at the age of two years (OR = 4.7 [95% CI 1.1–22.7], p = 0.043).

The study established that for premature infants with low birth weight (LBW) who experienced intraventricular hemorrhages (IVH) and intrauterine growth retardation (IUGR) were more likely to develop pathological abnormalities in multiple functional systems during early childhood.

IVH increased the likelihood of developing transient hypothyroidism among premature infants with LBW by 4 times at the age of one year (OR = 4.1 [95% CI 1.0–16.0], p = 0.034) and by 10 times at the age of two years (OR=10.3 [95% CI 1.7–64.0], p = 0.020), as well as anemia at one year by 11 times (OR = 11.1 [95% CI 2.2–56.9], $p < 0.001$). Premature infants with a birth weight of 1,500 to 2,500 grams with IUGR were statistically significantly more likely to have malnutrition at the age of one year (OR = 5.1 [95% CI 1.0–25.8], p = 0.033) and psychomotor developmental delay (OR = 16.0 [95% CI 1.6–155.0], p = 0.003). IUGR also increased the likelihood of a neurological diagnosis among children with LBW by 9 times at the age of three years (OR=8.7 [95% CI 1.3–58.9], p = 0.014).

When analyzing the results of instrumental studies among premature infants in the studied cohort, it was revealed that a significant predictor of nervous system pathology in early childhood was dilation of the lateral ventricles, as identified by neurosonography (NSG) during infancy (from 1 to 12 months of age), even without the development of hydrocephalus and hypertension syndrome. This deviation increased the likelihood of a neurological diagnosis among children with LBW by 5 times at the age of one year (OR = 5.0 [95% CI 1.2–20.5], p = 0.017) and two years (OR = 5.4 [95% CI 1.1–26.5], p = 0.028). Additionally, dilation of the lateral ventricles according to neurosonography results increased the chance of persistent transient hypothyroidism among children with ELBW by 5 times (OR = 4.6 [95% CI 1.1–20.2, p = 0.034) at the age of two years.

Social predictors determining the health status of premature infants in early childhood

The study analyzed a number of socioeconomic risk factors (such as age, anthropometric data, education and social status of parents, bad habits, chronic diseases, and income of parents) and identified several predictors associated with an increased likelihood of developing pathological abnormalities in individual functional systems among premature infants.

When analyzing the anthropometric data of parents, it was found that one of the significant predictors of nervous system pathology among premature infants in all groups included in the study was the mother's body mass index (BMI) > 25. At the age of one year, among premature infants with ELBW at birth, whose mothers had a BMI over 25 at the beginning of pregnancy. There was a statistically significant increase in the occurrence of a combination of four or more pathological deviations in different functional systems (OR = 3.8 [95% CI 1.1–13.3]), p = 0.047), psychomotor developmental delay (OR = 3.7 [95% CI 1.0–13.1]), p = 0.037), visual pathology (OR = 6.6 [95% CI 1.8–23.2]), p = 0.002), and the course of bronchopulmonary dysplasia (BPD) (OR = 5.4 [95% CI 1.5–19.0]), p = 0.013). At the age of two years, among premature infants, regardless of birth weight, whose mothers had a BMI over 25, there was a statistically significant increase in delayed speech development (OR = 2.5 [95% CI 1.0–5.9], p = 0.041). This predictor was also associated with the likelihood of a longer course of transient hypothyroidism at the age of two years among children with LBW (OR = 9.7 [95% CI 1.1–89.9]), p = 0.022).

The study established that one of the significant factors in maternal history, as a predictor of an unfavorable clinical prognosis for the group of children with a birth weight of less than 1,000 grams, is maternal smoking before and during pregnancy. This bad habit in the mother increased the likelihood of BPD persistence by 5 times (OR = 4.7 [95% CI 1.5–15.3], p = 0.007), visual pathology (OR = 4.1 [95% CI 1.3–12.6], p = 0.013), and the development of anemia at the age of one year (OR = 3.5 [95% CI 1.2–10.7], p = 0.023) by 4 times among premature infants with ELBW at birth.

Another significant social predictor of the development of pathological deviations in premature infants with a birth weight of less than 1,500 grams, according to the results of our study, was the mother's lack of higher education. Children with VLBW, whose mothers did not receive a higher education before the child's birth, were significantly more likely to have BPD at the age of one year (OR = 5.4 [95% CI 1.8–15.8]), p = 0.002). The lack of mother's higher education increased the likelihood of a neurological diagnosis in a child with VLBW at the age of two years by 6 times (OR = 6.1 [95% CI 1.2–30.1]), p = 0.021) and a combination of four or more pathological deviations among children with VLBW at the age of three years by 4 times (OR = 4.2 [95% CI 1.2–15.4], p = 0.026).

One more significant factor in parental anamnestic data associated with the clinical prognosis of premature infants in the study sample was age over 35 years. This predictor, both on the mother's and father's side, increased the likelihood of transient hypothyroidism among children with LBW at the age of two and three years.

DISCUSSION

The study found that children born with ELBW have different health statuses and are more likely to have pathological abnormalities in their nervous, endocrine, respiratory, and visual systems compared to premature infants with a birth weight of over 1,000 grams. This is consistent with previous studies [12, 13].

These pathological deviations that occur in the neonatal period largely determine the health status of premature infants at an older age. According to our study results, significant perinatal risk factors for the development of diseases of the nervous system, visual pathology, anemia, persistence of pulmonary pathology in premature infants depending on birth weight in early childhood include IUGR, IVH, severe anemia in the neonatal period, and dilation of the lateral ventricles. According to A.I. Safina et al. (2020) and A.K. Mironova et al. (2023), predictors of the development of disabling conditions in early childhood include gestational age less than 28 weeks, extremely low birth weight, oxygen dependence for more than 28 days after birth, grade 3 intraventricular hemorrhage, and periventricular leukomalacia [5, 13, 14].

A long-term cohort study by Yu.E. Shmatova et al. (2022) examined risk factors for children's health from the mother before and during pregnancy and found that maternal age over 40, smoking, single mother status, and mother's thyroid pathology can worsen the clinical prognosis in healthy full-term children in early childhood and primary school age [15]. During our study, for the first time in Russia, we obtained the data on the role of socioeconomic predictors associated with the clinical prognosis of premature infants based on birth weight. We found that the age of the parents over 35 years, the mother's BMI over 25, the level of mother's education, and parental smoking affect the health of premature infants throughout early childhood. A number of international studies have noted that maternal obesity affects the perinatal development of the brain in premature infants according to diffusion tensor imaging, which may be a pathogenetic rationale for the indirect effect of maternal obesity on the neuropsychic development of children [16, 17]. J.T. Bangma et al. (2020) in a systematic review found that maternal obesity and low socioeconomic status increase neonatal systemic inflammation and disrupt placental programming, which further worsens the health prognosis of premature infants with ELBW [18]. Our results can be used to carry out preventive measures among women planning pregnancy, pregravid preparation, as well as to create an individual plan for regular health check-up of premature infants whose mothers have these risk factors [19].

As a result of our study, it was established for the first time that the mother's higher education and the presence of siblings were significant predictors of a favorable clinical prognosis for premature infants, especially with ELBW, during a three-year follow-up. It was also established that the more children in the family, the greater the likelihood of improving the health of the premature infant in early childhood.

This fact may be associated with a fact that woman with higher education and experience raising her own children is more aware of the importance and possibility of regular health check-up and rehabilitation measures.

CONCLUSION

The conducted cohort prospective observational study of premature infants has provided unique data on the health status of children during the follow-up period. The study has also allowed us to identify key perinatal and social predictors of prognosis.

To implement a health-preserving strategy for the group of premature infants, it is crucial to improve not only the methods of nursing and neonatal care, but also to promote the principles of pre-conception preparation. This includes increasing awareness among the female population of childbearing age about the need to quit smoking, prevent and treat obesity, promote higher education among women, and support large families.

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Authors' contribution

Fedorova O.S. – conception and design, study coordination, drafting of the manuscript, critical revision of the manuscript for important intellectual content, and final approval of the manuscript for publication. Deev I.A. – conception and design, study coordination, drafting of the manuscript, and final approval of the manuscript for publication. Kulikova K.V. – database compilation, face-to-face visits, obtaining and interpreting clinical data, critical revision of the manuscript for important intellectual content, and final approval of the manuscript for publication. Khodkevich P.E. – literature analysis, database compilation, face-to-face visits, obtaining and interpreting clinical data, statistical processing of study results, and final approval of the manuscript for publication.

Authors' information

Khodkevich Polina E. – Neonatologist, Neonatal Pathology Department, Children's Hospital No. 1; Assistant Professor of the Intermediate-Level Pediatrics Division with a Pediatric Diseases Course, General Medicine Department, Siberian State Medical University, Tomsk, pkhodkevich@mail.ru, http://orcid.org/0000-0001-7639-1747.

Fedorova Olga S. – Dr. Sci. (Med.), Vice-Rector for Postgraduate Education and Research, Head of the Intermediate-Level Pediatrics Division with a Pediatric Diseases Course, General Medicine Department, Siberian State Medical University, Tomsk, olga.sergeevna.fedorova@gmail.com, https://orcid.org//0000-0002-7130-9609.

Kulikova Kristina V. – Cand. Sci. (Med.), Head of the Neonatal Intensive Care Unit, I.D.Evtushenko Regional Perinatal Center, Tomsk, kristina.v.kulikova@gmail.com, http://orcid.org/0000-0001-8926-5918.

Deev Ivan A. – Dr. Sci. (Med.), Professor of the Department of Management, Healthcare Economics and Health Insurance, Pirogov Russian National Research Medical University, Moscow, ivandeyev@yandex.ru, http://orcid.org/0000-0002-4449-4810.

(✉) **Khodkevich Polina E.,** pkhodkevich@mail.ru

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Study of the spectrum of unsaturated fatty acids in the blood of men with diabetes mellitus in Novosibirsk (ESSE-RF3 in the Novosibirsk region)

Shramko V.S.[1], Simonova G.I.[1], Shcherbakova L.V.[1], Afanasieva A.D.[1], Balanova J.A.[2], Imaeva A.E.[2], Shalnova S.A.[2], Ragino Yu.I.[1]

[1]Research Institute of Internal and Preventive Medicine – Branch of Federal Research Center Institute of Cytology and Genetics, Siberian Division of the Russian Academy of Sciences (IIPM – Branch of IC&G SD of RAS)
175/1 B. Bogatkova Str., Novosibirsk, 630089, Russian Federation

[2] National Medical Research Center for Therapy and Preventive Medicine of the Ministry of Healthcare of the Russian Federation
10, Bldg. 3, Petroverigsky Lane, Moscow, 101990, Russian Federation

ABSTRACT

Aim. To study the content of unsaturated fatty acids (FAs) in blood plasma in men from Novosibirsk (*ESSE-RF3* in the Novosibirsk region) with established type 2 diabetes mellitus (DM2), newly diagnosed diabetes, and prediabetes, as well as to evaluate the associations of various types of FAs with the presence or absence of diabetes and fasting glucose levels.

Materials and methods. Within the framework of the multicenter, single-stage epidemiological ESSE-RF3 study in the Novosibirsk region, 1,200 residents of Novosibirsk (600 men, 600 women) aged 35–74 years were examined. The present study included 563 men with an average age of 54.4 ± 11.48 years, comprising: 61 individuals diagnosed with DM2 based on anamnestic data (Group I); 65 men with newly diagnosed diabetes (Group II); 46 men with conditional prediabetes (Group III); and 391 men without diabetes – (Group IV). The levels of unsaturated FAs in blood plasma were determined via high-performance liquid chromatography.

Results. An increase in omega-3, -6, and -9 FA levels was revealed in Group I compared to Group IV. An increase in the level of oleic acid ($p = 0.040$) was found in Group II compared to Group IV. The relative chance of DM2 is directly associated with an increase in the levels of omega-3 alpha-linolenic acid (odds ratio (OR) = 1.030, 95 confidence interval (CI) 1.002–1.058; $p = 0.034$) and omega-6 gamma-linolenic acid (OR = 1.026, 95 CI 1.001–1.051; $p = 0.044$). Newly diagnosed diabetes is inversely associated with the level of linoleic acid in blood plasma (OR = 0.545, 95 CI 0.301–0.996; $p = 0.048$), as well as directly associated with the level of oleic acid (OR = 1.961, 95 CI 1.054–3.648; $p = 0.034$). Prediabetes is inversely associated with the level of hexadecenoic acid (OR = 0.969, 95 CI 0.943–0.996; $p = 0.025$).

Conclusion. Detection of changes in the profile of unsaturated FAs in blood plasma can be used as an additional prognostic biomarker to identify patients at risk of developing DM.

Keywords: diabetes mellitus, fatty acids, blood, risk factors

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 participants of the study signed an informed consent. The study was approved by the local Ethics committee of IIPM – Branch of IC&G SD of RAS (Protocol No. 69 of 29.09.2020).

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✉ *Shramko Viktoriya S.,* Nosova@211.ru

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Исследование спектра ненасыщенных жирных кислот крови у мужчин с сахарным диабетом г. Новосибирска («ЭССЕ-РФ3» в Новосибирской области)

Шрамко В.С.[1], Симонова Г.И.[1], Щербакова Л.В.[1], Афанасьева А.Д.[1], Баланова Ю.А.[2], Имаева А.Э.[2], Шальнова С.А.[2], Рагино Ю.И.[1]

[1] Научно-исследовательский институт терапии и профилактической медицины – филиал Института цитологии и генетики СО РАН (НИИТПМ – филиал ИЦиГ СО РАН)
Россия, 630089, г. Новосибирск, ул. Б. Богаткова, 175/1

[2] Национальный медицинский исследовательский центр терапии и профилактической медицины (НМИЦ ТПМ)
Россия, 101990, г. Москва, Петроверигский пер., 10/3

РЕЗЮМЕ

Цель. Изучить содержание ненасыщенных жирных кислот (ЖК) плазмы крови у мужчин г. Новосибирска («ЭССЕ-РФ3» в Новосибирской области) с установленным сахарным диабетом 2-го типа (СД2), впервые диагностированным диабетом и «предиабетом», а также оценить ассоциации различных типов ЖК с наличием или отсутствием СД и уровнем глюкозы натощак.

Материалы и методы. В рамках многоцентрового одномоментного эпидемиологического исследования ЭССЕ-РФ3 по Новосибирской области были обследованы 1200 жителей г. Новосибирска (мужчин – 600, женщин – 600) в возрасте 35–74 лет. В исследование были включены 563 мужчины (средний возраст 54,4 ± 11,48 лет), из них: 61 человек с диагнозом СД2 на основании анамнестических данных – группа (I); 65 мужчин с впервые выявленным диабетом – группа (II); 46 мужчин с условным «предиабетом» – группа (III); 391 человек без СД – группа (IV). В плазме крови всем определяли уровень ненасыщенных ЖК методом высокоэффективной жидкостной хроматографии.

Результаты. В группе (I) выявлено увеличение содержания омега-3, -6, -9 ЖК при сравнении с группой (IV). В группе (II) установлено увеличение уровня олеиновой ЖК ($p = 0,040$) при сравнении с группой (IV). Наличие СД2 прямо ассоциировано с повышением уровня омега-3 альфа-линоленовой (отношение шансов (ОШ) = 1,030; 95-й доверительный интервал (ДИ) 1,002–1,058; $p = 0,034$) и омега-6 гамма-линоленовой ЖК (ОШ = 1,026; 95-й ДИ 1,001–1,051; $p = 0,044$). Наличие впервые выявленного СД обратно ассоциировано с содержанием в плазме крови линолевой кислоты (ОШ = 0,545; 95-й ДИ 0,301–0,996; $p = 0,048$) и прямо ассоциировано с уровнем олеиновой ЖК (ОШ = 1,961; 95-й ДИ 1,054–3,648; $p = 0,034$). Наличие «предиабета» обратно ассоциировано с содержанием гексадеценовой кислоты (ОШ = 0,969; 95-й ДИ 0,943–0,996; $p = 0,025$).

Заключение. Выявление изменений профиля ненасыщенных ЖК в плазме крови может использоваться в качестве дополнительного прогностического биомаркера для выявления пациентов с риском развития СД.

Ключевые слова: сахарный диабет, жирные кислоты, кровь, факторы риска

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

Источник финансирования. Работа проведена в рамках «ЭССЕ-РФ3» по Новосибирской области и бюджетной темы по государственному заданию № FWNR-2024-0004.

Соответствие принципам этики. Все участники исследования подписали информированное согласие. Исследование одобрено локальным этическим комитетом НИИТПМ – филиал ИЦиГ СО РАН (протокол № 69 от 29.09.2020).

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INTRODUCTION

According to the International Diabetes Federation, by 2021 there were about 537 million patients with diabetes mellitus (DM) in the world, and by 2045 their number is expected to increase to 783 million [1]. At the same time, type 2 DM (DM2) accounts for 90–95% of all cases of diabetes [2]. DM2 poses a significant risk to people's health and life expectancy. For example, the average life expectancy of a 50-year-old person with DM2 is six years shorter compared to people without DM2 [3].

According to the hypothesis of lipotoxicity, chronically elevated levels of free fatty acids (FA) may cause dysfunction of pancreatic β-cells and, thereby, disrupt insulin secretion, accelerating two major defects underlying the pathophysiology of DM2 [4]. However, the data on the association between different types of FA (omega-3 /-6 /-9) and the risk of developing DM2 are quite contradictory. For example, in the study by J. Shanta et al. [5], a direct effect of arachidonic acid (20 : 4, omega-6), and not its metabolites, was noted on the regulation of insulin secretion by β-cells. In the study by M.A.Lankinen et al. [6] levels of digomo-γ-linolenic acid (20 : 3, omega-6) in phospholipids and cholesterol esters, but not in triglycerides, were positively associated with the risk of developing DM2. In contrast, according to a recent meta-analysis, higher intake of linoleic acid (18 : 2, omega-6) and its higher concentrations in adipose tissue and blood are associated with a decrease in the risk of developing DM2 [7].

In experimental studies, with regard to supplements and/or a diet high in omega-3 polyunsaturated fats (PUFA), it has been shown that omega-3 FA (in particular, α-linolenic acid 18 : 3) can increase fasting glucose levels [8] and is directly associated with the risk of developing DM2 [9]. However, in a number of meta-analyses, the level of docosahexaenoic acid (20 : 3, omega-3) was lower in patients with DM2 compared with the control group of healthy subjects [10]. In total, it can be said that the composition of FA, rather than their total level, has a greater effect on insulin-glucose homeostasis.

The aim of the study was to study the levels of unsaturated FA in blood plasma in men from Novosibirsk (Epidemiology of Cardiovascular Diseases and Their Risk Factors in the Regions of the Russian Federation (ESSE–RF3) in the Novosibirsk region) with an established DM2 diagnosis, newly diagnosed diabetes, and prediabetes, as well as to evaluate the associations of various types of unsaturated FA with the presence/absence of DM and fasting glucose levels.

MATERIALS AND METHODS

The recruitment and examination of participants took place within the framework of the multicenter single-stage epidemiological *ESSE-RF3* study in 2020–2022. The study was approved by the Independent Ethics Committee of the National Medical Research Center for Therapy and Preventive Medicine [11]. Within the framework of this study, 1,200 residents of the Novosibirsk region aged 35–74 years (600 men, 600 women) were examined at the premises of the IIPM – Branch of IC&G SB RAS.

The diagnosis of DM2 was established on the basis of anamnestic data and included the study participants who positively answered the questions: "Has your doctor ever told you that you have/had the following diseases – diabetes mellitus?" and "Is the type of diabetes mellitus type 2?". The individuals with fasting glucose levels in plasma of ≥ 7.0 mmol/l, without a previous history of diabetes, and who were not taking hypoglycemic drugs were classified as patients with newly diagnosed diabetes. The respondents who answered positively to the question: "Has a doctor or other medical professional told you that you have an elevated sugar level in blood?", but whose blood glucose level at the moment of screening was < 7.0 mmol/l and who were not taking hypoglycemic drugs, were classified as patients with conditional prediabetes.

The blood samples for laboratory tests (namely, levels of total cholesterol, cholesterol, high-density and low-density lipoprotein cholesterol, triglycerides, and glucose) were collected from all participants under fasting conditions by venipuncture from the cubital vein. The tests were performed in the laboratory of the National Medical Research Center for Therapy and Preventive Medicine (Moscow) [12]. The levels of the parameters in blood serum were determined using an Abbot Architect c8000 biochemical analyzer (USA) and diagnostic kits from Abbot Diagnostic (USA). Recalculation of serum glucose into plasma glucose was carried out using The European Association for the Study of Diabetes (2005) formula: plasma glucose (mmol/l) = –0.137 + 1.047 × serum glucose (mmol/l).

Additionally, the following levels of omega-3 PUFA were determined in the blood plasma: alpha-linolenic acid (C 18 :3), eicosapentaenoic acid (C 20 : 5), docosahexaenoic acid (C 22 : 6); omega-6 PUFA: linoleic acid (C 18 : 2), gamma-linolenic acid (C 18 : 3), dihomo-gamma-linolenic acid (C 20 : 3), arachidonic acid (C 20 : 4), docosatetraenoic acid (C 22 : 4), docosapentaenoic acid (C 22 : 5); omega-9 FA: hexadecenoic acid (C 16 : 1), oleic acid (C 18 : 1), mead acid (C 20 : 3), nervonic acid (C 24 : 1). These levels were determined by the method of high-performance liquid chromatography at the premises of the IIPM – Branch of IC&G SB RAS (Novosibirsk).

The values obtained during the study were statistically processed using the SPSS 13.0 statistical software package. The Kolmogorov – Smirnov test was used to assess the distribution of features. Due to their abnormal distribution, descriptive statistics for continuous features is presented in the form of *Me* [25%; 75%]. The nonparametric Mann – Whitney *U*-test was used to compare the groups. The Pearson Criterion ($\chi 2$) was used to determine the statistical significance of differences in qualitative characteristics. The associations of FA with DM/newly diagnosed DM/prediabetes were studied using a multifactorial logistic regression model (with standardization by age, body mass index (BMI), and plasma glucose levels). The results are presented as the odds ratio (OR) and 95% confidence interval (CI). The differences were considered statistically significant at $p < 0.05$.

RESULTS

The present study included 563 men, with an average age of 54.4 ± 11.48 years. The study participants were divided into four groups, depending on the presence/absence of a DM2 diagnosis and fasting glucose levels above/below 7.0 mmol/l: group (I) – 61 men with an established diagnosis of DM2; group (II) – 65 men with newly diagnosed diabetes; group (III) – 46 men with conditional prediabetes; group (IV) – 391 people without diabetes. Table 1 shows the distribution of blood plasma FA by groups.

Table 1

The content of FA in blood plasma in men of the study groups

Fatty acid	Group (I), $n = 61$	Group (II), $n = 65$	Group (III), $n = 46$	Group (IV), $n = 391$	p_{I-IV}	p_{II-IV}	p_{III-IV}
Alpha-linolenic acid 18 : 3 omega-3	86.0 [64.5; 114.5]	69.0 [55.5; 92.0]	74.0 [52.0; 94.25]	68.0 [54.0; 92.0]	0.001	0.581	0.955
Eicosapentaenoic acid 20 : 5 omega-3	40.0 [24.5; 62.0]	31.0 [22.0; 52.5]	32.0 [19.75; 62.75]	30.0 [19.0; 48.0]	0.002	0.169	0.270
Docosahexaenoic acid 22 : 6 omega-3	124.5 [80.0; 171.5]	124.0 [55.5; 178.5]	96.5 [52.25; 176.0]	10.0 [53.0; 159.0]	0.040	0.230	0.899
Linoleic acid * 18 : 2 omega-6	3.52 [3.07; 3.8]	3.13 [1.94; 3.72]	3.14 [1.6; 3.98]	3.22 [1.89; 3.76]	0.021	0.823	0.738
Gamma-linolenic acid 18 : 3 omega-6	88.0 [48.5; 112.0]	64.0 [37.5; 98.0]	48.0 [27.75; 101.5]	59.0 [33.0; 86.0]	0.001	0.480	0.786
Dihomo-gamma-linolenic acid 20 : 3 omega-6	139.0 [74.0; 216.5]	98.0 [54.5; 153.0]	111.0 [49.0; 190.25]	91.0 [53.0; 155.0]	0.001	0.712	0.805
Arachidonic acid * 20 : 4 omega-6	1.13 [0.72; 1.33]	1.06 [0.42; 1.3]	0.90 [0.35; 1.32]	0.97 [0.41; 1.26]	0.015	0.374	0.783
Docosatetraenoic acid 22 : 4 omega-6	27.5 [19.5; 32.75]	27.0 [19.0; 34.0]	21.0 [12.75; 33.0]	25.0 [14.0; 33.0]	0.086	0.118	0.499
Docosapentaenoic acid 22 : 5 omega-6	32.0 [20.0; 44.0]	28.0 [8.0; 43.5]	24.0 [7.75; 41.25]	26.0 [8.0; 40.0]	0.041	0.427	0.839
Hexadecenoic acid 16 : 1 omega-9	46.0 [29.5; 75.0]	40.0 [19.0; 58.0]	32.0 [17.0; 59.25]	39.0 [19.0; 62.0]	0.004	0.994	0.330
Oleic acid * 18 : 1 omega-9	2.09 [1.63; 2.88]	2.14 [1.19; 3.04]	1.44 [0.77; 2.51]	1.66 [0.96; 2.64]	0.007	0.040	0.204
Mead acid 20 : 3 omega-9	25.0 [12.5; 33.0]	23.0 [5.5; 34.0]	12.5 [4.0; 30.0]	18.0 [4.0; 29.0]	0.017	0.072	0.664
Nervonic acid 24 : 1 omega-9	73.0 [56.0; 95.5]	67.0 [43.5; 94.5]	60.0 [42.75; 95.25]	63.0 [46.0; 89.0]	0.025	0.602	0.845

* units of measurement for FA in μmol/ml.

In group (I) of men with established DM2, an increase in the relative content of omega-3 PUFA was revealed: alpha-linoleic acid by 26% (p = 0.001), eicosapentaenoic acid by 33% (p = 0.002), docosahexaenoic acid by 17% (p = 0.040); omega-6 PUFA: linoleic acid by 9% (p = 0.021), gamma-linolenic acid by 49% (p = 0.001), dihomo-gamma-linolenic acid by 52% (p = 0.001), arachidonic acid by 16% (p = 0.015), docosapentaenoic acid by 23% (p = 0.041); and omega-9 FA: hexadecenoic acid by 18% (p = 0.004), oleic acid by 26% (p = 0.007), mead acid by 38% (p = 0.017), nervonic acid by 15% (p = 0.040), when compared with group (IV) of men without diabetes (Table 1).

In group (II) of men with newly diagnosed diabetes, an increase in the relative content of only omega-9 oleic acid by 29% (p = 0.040) was found, when compared with group (IV) of men without diabetes (Table 1).

The levels of unsaturated FA in men with conditional prediabetes (group III) were similar to the levels in samples obtained from men of the same age without diabetes (group IV). When comparing groups III and IV, no significant difference was found (Table 1).

Table 2

The results of a multifactorial logistic regression analysis of DM2 association depending on the level of FA, μmol/ml

Fatty acid	OR (95% CI for OR), p
Alpha-linolenic acid, 18 : 3 omega-3	1.030 (1.002–1.058), 0.034
Eicosapentaenoic acid, 20 : 5 omega-3	0.993 (0.962–1.025), 0.685
Docosahexaenoic acid, 22 : 6 omega-3	0.986 (0.967–1.006), 0,.60
Linoleic acid, 18 : 2 omega-6	0.639 (0.287–1.422), 0.272
Gamma-linolenic acid, 18 : 3 omega-6	1.026 (1.001–1.051), 0.044
Dihomo-gamma-linolenic acid, 20 : 3 omega-6	1.003 (0.990–1.015), 0.680
Arachidonic acid, 20 : 4 omega-6	1.002 (0.999–1.005), 0.300
Docosatetraenoic acid, 22 : 4 omega-6	0.956 (0.874–1.045), 0.319
Docosapentaenoic acid, 22 : 5 omega-6	1.019 (0.971–1.068), 0.447
Hexadecenoic acid, 16 : 1 omega-9	1.007 (0.971–1.045), 0.694
Oleic acid, 18 : 1 omega-9	1.848 (0.691–4.940), 0.221
Mead acid, 20 : 3 omega-9	0.943 (0.861–1.032), 0.200
Nervonic acid, 24 : 1 omega-9	0.998 (0.973–1.023), 0.885

Note: CI – confidence interval; OR – odds ratio.

Then, the probability of having DM2 was estimated via the Model 1 of multivariate logistic regression analysis (Table 2). The dichotomous parameter – the presence or absence of DM2 (according to the anamnestic data) was used as a dependent variable; the studied unsaturated FA were used as independent variables. The analysis of Model 1 was carried out with standardization by age, BMI, and plasma glucose content.

According to the results, the relative odds of having DM2 are directly associated with an increase in the levels of omega-3 alpha-linolenic acid (OR = 1.030, 95% CI 1.002–1.058; p = 0.034) and omega-6 gamma-linolenic acid (OR = 1.026, 95% CI 1.001–1.051; p = 0.044). With an increase in their level by 1 nmol/ml, the probability of having DM2 in men increases by 3 and 2.6%, respectively.

The subsequent stepwise multivariate logistic regression analysis showed independent associations of some of the FA studied with the odds of having newly diagnosed diabetes (Model 2), with standardization by age and BMI (Table 3).

Table 3

The results of a multivariate logistic regression analysis of associations of newly diagnosed diabetes depending on the level of FA, μmol/ml

Fatty acid	OR (95% CI for OR), p
Alpha-linolenic acid, 18 : 3 omega-3	1.013 (0.995—1.031), 0.161
Eicosapentaenoic acid, 20 : 5 omega-3	0.987 (0.961—1.014), 0.342
Docosahexaenoic acid, 22 : 6 omega-3	1.004 (0.990—1.019), 0.553
Linoleic acid, 18 : 2 omega-6	0.545 (0.301—0.996), 0.048
Gamma-linolenic acid, 18 : 3 omega-6	1.004 (0.989—1.020), 0.590
Dihomo-gamma-linolenic acid, 20 : 3 omega-6	0.998 (0.989—1.008), 0.744
Arachidonic acid, 20 : 4 omega-6	1.431 (0.147—3.896), 0.757
Docosatetraenoic acid, 22 : 4 omega-6	1.001 (0.937—1.069), 0.979
Docosapentaenoic acid, 22 : 5 omega-6	0.994 (0.958—1.032), 0.760
Hexadecenoic acid, 16 : 1 omega-9	0.988 (0.962—1.016), 0.401
Oleic acid, 18 : 1 omega-9	1.961 (1.054—3.648), 0.034
Mead acid, 20 : 3 omega-9	0.998 (0.939—1.061), 0.949
Nervonic acid, 24 : 1 omega-9	1.002 (0.985—1.019), 0.841

Note: CI – confidence interval; OR – odds ratio.

Newly diagnosed diabetes in men aged 35–74 years is inversely associated with the level of linoleic acid in blood plasma (OR = 0.545, 95% CI 0.301–0.996; p = 0.048), as well as directly

associated with the level of oleic acid (OR = 1.961, 95% CI 1.054–3.648; p = 0.034). Thus, with a decrease in the concentration of omega-6 linoleic PUFA by 1 μmol/ml, the chance of having newly diagnosed diabetes increases by 45%; with an increase in the level of omega-9 oleic acid by 1 μmol / ml, the chance of having newly diagnosed diabetes is 1.96 times higher.

In the Model 3 of multivariate logistic regression analysis, the probability of having prediabetes depending on the level of the studied unsaturated FA was estimated (Table 4). The analysis of Model 3 was carried out with standardization by age and BMI.

Table 4

The results of a multivariate logistic regression analysis of associations of prediabetes depending on the level of FA, μmol / ml

Fatty acid	OR (95% CI for OR), p
Alpha-linolenic acid, 18 : 3 omega-3	1.001 (0.984—1.019), 0.887
Eicosapentaenoic acid, 20 : 5 omega-3	1.023 (0.997—1.049), 0.080
Docosahexaenoic acid, 22 : 6 omega-3	1.007 (0.994—1.020), 0.278
Linoleic acid, 18 : 2 omega-6	1.042 (0.606—1.793), 0.881
Gamma-linolenic acid, 18 : 3 omega-6	1.004 (0.990—1.017), 0.592
Dihomo-gamma-linolenic acid, 20 : 3 omega-6	1.002 (0.994—1.011), 0.579
Arachidonic acid, 20 : 4 omega-6	0.814 (0.102—3.510), 0.846
Docosatetraenoic acid, 22 : 4 omega-6	0.951 (0.895—1.010), 0.100
Docosapentaenoic acid, 22 : 5 omega-6	0.996 (0.962—1.031), 0.827
Hexadecenoic acid, 16 : 1 omega-9	0.969 (0.943—0.996), 0.025
Oleic acid, 18 : 1 omega-9	0.657 (0.344—1.256), 0.204
Mead acid, 20 : 3 omega-9	1.007 (0.953—1.064), 0.801
Nervonic acid, 24 : 1 omega-9	1.008 (0.992—1.024), 0.334

Note: CI – confidence interval; OR – odds ratio.

When studying the results, it was found that prediabetes, regardless of age and BMI, was inversely associated with the level of hexadecenoic acid (OR = 0.969, 95% CI 0.943–0.996; p = 0.025). A decrease in the level of omega-9 hexadecenoic FA by 1 nmol / ml increases the chance of having prediabetes by 3.1%.

DISCUSSION

Presently, almost one in eleven people between the ages of 20 and 79 years suffers from diabetes [1]. Moreover, diabetes often remains undiagnosed for a long time, since an increase in blood glucose levels develops gradually. In addition, clinical manifestations in the early stages are often not severe enough for the patient to notice the classic symptoms of diabetes. Nevertheless, even undiagnosed patients have an increased risk of developing macro- and microvascular complications [2]. This, in turn, stresses the expediency of conducting an early diagnostic examination to detect diabetes, as well as timely initiation of drug treatment to reduce the risk of complications.

There is evidence that DM is closely associated with lipid metabolism disorders, especially with the involvement of free FA. Increased levels of free FA in blood can cause insulin resistance, disruption of insulin signaling pathways, and destruction of β-cells. However, different types of FA have different and even opposite effects during the development and course of insulin resistance and DM2 [4]. Therefore, studying the relationship between specific types of FA and DM is more important than studying the general level of FA.

Linoleic acid (18 : 2) belongs to the family of essential omega-6 PUFAs. It is found in significant amounts in vegetable oils and nuts [13]. It is known that linoleic PUFA is a biochemical precursor of pro-inflammatory metabolites, especially eicosanoids and endocannabinoids. Therefore, it is generally accepted that its excess in the diet is directly or indirectly associated with markers of inflammation and metabolic diseases (such as obesity). However, pro-inflammatory effects may depend on the complex interaction of metabolic factors and have not been definitively proven [14].

Similar contradictions are also observed in the studies of linoleic acid as a biomarker. Some studies show that higher 18 : 2 levels in blood or adipose tissue are associated with a lower risk of developing DM2 [15, 16]. Other studies do not show any significant association, or contain contradictory data about the association between linoleic acid in blood and DM2 [17, 18]. In this study, in men aged 35–74 years, the level of linoleic acid was inversely associated with

newly diagnosed diabetes and was significantly higher in the group with an established diagnosis of DM2 (based on anamnestic data). According to a meta-analysis of prospective studies by S.M. Mousavi et al. [7], each 5% increase in linoleic PUFA intake is associated with a 10% decrease in the risk of developing DM2, while a 15% decrease in the risk of developing diabetes is observed with an increase in the 18 : 2 level as a biomarker. The results of the EPIC-InterAct Case-Cohort Study [15] also provide convincing evidence of a strong inverse association between linoleic FA levels in blood and DM2. In this study, we only analyzed linoleic acid levels at baseline and did not assess changes over time from newly diagnosed to established diabetes, which could have led to these differences. In addition, it is necessary to take into account the total amount of dietary intake of linoleic PUFA, which was not evaluated in this study and needs further confirmation.

Gamma-linolenic PUFA (18 : 3, omega-6) belongs to the class of essential omega-6 FA. It usually gets to the human body as part of dietary supplements. Due to the rapid conversion to omega-6, dihomo-gamma-linolenic PUFA is found in low concentrations in blood and tissues [19]. Therefore, the increased content of gamma-linolenic acid cannot be associated with high food consumption. In a prospective study by Z. Miao et al. [17], it was found that baseline levels of 18 : 3 (omega-6) in erythrocytes were positively associated with DM2, regardless of BMI and other potential factors influencing the result. The authors suggested that high levels of circulating gamma-linolenic acid may be a risk factor for developing DM2.

As part of the Kuopio Ischaemic Heart Disease Risk Factor Study [20], it was also found that higher concentrations of gamma-linolenic and dihomo-gamma-linolenic PUFA may be associated with a higher risk of developing DM2. In this study, the levels of gamma-linolenic acid were the highest in people with an established diagnosis of DM2. In addition, regardless of risk factors (age, BMI, plasma glucose levels), direct associations were found between gamma-linolenic acid and DM2, which may indicate the importance of this PUFA in the development of the disease.

Alpha-linolenic acid (18 : 3, omega-3) is the most common PUFA of omega-3 class. However, the human body cannot synthesize this PUFA; therefore, it must be obtained from food [13]. Among the well–known edible oils, linseed oil has the highest content of alpha-linolenic acid and is an excellent source of supplements. In experimental animal studies, it has been shown that alpha-linolenic acid intake can improve the lipid profile, treat diabetes and reduce complications caused by diabetes, as well as reduce the risk of developing diseases of the circulatory system [21]. However, one large systematic review registered on PROSPERO found no evidence that alpha-linolenic PUFA alters glucose metabolism or affects the risk of developing diabetes [8].

According to 20 prospective cohort studies (https://force.nutrition.tufts.edu/), the levels of alpha-linolenic acid in blood/adipose tissue (of plant origin) also were not significantly associated with DM2 [22]. However, some of the data presented in large meta-analyses on the effects of alpha-linolenic acid are either limited or of low quality. We established a higher level of alpha-linolenic PUFA in the group of men with DM2 (based on anamnestic data), and in Model-1 of the regression analysis it was a significant indicator affecting the presence of diabetes, which contradicts most literature data and, of course, requires further study.

Oleic acid (18 : 1) is one of the most common omega-9 monounsaturated FA (MUFA), found in both animal and vegetable oils. It accounts for approximately 80% of MUFA in the composition of total plasma lipids [13]. It is believed that oleic acid can stimulate the secretion of glucagon-like peptide-1, thereby showing its protective effect in patients with DM2 [23]. Moreover, it can increase insulin sensitivity by suppressing the production of reactive oxygen species and regulating the activity of matrix metalloproteinases-2 [24]. In contrast, a large prospective cohort study [25] did not show any significant overall association between circulating oleic acid and diabetes. The study by T. Plotz et al. [26] records the toxicity of

oleic acid and its inability to neutralize the toxic effects of palmitic acid. A cross-sectional study by M. Kang et al. [27] found that an increase in 18 : 1 and desaturase activity can lead to impaired metabolism of FA (including MUFA) and dysfunction of adipose tissue. Consequently, this promotes hypersecretion of proatherogenic, proinflammatory, and prodiabetic adipocytokines that contribute to the development of DM2. In this study, higher levels of oleic acid in men with established and newly diagnosed diabetes, as well as direct associations with the chance of having newly diagnosed diabetes are probably associated with the characteristic accumulation of fat in the anterior abdominal wall and viscerally, which is often found in men.

Hexadecenoic FA (16 : 1 omega-9) is another omega-9 MUFA that can be produced by partial β-oxidation of oleic acid. It is mainly found in neutral lipids (triglycerides and cholesterol esters) and monocytes [28]. Very little information is available about the hexadecenoic FA. This MUFA is either barely mentioned in studies or occurs in epidemiological articles, where FA are simply indicated as a secondary component of certain tissues, without taking into account possible biological or biochemical phenomena [29]. Recent studies have shown that omega-9 hexadecenoic FA exhibits a strong anti-inflammatory effect *in vitro* and *in vivo*, which differs from the effect of omega-7 palmitoleic acid (16 : 1), and is comparable in magnitude to the effect of omega-3 PUFA [29]. In our case, the concentrations of hexadecenoic MUFA did not differ in the studied groups, however, in the Model-3 regression analysis, an inverse association with prediabetes was revealed. Despite the fact that we did not evaluate the activity of this omega-9 MUFA, the results obtained can be used in new areas of research.

CONCLUSION

In the course of the comparative study, it was shown that the levels of unsaturated FA in the blood plasma of men with established DM2 significantly differ from the levels of FA in individuals with newly diagnosed DM, prediabetes, and without diabetes. In men aged 35–74 years, DM2 is directly associated with the level of alpha-linolenic and gamma-linolenic PUFA in blood plasma. Newly diagnosed DM is inversely associated with the level of linoleic acid and directly associated with the level of oleic acid. Prediabetes is inversely associated with the content of hexadecenoic acid. Thus, detection of changes in the profile of unsaturated FA can be used as an additional prognostic biomarker for identifying patients at risk of developing DM.

Limitations. Insulin sensitivity and secretion, which are important mechanisms contributing to the risk of developing DM2 and/or worsening glycaemia, were not assessed.

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Authors' contribution

V.S. Shramko – concept and design development; data analysis and interpretation; writing the article. Simonova G.I., Afanasieva A.D. – revisions to the manuscript to improve the scientific value of the article. Shcherbakova L.V. – data analysis and interpretation.

Balanova Yu.A. – final approval for the publication of the manuscript. Imaeva A.E., Shalnova S.A. – critically revision of the manuscript for important intellectual content. Ragino Yu.I. – concept and design development; final approval for the publication of the manuscript. All the authors approved the final version of the article before publication.

Authors' information

Shramko Viktoriya S. – Cand. Sci. (Med.), Researcher, Laboratory of Clinical Biochemical and Hormonal Studies of Therapeutic Diseases, Head of the Department of Clinical-Biochemical and Molecular Genetic Research Methods, IIPM – Branch of IC&G SB RAS, Novosibirsk, Russian Federation, Nosova@211.ru, https://orcid.org/0000-0002-0436-2549

Simonova Galina I. – Dr. Sci. (Med.), Professor, Honored Scientist of the Russian Federation, Chief Researcher, Laboratory of Etiopathogenesis and Clinic of Internal Diseases, IIPM – Branch of IC&G SB RAS, Novosibirsk, Russian Federation; g.simonova2019@gmail.com, https://orcid.org/0000-0002-4030-6130

Shcherbakova Liliya V. – Senior Researcher, Laboratory of Clinical, Population and Preventive Research of Therapeutic and Endocrine Diseases, Head of the Department of Extra-budgetary works, IIPM – Branch of IC&G SB RAS, Novosibirsk, Russian Federation, 9584792@mail.ru, https://orcid.org/0000-0001-9270-9188

Afanasieva Alena D. – Cand. Sci. (Med.), Head of the Laboratory of Genetic and Environmental Determinants of the Human Life Cycle IIPM – Branch of IC&G SB RAS, Novosibirsk, Russian Federation, alene.elene@gmail.com, 0000-0001-7875-1566

Balanova Julia A. – Dr. Sci. (Med.), Senior Researcher, Department of Epidemiology of Chronic Non-Communicable Diseases, National Health and Research Center of Preventive Healthcare of the Ministry of Health of Russia, Moscow, Russian Federation, jbalanova@gnicpm.ru, https://orcid.org/0000-0001-8011-2798

Imaeva Asiya E. – Dr. Sci. (Med.), Senior Researcher, Department of Epidemiology of Chronic Non-Communicable Diseases, National Health and Research Center of Preventive Healthcare of the Ministry of Health of Russia, Moscow, Russian Federation, AImaeva@gnicpm.ru, https://orcid.org/0000-0002-9332-0622

Shalnova Svetlana A. – Dr. Sci. (Med.), Professor, Head of the Department of Epidemiology of Chronic Non-Communicable Diseases, FGBU National Health and Research Center of Preventive Healthcare of the Ministry of Health of Russia, Moscow, Russian Federation, SShalnova@gnicpm.ru, https://orcid.org/0000-0003-2087-6483

Ragino Yulia I. – Dr. Sci. (Med.), Professor, Corresponding Member of the Russian Academy of Sciences, Head of the IIPM – Branch of IC&G SB RAS, Chief Researcher at the Laboratory of Clinical Biochemical and Hormonal Studies of Therapeutic Diseases; IIPM – Branch of IC&G SB RAS, Novosibirsk, Russian Federation, ragino@mail.ru, https://orcid.org/0000-0002-4936-8362

(✉) **Shramko Viktoriya S.,** Nosova@211.ru

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Efficiency of two available kits for amplification of three EGFR SNPs in patients with NSCLC: 181946 G/A (rs2293347), –191 C/A (rs712830) and –216G/T (rs712829) with GC-rich regions

Jurišić V.[1], Obradović J.[2], Tošić N., Pavlović S.[3], Gulaeva L.F.[4], Gershtein E.S.[5], Kushlinskii N.E.[5]

[1]*Faculty of Medical Sciences, Unversity of Kragujevac*
69, Svetozar Markovic Str., Kragujevac, 34000, Serbia

[2]*Institute of Biology and Ecology, Faculty of Science, University of Kragujevac,*
12, Radoja Domanovicha Str., Kragujevac, 34000, Serbia

[3]*Institute of Molecular Genetics and Genetic Engineering, University of Belgrade*
1, Student Ave., Belgrade, 11000, Serbia

[4]*University of Medicine,*
52, Krasny Ave., Novosibirsk, 630091, Russian Federation

[5]*N.N. Blokhin National Medical Research Center of Oncology,*
24, Kashirskoye Highway, Moscow, 115522, Russian Federation

ABSTRACT

Aim. To conduct a comparative analysis of two available kits that contain all necessary reagents and additives in a single reaction mixture, including 100 mM Tris-HCl, 100 mM KCL, 4 mM MgSO4, 0.2% of Tween 20, for the amplification of the three most common EGFR (epidermal growth factor receptor) gene polymorphisms in patients with non-small cell lung cancer (NSCLC): 181946 G/A (rs2293347), -191 C/A (rs712830), and -216G/T (rs712829).

Materials and methods. The protocol for genotyping 181946C>T, 191C>A and -216G/T was refined according to previously reported data. Polymerase chain reaction (PCR) products measuring 197 bp were detected using electrophoresis in a 2% agarose gel, followed by staining with ethidium bromide.

Results. The Biomaster HS Taq-PCR Color 2× and Biomaster LR HS PCR 2× reagent kits were effective for amplification of 181946 G/A polymorphism located in the intron of the *EGFR* gene. Additionally, polymorphisms -191 C/A (rs712829) and -216G/T (rs712829), located in the promoter region and containing a high GC content, were successfully amplified using the Biomaster LR HS PCR 2× kit.

Conclusion. The present study shows that the Biomaster HS Taq-PCR Color 2× and Biomaster LR HS PCR 2× reagent kits are effective for amplification of 181946 G/A polymorphism located in the intron of the *EGFR* gene. Furthermore, the EGFR SNP -191 C/A, located in the promoter region with a high GC content, was successfully amplified using the Biomaster LR HS PCR 2× reagent kit.

Keywords: additives, amplification, EGFR, NSCLC, polymorphism, PCR, reagents

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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✉ *Jurisic Vladimir,* jurisicvladimir@gmail.com

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 (Protocol No. 5 of 02.10.2022).

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Эффективность двух доступных наборов для амплификации трех нуклеотидных полиморфизмов, содержащих GC-богатые участки: 181946 G/A (rs2293347), –191 C/A (rs712830) и –216G/T (rs712829), у больных немелкоклеточным раком легкого

Юришич В.[1], Обрадович Я.[2], Павлович С.[3], Тошич Н.[3], Гуляева Л.Ф.[4], Герштейн Е.С.[5], Кушлинский Н.Е.[5]

[1] Факультет медицинских наук, Крагуевацкий университет
Сербия, 34000, г. Крагуевац, ул. Светозара Марковича, 69

[2] Институт биологии и экологии, Крагуевацкий университет
Сербия, 34000, г. Крагуевац, ул. Радоя Домановича, 12

[3] Институт молекулярной генетики и генной инженерии, Белградский университет
Сербия, 11000, г. Белград, ул. Студенческий пр., 1

[4] Федеральный исследовательский центр фундаментальной и трансляционной медицины (ФИЦ ФТМ)
Россия, 630117, г. Новосибирск, ул. Тимакова, 2

[5] Национальный медицинский исследовательский центр (НМИЦ) онкологии им. Н.Н. Блохина
Россия, 115522, г. Москва, Каширское шоссе, 24

РЕЗЮМЕ

Цель исследования – сравнительный анализ двух доступных наборов, содержащих в одной реакционной смеси все реагенты и добавки, включая 100 mM Tris-HCl, 100 mM KCL, 4 mM MgSO4, 0,2% of Tween 20, необходимых для амплификации трех наиболее часто встречающихся у больных немелкоклеточным раком легкого (НМРЛ) полиморфизмов гена рецептора эпидермального фактора роста EGFR: 181946 G/A (rs2293347), –191 C/A (rs712830) и –216G/T (rs712829).

Материалы и методы. Протокол для генотипирования 181946C>T, 191C>A и -216G/T был уточнен в соответствии с ранее представленными данными. Продукты полимеразной цепной реакции (ПЦР) размером 197 bp детектировали с помощью электрофореза в 2%-м агарозном геле с последующим окрашиванием этидиум бромидом.

Результаты. Наборы реактивов Biomaster HS Taq-PCR Color 2× и Biomaster LR HS PCR 2× были эффективны для амплификации 181946 G/A, локализованной в интроне гена *EGFR*. Кроме того, полиморфизмы –191 C/A (rs712829) и –216G/T (rs712829), расположенные в промоторном участке и содержащие чрезвычайно высокое количество GC, успешно амплифицировались с помощью набора Biomaster LR HS PCR 2×.

Заключение. В настоящем исследовании показано, что наборы реактивов Biomaster HS Taq-PCR Color 2× и Biomaster LR HS PCR 2× эффективны для амплификации 181946 G/A, локализованного в интроне гена *EGFR*. Кроме того, EGFR SNP -191 C/A, локализованный в промоторном участке с крайне высоким содержанием GC-пар, успешно амплифицировался с помощью набора реактивов Biomaster LR HS PCR 2x.

Ключевые слова: добавки, амплификация, EGFR, НМРЛ, полиморфизм, ПЦР, реагенты

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

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Соответствие принципам этики. Все пациенты подписали информированное согласие на участие в исследовании. Исследование одобрено локальным этическим комитетом НМИЦ онкологии им. Н.Н. Блохина (протокол № 5 от 02.10.2022).

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INTRODUCTION

The epidermal growth factor receptor (EGFR) belongs to the receptor tyrosine kinase family. It has a strong impact on cell growth and differentiation of both healthy and lung cancer cells and its oncogenic potential is recognized and established [1–3]. Several previously described *EGFR* single nucleotide polymorphisms (SNPs) are associated with regulation of receptor protein synthesis [1, 2, 4]. Based on the *EGFR* role in carcinogenesis, proliferation, and differentiation, it is important to study *EGFR* SNPs in non-small cell lung cancer (NSCLC) patients [3]. However, *EGFR* promoter region that we have studied is one of the extremely guanine-cytosine (GC) rich regions with up to 75.45% GC base pairs [5], so it required special amplification conditions.

Polymerase chain reaction (PCR) is a worldwide used technique for EGFR mutation detection. However, unique optimization of each PCR protocol is required, especially when GC rich regions are amplified, because they tend to form secondary structures that interrupt standard PCR amplification [6–8]. We have shown previously that a laborious PCR optimization strategy was especially necessary for SNPs amplification in the promoter region of *EGFR* [5, 9]. Until recently, there was no available complete reagent mixtures for amplification of these complex GC rich regions. In this study, for the first time, we have tested Biomaster LR HS PCR 2× (BiolabMix, Russia) and HS Taq-PCR Color 2× (BiolabMix, Russia) kits with reaction mixtures for amplification of three SNPs: 181946 G/A, –191 C/A, and –216 G/T. The tests were conducted via a polymerase chain reaction with restriction of fragment length polymorphism (PCR-RFLP) method.

MATERIALS AND METHODS

The protocol for genotyping 181946C>T, 191C>A and –216 G/T was refined according to previously reported data. PCR products measuring 197 bp were detected using electrophoresis in a 2% agarose gel, followed by staining with ethidium bromide.

Sample preparation. QIAamp DNA Blood Mini Kit (Qiagen, Germany) and DNA Kits (Invitrogen/Life Technologies, Carlsbad, CA, USA) where used for isolation of DNA sample from blood of healthy volunteers and formalin-fixed paraffin-embedded (FFPE) blocks from NSCLC tumor tissue. At least three analyses were performed in each group of respondents. An identical sample from each group is the sample used to determine the same polymorphism. The material was divided so that the test reagents were always tested on the same sample in order to avoid errors or false findings.

Concentration of both DNA samples was measured by Qubit® Fluorometer (Invitrogen/Life Technologies, Carlsbad, CA, USA). Local Ethics Committee approved of this study and the usage of tissue samples for scientific study.

Genotyping protocol. The protocol for 181946C>T genotyping was adjusted according to the previously reported data [4] with a few modifications. Namely, the temperature profile of PCR using KAPA Taq Hot Start PCR Kits was as follows: initial denaturation at 95 °C for 5 min; denaturation at 94 °C for 30s; annealing at 55 °C for 30s; extension at 72 °C for 60 s (in 45 cycling steps); and final extension at 72 °C for 7 min. The total volume of the PCR reaction mixture was 25 µl, with 0.5 µl of genomic DNA, 0.4 µM of each primer, 0.2 mM of each dNTPs, magnesium concentration was adjusted for 1.7 mM $MgCl_2$, and 1 U KAPA Taq DNA polymerase in 1× PCR buffer

A. 244 bp PCR products of were detected via 2% agarose gel electrophoresis with ethidium bromide staining.

For 191C>A and –216 G/T *EGFR* polymorphisms genotyping, we have applied the previously described protocol [1] with several modifications [5, 9]. All PCR reactions were performed via KAPA Taq Hot Start PCR Kits (Kapa biosystems, Boston, MA, USA). The temperature profile of PCR was as follows: initial denaturation at 95 °C for 5 min; denaturation at 94 °C for 30 s, annealing at 63 °C for 30 s, extension at 72 °C for 60s (in 45 cycling steps); and final extension at 72 °C for 7 min. The total volume of the PCR reaction mixture was 25 µl, with 0.4 µl genomic DNA, 0.4 µl of each primer, 0.2 mM of each dNTPs, 1.5 M betaine, and 1U KAPA Taq DNA polymerase in 1 × PCR buffer A (with 1.5 mM $MgCl_2$). 197 bp PCR products were detected via 2% agarose gel electrophoresis with ethidium bromide staining.

Biomaster HS Taq-PCR Color 2× (BiolabMix, Russia) and Biomaster LR HS PCR 2× (BiolabMix, Russia) containing 100 mM Tris-HCl, 100 mM KCL, 4 mM MgSO4, 0,2% of Tween 20 and DMSO were tested according to recommendations of the manufacturer and adjusted via the same PCR-RFLP protocols for both SNPs. PCRs were performed in total volume of 25 µl, with 0.4 or 0.5 µl genomic DNA and 0.4 µM of each primer. Temperature profile of the reaction was the same as previously described.

PCRs performed with this mixture had initial denaturation at 94 °C for 4 min, and the rest of the temperature profile of the reaction was the same and previously described.

Cfr42I restriction enzyme, (Fermentas/Thermo Fisher Scientific, Vilnius, Lithuania) was used for –191C>A RFLP digestion. Fast Digest TfiI, (PfeI) restriction enzyme (Fermentas/Thermo Fisher Scientific, Vilnius, Lithuania) was used for 181946C>T RFLP digestion. Products of the reaction were detected via a 3% agarose gel electrophoresis.

RESULTS

This study showed effectiveness of the studied kits for amplification of three most important *EGFR* SNPs in tissue samples of NSCLC patients containing GC rich region. For 181946 G/A, PCR analyses with both Biomaster kits produced 244 bp sized fragments (Fig. 1). Neither of the tested Biomaster kits affected subsequent RFLP analyses. Namely, RFLP products were as follows: 244 bp for 181946G and 171 bp for 181946 A (Fig. 2).

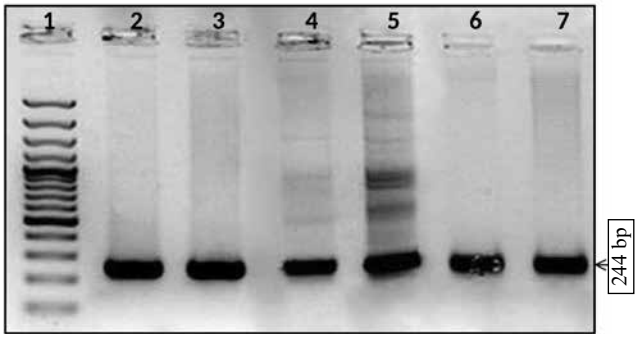

Fig. 1. PCR products of 181946G/A (D994D) (rs2293347) polymorphism amplification using Biomaster HS. Taq-PCR Color (2×) and Biomaster LR HS PCR (2×) in separate reaction mixtures. Lane 1 – markers, lane 2 – control DNA, lane 3 – NSCLC DNA, lane 4 – control DNA with Biomaster HS Taq-PCR Color (2×), lane 5 – NSCLC DNA with Biomaster HS Taq-PCR Color (2×), lane 6 – control DNA with Biomaster LR HS PCR (2×), lane 7 – NSCLC DNA with Biomaster LR HS PCR (2×)

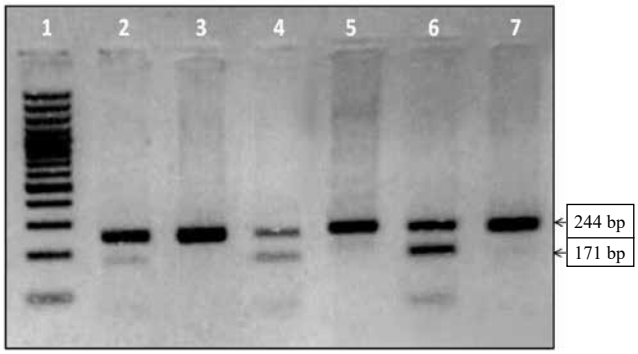

Fig. 2. PCR-RFLP products for 181946G/A (D994D) (rs2293347) polymorphism with Biomaster HS Taq-PCR. Color (2×) and Biomaster LR HS PCR (2×) in separate reaction mixtures. Lane 1 – standard, lane 2 – control DNA, clane 3 – NSCLC DNA, lane 4 – control DNA with Biomaster HS Taq-PCR Color (2×), lane 5 – NSCLC DNA with Biomaster HS Taq-PCR Color (2x), lane 6 – control DNA with Biomaster LR HS PCR (2×), lane 7 – NSCLC DNA with Biomaster LR HS PCR (2×)

Biomaster LR HS PCR 2× was tested for –191 C/A and the appropriate PCR amplification was detected (Fig. 3). Biomaster HS Taq-PCR Color 2× produced no desired 197 bp PCR products (Fig. 3). Instead, a lot of smears and extra bands above the expected band size were visible in the

agarose gel. Since there was no amplification for Biomaster HS Taq-PCR Color 2×, we have performed RFLP analysis only with Biomaster LR HS PCR 2× kit and visualized the 191 bp fragment that corresponds to wild type homozygote for both DNA samples (–-191GG) (Fig. 4). Results of PCR-RFLP products for -216G/T (rs712829) polymorphism using Biomaster LR HS PCR (2×) reaction mixture are shown in Fig. 5.

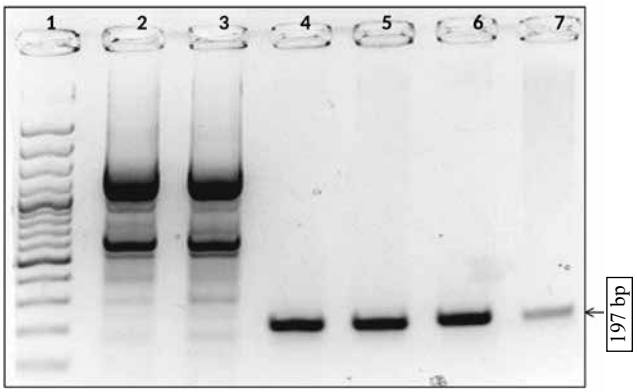

Fig. 3 PCR products for –191C/A (rs712830) and –216G/T (rs712829) polymorphisms with Biomaster HS.Taq-PCR Color (2x) and Biomaster LR HS PCR (2×) in separate reaction mixtures. Lane 1 – standard, lane 2 – control DNA with Biomaster HS Taq-PCR Color (2×), lane 3 – NSCLC DNA with Biomaster HS Taq-PCR Color (2×), lane 4 – control DNA with Biomaster LR HS PCR (2×), lane 5 – NSCLC DNA with Biomaster LR HS PCR (2×), lane 6 –control DNA,lane 7 – NSCLC DNA

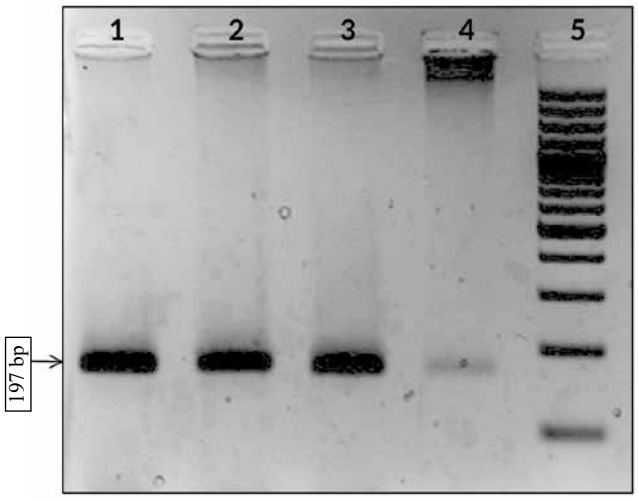

Fig. 4 PCR-RFLP products for –191C/A (rs712830) polymorphism with Biomaster LR HS PCR reaction. Lane 1 – control DNA with Biomaster LR HS PCR (2×), lane 2 – NSCLC DNA with Biomaster LR HS PCR (2×), lane 3 – control DNA, lane 4 – NSCLC DNA, lane 5 – marker

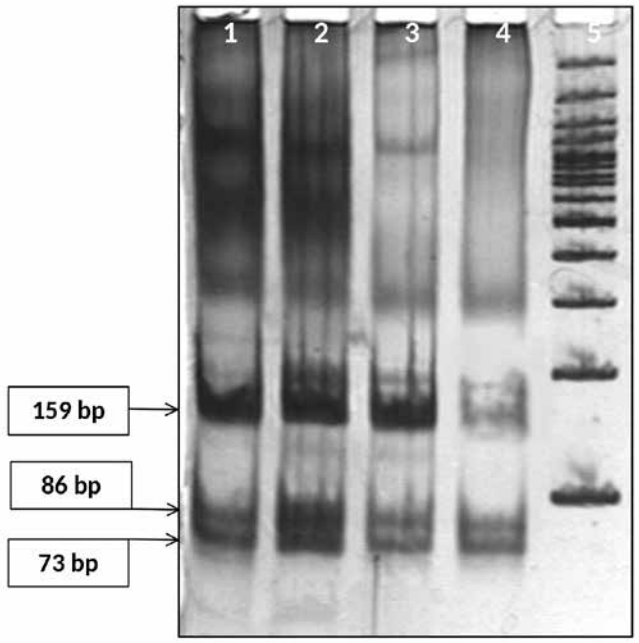

Fig. 5 PCR-RFLP products for –216G/T (rs712829) polymorphism with Biomaster LR HS PCR reaction mixture (2×). Lane 1 – control DNA with Biomaster LR HS PCR (2×), lane 2 – NSCLC DNA with Biomaster LR HS PCR (2×), lane 3 – control DNA, lane 4 – NSCLC DNA, lane 5 – marker

DISCUSSION

Conventional PCR protocol is essential for a wide range of biological experiments, including the study of EGFR, but every single PCR requires specific set of conditions. Although it might be time- and cost-consuming – from designing primers, setting up materials, reagents, reaction mixtures, thermal cycling conditions etc. to troubleshooting strategy – optimization of PCR is inevitable, especially when GC rich regions are amplified [3, 5, 10]. These regions with a high number of GC base pairs form secondary structures, show resistance to denaturation, and lead to incorrect primer attachment.

Several attempts were made to resolve these problems, including modification of primers or temperature conditions [11], and usage of different additives [7, 9]. Enhancing reagents are usually used to increase yield and specificity of the reaction in such complex DNA templates [6, 8, 11–13]. Previously we have adjusted appropriate DNA template concentration, thermal cycling conditions, and optimal $MgCl_2$ concentration for PCR amplification of *EGFR* SNPs in promoter regions with GC content up to 75.45% [3, 5] and tested the effects of different additives that enhance PCR specificity [7, 9].

In this study, we reported the benefits of Biomaster kits usage since both of them include all of the components necessary for PCR reaction including additive and all reagents in one mixture and, thus, minimize the risk of contamination. In addition, the master mix preparation time is reduced. The results of this study showed that both of the Biomaster kits produced a more successful PCR reaction when compared to PCR reactions without them. Biomaster LR HS PCR 2× has a combination of HS-Taq and Pfu DNA polymerases with stabilizers of DNA polymerases and appropriated additives that help to conduct a PCR reaction. It even contains 0.2% of Tween 20 that was shown to reduce the number of unspecific bands [14]. The manufacturer suggested adding a little more DMSO if necessary, as it was previously confirmed to be an important part of a PCR reaction. PCR buffers used for master mix usually include $MgCl_2$, while Biomaster LR HS PCR 2× buffer contains $MgSO_4$, which might increase polymerase activity [8].

This kit is suitable for long, complex regions and for GC rich fragments. We also demonstrate that the expected PCR-RFLP products for −191C>A from lung cancer sample were visualized via gel electrophoresis (Fig. 3 and 4). It was not even a long PCR fragment (244 bp). This kit, as well as Biomaster HS Taq-PCR Color 2×, worked properly for 181946 G/A (Fig. 1 and 2).

Biomaster HS Taq-PCR Color 2× is used for conventional PCR and for templates up to 5 kbp in length. It already contains dyes so there is no need to load a buffer during gel electrophoresis. Due to its high density, reaction mixture sinks in the well of the agarose gel easier. This kit is not suitable for −191C>A because it lacked to produce desired PCR products (Fig. 3).

CONCLUSION

In this study, we have shown that Biomaster HS Taq-PCR Color 2× and Biomaster LR HS PCR 2× are effective for 181946 G/A amplification located in the introne of the *EGFR* gene. In addition, *EGFR* SNP –191C/A, located in the promoter region with extremely high GC content, was successfully amplified with Biomaster LR HS PCR 2×.

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Authors' contribution

Jurisic V. – concept and design development of the article, sample preparation, collection of material, performing the biochemical section of the study; literature analysis; data analysis and interpretation; writing and designing the text of the manuscript. Obradovic J. – sample preparation, performing the biochemical section of the research. Tosic N. – performing the section of radiation studies. Pavlovic S. – performing the biochemical section of the study. Gulyaeva L.F. – collection of material. Gershtein E.S. – translation of the article; participation in the concept development of the article. Kushlinskii N.E. – scientific content editing; approval of the final version of the manuscript.

Authors' information

Jurisic Vladimir – DM, PhD, Professor, University of Kragujevac, Faculty of Medical Sciences, Kragujevac, Serbia, jurisicvladimir@gmail.com, https://orcid.org/0000-0001-6525-128X

Obradovic Jasmina – PhD (Biol.), Research Associate, Department of Science, Institute for Information Technologies, University of Kragujevac, Serbia, jasmina.m.obradovic@gmail.com, https://orcid.org/0000-0002-8853-6525

Tosic Natasha – PhD (Biol.), Researcher Associate, Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Serbia, natasa.tosic@imgge.bg.ac.rs, https://orcid.org/0000-0002-1293-6215

Pavlovic Sonja – PhD (Biol.), Professor, Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Serbia, sonya@sezampro.rs, https://orcid.org/0000-0002-2915-1641

Gulaeva Lyudmila F. – Dr. Sci. (Biol.), Professor, Leading Researcher, Fundamental and Translational Medicine Federal Research Center, Novosibirsk, lfgulyaeva@gmail.com, https://orcid.org/0000-0002-7693-3777

Gershtein Elena S. – Dr. Sci. (Biol.), Professor, Leading Researcher, Laboratory for Clinical Biochemistry, N.N. Blokhin National Medical Research Center of Oncology, Moscow, esgershtein@gmail.com, https://orcid.org/0000-0002-3321-801X

Kushlinskii Nikolay E. – Dr. Sci. (Med.), Professor, Academician of Russian Academy of Sciences, Head of the Laboratory for Clinical Biochemistry, N.N. Blokhin National Medical Research Center of Oncology, Moscow, biochimia@yandex.ru, https://orcid.org/0000-0002-3898-4127

(✉) **Jurisic Vladimir,** jurisicvladimir@gmail.com

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Atherosclerosis and inflammation – from pathogenesis to treatment: current state of affairs (Part I)

Avagimyan A.A.[1], Kaktursky L.V.[2], Urazova O.I.[3], Trofimenko A.I.[4], Sukiasyan L.M.[1], Kogan E.A.[5], Demura T.A.[5], Pogosova N.V.[6, 7]

[1] Mkhitar Heratsi Yerevan State Medical University
2a, Koryuna Str., Yerevan, 0025, Republic Armenia

[2] Avtsyn Research Institute of Human Morphology of the Federal state budgetary scientific institution "Petrovsky National Research Center of Surgery"
3, Tsuryupy Str., Moscow, 117418, Russian Federation

[3] Siberian State Medical University
2, Moscow Trakt, Tomsk, 634050, Russian Federation

[4] Kuban State Medical University (KubSMU)
4, Mitrofana Sedina Str., Krasnodar, 350063, Russian Federation

[5] I.M.Sechenov First Moscow State Medical University (Sechenov University)
8, Build. 2, Trubetskaya Str., Moscow, 119048, Russian Federation

[6] E.I.Chazov National Medical Research Center for Cardiology
15a, Build. 6, Akademika Chazova Str., Moscow, 121552, Russian Federation

[7] People's Friendship University of Russia (RUDN University)
6, Miklukho – Maklaya Str., Moscow, 117198, Russian Federation

ABSTRACT

Atherosclerosis and atherosclerosis-related cardiovascular diseases are a significant public health concern and a rapidly evolving area of research in both fundamental and clinical medicine. Despite the extensive history of studying, many aspects of atherosclerosis etiology and pathogenesis remain unclear.

Traditionally, the pathogenesis of atherosclerosis has been viewed in terms of the localized accumulation of specific lipoprotein fractions in the arterial wall. However, both innate and adaptive immunity play active roles in atherogenesis. Cells and mediators of the immune system engage in intricate interactions with cellular and extracellular components in all layers of the vascular wall. For this reason, scientific community have reached a consensus on the crucial role of inflammation in the onset, progression, and destabilization of an atherosclerotic plaque.

Therefore, atherogenesis can be considered not only as a metabolic disorder, but also as an immunoinflammatory process. The aim of this lecture was to summarize contemporary data regarding the role of inflammation at various stages of the atherosclerotic continuum.

Keywords: atherosclerosis, atherogenesis, lipoproteins, inflammation, atherosclerotic plaque, atheroma

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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✉ *Avagimyan Ashot A.,* avagimyan.cardiology@mail.ru

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Атеросклероз и воспаление – путь от патогенеза к терапии: обзор современного состояния проблемы (часть 1)

Авагимян А.А.[1], Кактурский Л.В.[2], Уразова О.И.[3], Трофименко А.И.[4], Сукиасян Л.М.[1], Коган Е.А.[5], Демура Т.А.[5], Погосова Н.В.[6, 7]

[1] Ереванский государственный медицинский университет им. Мхитара Гераци (ЕГМУ им. М. Гераци) Республика Армения, 0025, г. Ереван, ул. Корюна, 2а

[2] Научно-исследовательский институт морфологии человека им. акад. А.П. Авцына Федерального государственного бюджетного научного учреждения «Российский научный центр хирургии им. академика Б.В. Петровского» (НИИМЧ им. акад. А.П. Авцына ФГБНУ «РНЦХ им. акад. Б.В. Петровского») Россия, 117418, г. Москва, ул. Цюрупы, 3

[3] Сибирский государственный медицинский университет (СибГМУ) Россия, 634050, г. Томск, Московский тракт, 2

[4] Кубанский государственный медицинский университет (КубГМУ) Россия, 350063, г. Краснодар, ул. Митрофана Седина, 4

[5] Первый Московский государственный медицинский университет им. И.М. Сеченова (Первый МГМУ им. И.М. Сеченова) (Сеченовский Университет) Россия, 119048, г. Москва, ул. Трубецкая, 8, стр. 2

[6] Национальный медицинский исследовательский центр кардиологии им. акад. Е.И. Чазова (НМИЦК им. акад. Е.И. Чазова) Россия, 121552, г. Москва, ул. Академика Чазова, 15а, стр. 6

[7] Российский университет дружбы народов им. Патриса Лумумбы (РУДН им. Патриса Лумубы) Россия, 117198, г. Москва, ул. Миклухо-Маклая, 6

РЕЗЮМЕ

Атеросклероз и ассоциированные с ним болезни системы кровообращения являются актуальной проблемой общественного здравоохранения, а также активно развивающимся направлением исследований как в фундаментальной, так и клинической медицине. Несмотря на многолетнюю историю изучения заболевания, до сих пор многие аспекты этиологии и патогенеза атеросклероза остаются неясными.

Традиционно патогенез атеросклероза рассматривают с позиции очагового накопления отдельных фракций липопротеинов в сосудистой стенке артерий. Однако в атерогенезе активное участие принимают факторы как врожденного, так и адаптивного иммунитета. Клетки и медиаторы иммунной системы вступают в сложное взаимодействие с клеточным и внеклеточным компонентами всех слоев сосудистой стенки. Вследствие этого большинство исследователей пришли к консенсусу о ключевой роли воспаления в инициации, прогрессировании, а также дестабилизации атеросклеротической бляшки.

Таким образом, атерогенез можно рассматривать не только как дисметаболический, но и как иммуновоспалительный процесс. Целью настоящей лекции является обобщение новейших данных о роли воспаления на разных этапах атеросклеротического континуума.

Ключевые слова: атеросклероз, атерогенез, липопротеины, воспаление, атеросклеротическая бляшка, атерома

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INTRODUCTION

Cardiovascular diseases (CVD) are the leading cause of premature death and disability in economically developed countries,and impose a significant burden on healthcare systems, resulting in substantial economic consequences [1].

Diseases associated with atherosclerosis contribute significantly to CVD mortality [2–4]. Despite advancements in modern lipidology, including the implementation of high-intensity statin therapy, its combination with ezetimibe and proprotein convertase subtilisin / kexin type 9 (PSCK9) inhibitors in clinical practice, the challenge of achieving target lipid profile values and addressing residual cardiovascular risk remains pertinent [5]. Notably, approximately half of the patients receiving high-intensity statin therapy in combination with ezetimibe continue to exhibit an elevated risk of adverse cardiovascular outcomes [6]. As of 2024, atherosclerosis remains an idiopathic, multifactorial disease. Concurrently, a consensus has been reached among researchers regarding the pivotal role of inflammation in the pathogenesis of atherosclerosis [7].

Historically, the initiation of atherogenesis was conceptualized through the lens of endothelial dysfunction within the framework of the «endothelial response to injury» hypothesis. However, subsequent investigations revealed no damage, but rather activation of the endothelium due to biomechanical stress and the initiation of associated molecular cascades [8]. These findings do not negate but rather complement and expand upon the involvement of inflammation in the pathogenesis of all stages of atherogenesis [9].

CVD prevention encompasses a complex algorithm comprising a set of measures aimed at lifestyle modification and management of modifiable risk factors [10]. However, optimal pharmacological intervention and reduction of low-density lipoprotein cholesterol (LDL-C) and blood pressure (BP) do not provide comprehensive protection against cardiovascular complications [11].

It is imperative to consider comorbid conditions represented by relatively novel (non-classical) risk factors for CVD, such as cancer and associated chemotherapy [12], HIV infection (irrespective of viral load levels) [13], *Helicobacter pylori* [14], and oral microbiome dysbiosis [15].

Observational studies have demonstrated the potential anti-atherogenic properties of disease-modifying and/or targeted anti-inflammatory drugs used to treat autoimmune diseases [16]. It has been established that the beneficial effect of statins in reducing cardiovascular risk is associated not only with their primary lipid-lowering effect, but also with anti-inflammatory effects [17].

In contemporary clinical lipidology, the critical importance of inflammation in atherogenesis is directly confirmed by the inclusion of colchicine in the clinical guidelines for cardiovascular prevention prepared by the Russian National Society of Preventive Cardiology in collaboration with the Russian Society of Cardiology [5]. Low-dose colchicine therapy in patients with coronary artery disease has been approved by the U.S. Food and Drug Administration as an efficacious method for reducing residual cardiovascular risk [18].

A subsequent step in improving therapeutic algorithms is the use of nanotechnology to produce novel dosage forms of drugs that can be delivered to specific tissues or cell populations. Thus, a comprehensive study of the pathogenesis of atherosclerosis in combination with the application of a multi-omics approach is highly relevant, as a detailed understanding of the molecular mechanisms of atherogenesis forms the basis for developing

a «signature,» the impact on which will provide more precise control of atherogenic inflammation and facilitate the development of a vaccine against atherosclerosis [19, 20], which will likely require more than one decade.

The aim of this lecture was to synthesize potentially clinically significant data on the role of inflammation at different stages of atherogenesis, including destabilization of atherosclerotic plaques. An international multidisciplinary team of experts prepared the materials. Within the concept «from pathogenesis to therapy,» the presented lecture is divided into two parts.

CONTRIBUTION OF IMMUNE CELLS TO THE DEVELOPMENT OF ATHEROGENIC ENDOTHELIAL DYSFUNCTION

Advances in fundamental scientific research have enabled to reevaluate traditional perspectives on the endothelium, which was previously regarded in the scientific community solely as a conventional layer of flat cells of mesenchymal origin lining the inner surface of blood and lymphatic vessels as well as cardiac cavities [21, 22]. Contemporary understanding considers the endothelium as an active morphological subunit (or, according to some authoritative scientific schools, a distinct organ) that produces a diverse array of biologically active substances with autocrine, paracrine, and juxtacrine activity [23, 24].

Under physiological conditions, the endothelium predominantly exhibits antithrombotic, anti-inflammatory, and vasoactive properties, regulating vascular wall permeability for circulating biologically active molecules and vascular tone through the balance between the release of vasodilators (e.g., nitric monoxide (NO) and prostaglandin E2 (PgE2)) and vasoconstrictors (e.g., endothelin-1 and thromboxane (Tx) A2) [25]. The development of proinflammatory and vasospastic endothelial dysfunction leads to a pathological increase in vascular permeability and a decrease in the bioavailability of atheroprotective NO, contributing to the subendothelial accumulation of atherogenic (ApoB100-containing) lipoprotein fractions (primarily low-density lipoproteins [LDL]) and the development of so-called sterile inflammation [26–30].

Recent research has led to a re-evaluation of the role of perivascular adipose tissue. It has been demonstrated that perivascular adipose tissue produces a diverse group of cytokines and biologically active substances, including tumor necrosis factor α (TNFα), interleukins (IL) IL-1, IL-6, and IL-8, adipocyte-derived relaxing factor, macrophage chemotactic protein-1, plasminogen activator inhibitor-1, complement component C3, apelin, leptin, resistin, visfatin, carbon monoxide (CO), and hydrogen sulfide (H_2S). These biologically active substances can modulate the endothelial state and vascular tone, and exhibit both pro- and antiatherogenic effects [31–34]. Considering the contribution of perivascular adipose tissue to atherogenesis, it is important to note the role of the inflammatory microenvironment in its metabolic reorganization as well as in the development of structural and functional dysfunction of the vasa vasorum and significant dystrophic changes in the perivascular nerve plexus [35, 36]. Furthermore, functional dysregulation of stem/progenitor cells of perivascular adipose tissue (a stem cell niche located at the medial-adventitial interface) has been observed, including adipocyte progenitor cells, smooth muscle cells (SMCs), endothelial cells, mesenchymal stem cells (MSCs), and myeloid progenitor cells [37].

Chronic low-grade inflammation of the vascular wall also induces accumulation of senescent cells. In this context, the inflammatory microenvironment acquires a senescence-associated secretory phenotype, which plays a crucial role in the development of both early vascular aging syndrome and aging heart [38]. Senescent cells are characterized by mitochondrial damage, telomere shortening, epigenetic alterations, metabolic dysregulation (particularly protein metabolism), stem cell dysfunction, and impaired intercellular communication. The key molecules associated with the initiation of aging processes are NF-kB, C/EBP beta, GATA4, mTOR, and p38MAPK, as well as disturbances in the functioning of signaling mechanisms involving cyclic GMP-AMP synthetase (cGAS) and cyclic GMP-AMP (cGAMP) [39]. The accumulation of senescent cells leads to a decrease in the activity of antioxidant systems, particularly the inactivation of the Nrf2/ARE/sestrin-2 cascade [40]. These alterations underlie the development of the early aging syndrome of blood vessels, which presents a significant challenge in the field of internal medicine [41].

As a consequence of the interaction between positively charged amino acids, specifically

arginine and lysine in LDL, and negatively charged proteoglycans in the arterial wall, atherogenic lipoproteins are retained within the vessel wall [42]. Acute activation of the endothelium induces the expression of cell adhesion molecules, resulting in the attachment of monocytes to the endothelial cells [43–45].

Monocytes adhere to endothelial cells via PSGL-1/CD162 receptors (receptors for P-selectin and E-selectin on the surface of endothelial cells), CD11b and CD18 (subunits of the Mac-1 receptor for ICAM1), LFA-1/CD11a (receptor for ICAM1), CD29, and CD49d (subunits of the VLA-4 receptor for VCAM1) [46]. Following cell adhesion, MCP-1 stimulates monocyte migration and infiltration. Upon migration into the endothelium, monocytes differentiate into macrophages under the influence of macrophage colony-stimulating factor (M-CSF) [47].

Upon entering the subendothelial space, LDL undergoes not only oxidation, but also aggregation. In the context of an inflammatory microenvironment and the accompanying decrease in pH within the vascular wall, the composition of lipoproteins shifts from the large and medium fractions to the small and dense subfractions. These subfractions exhibit lower affinity for LDL receptors (which impede their removal), greater mobility in the extracellular matrix, and consequently, higher atherogenicity [48].

The accumulation of atherogenic LDL in the subendothelial space of monocytes and resident macrophages that have migrated from circulating blood results in the release of a wide range of proinflammatory cytokines (IL-1β, IL-6, IL-12, IL-15, IL-18, and TNFα) [49]. It is worth noting that under the pathological conditions of an atherogenic microenvironment, macrophages can acquire both proinflammatory and anti-inflammatory phenotypes, characterized by the release of corresponding molecules (IL-4, IL-10, IL-13, and transforming growth factor 1β (TGF-1β)) [50].

Therefore, irrespective of the causal relationship between oxidized LDL and atherosclerosis, atherosclerotic changes in arterial walls can develop in the presence of a normal lipid profile. The increasing prevalence of type 2 diabetes mellitus and metabolic syndrome in the population, coupled with the control of LDL levels through lipid-lowering therapy, has altered the lipid risk profiles. Notably, a significant contribution is observed from elevated levels of desialylated, electronegative, small dense, and multiply modified LDL [51, 52].

In the subendothelial space, modified lipoproteins are captured by macrophages and dendritic cells, which are mononuclear phagocytes that are resident in the normal arterial wall since fetal development. Additionally, circulating monocytes originating from the bone marrow or spleen adhere to the endothelial layer, migrate into the intima via diapedesis, and differentiate into macrophages [50]. In addition, endothelial cells can migrate into the intima and undergo endothelial – mesenchymal transition, thereby promoting thickening (intimal remodeling) and exacerbating inflammation [53].

The endothelial reaction, a key component of the inflammatory response, encompasses the coordinated activation of both innate immunity (macrophages) and adaptive immunity (T- and B lymphocytes). Upon entering the subendothelial space, recruited monocytes differentiate into macrophages and polarize, adopting diverse functional phenotypes in response to alterations in the microenvironment [54].

T lymphocytes transform monocytes into proinflammatory M1 macrophages, which produce proinflammatory cytokines (IL-1α, IL-1β, IL-6, IL-12, IL-15, IL-18, and TNFα) involved in the progression of atherosclerosis, or into «alternative» anti-inflammatory M2 macrophages, which produce anti-inflammatory cytokines (IL-4, IL-10, IL-13, and TGFβ) capable of modulating the inflammatory response. Consequently, T lymphocytes regulate the continuum of inflammation resolution [55]. Although macrophages are the primary source of cytokines, other cells, such as lymphocytes, polymorphonuclear leukocytes, and endothelial cells, which play a significant role in atherosclerosis, also contribute to cytokine production.

Neutrophilic granulocytes are directly implicated in the development of oxidative stress in endothelial cells and the formation of erosion on plaque surfaces [56]. Furthermore, neutrophils directly activate neighboring cells, such as macrophages and T lymphocytes, thereby intensifying the inflammatory reaction in atheroma [57].

T lymphocytes are present in atherosclerotic plaques to varying degrees at nearly all stages of their formation and associated complications. The process of atheroma development and weakening is accompanied by an immunoallergic reaction of delayed-type hypersensitivity, associated with

significant activation of the CD4+ T lymphocyte subpopulation and secretion of interferon γ (IFNγ) by the latter [13]. Direct receptor-mediated contact occurs between T lymphocytes and macrophages, promoting activation of the latter and increased production of proinflammatory cytokines and proteolytic enzymes, potentiating the development of destructive processes in the plaque [58].

IFNγ and TNFα mediate atherosclerotic changes, affecting macrophages and the endothelium and increasing the level of fractalkine (CX3CL1), a chemokine that regulates the migration and adhesion of leukocytes [59]. IFNγ destabilizes atherosclerotic plaques by inhibiting collagen synthesis, promoting SMC apoptosis, and increasing endothelial permeability [60]. By activating matrix metalloproteinases (MMPs), IFNγ and TNFα promote the degradation of collagen and extracellular matrix, thereby disrupting the stability of atherosclerotic plaques [61].

Typical atheromas contain a lipid core, dying macrophages that form a necrotic core, and a developing thick fibrous cap, which is facilitated by the production of collagen, elastin, fibronectin, and other components of the extracellular matrix by SMC [62]. Macrophage activation results in the release of numerous cytokines and their transformation into foam cells [63]. Activated macrophages release additional inflammatory stimuli and stimulate the formation of the necrotic core in the atherosclerotic plaque. Unstable atheromas are commonly classified into three types [64]:

1. Lipid type – fibroatheroma with a massive lipid core and a thin fibrous cap.

2. Inflammatory and erosive – atheromas with an increased content of proteoglycans and inflammatory or erosive damage to the cap.

3. Dystrophic necrotic type – atheromas with a calcified core and pronounced dystrophic and necrotic changes in the cap.

THE ROLE OF MACROPHAGES IN ATHEROSCLEROSIS

Macrophages catabolize oxidized LDL in the arterial wall to form foam cells. The fate of macrophages varies depending on the concentration of cytokines and their combination as well as the quantity of oxidized LDL [65, 66]. Deceased macrophages coalesce, forming a lipid-rich necrotic core that stimulates the migration of smooth muscle cells from the media to the intima, encapsulated by a collagen cap, with subsequent formation of fibroatheroma [67]. Under conditions of chronic low-grade inflammation, macrophages exhibit catabolic effects, degrading and thinning the fibrous cap, thereby thinning the fibroatheroma (< 65 μm) [68]. These pathological changes, characterized by the presence of a large lipid-rich necrotic core separated from the arterial lumen by a thin fibrous cap, render the plaque unstable and susceptible to rupture [69].

M1 macrophages are classically activated by proinflammatory cytokines, particularly INFγ and bacterial lipopolysaccharides, and produce, as previously mentioned, proinflammatory cytokines, such as IL-1β, IL-6, and TNFα, as well as inducible nitric oxide synthase (iNOS) and NADPH oxidase, with subsequent development of nitrosative and oxidative stress [70]. M2 macrophages, conversely, are alternatively activated by anti-inflammatory cytokines, such as IL-4 and IL-13, and produce elevated levels of IL-10 and TGF-1β [71]. M2 macrophages also express scavenger receptors, such as CD163 and CD206, which play a significant role in atherogenesis [72]. Notably, macrophages in the fibrous capsule of an atherosclerotic plaque express both proinflammatory and anti-inflammatory cytokines, indicating a mixed M1/M2 phenotype [73].

IL-6 plays a crucial role in atherogenesis; specifically, it stimulates the production of acute-phase response proteins and enhances the proliferation and differentiation of lymphocytes [74]. Furthermore, IL-6 activates cyclooxygenase-2 (COX-2), which increases the concentration of IL-1β, TNFα, and high-sensitivity C-reactive protein (hs-CRP) in blood plasma by augmenting the activity of NF-κB, JAK/STAT3, and MAPK transcriptional cascades [75].

Recent studies have identified several novel subtypes of macrophages that may be present in atherosclerotic plaques, including MMe, Mox, M(Hb), Mhem, M4, and HA-mac macrophages. Metabolically activated (MMe) macrophages predominantly reside in the adipose tissue. Their primary function is to eliminate the dead adipocytes [76].

Mox macrophages are inflammatory macrophages that produce high levels of the enzyme Hmox1 (heme oxygenase 1). The M1, MMe, and Mox macrophages are activated by LDL and INFγ [77].

M4 macrophages are proinflammatory macrophages that mature and are activated by the platelet chemokine CXCL-4 (arterial thrombosis companion) and can participate in the degradation of the fibrous cap and plaque rupture by producing the enzyme MMP-12 [78].

Macrophages HA-mac, M(Hb) (hemoglobin-stimulated), and Mhem are anti-inflammatory macrophages with pronounced atheroprotective effects activated by the hemoglobin – haptoglobin complex (hb – hp), which is involved in the clearance of hemoglobin from the hemorrhagic zones [79]. Macrophages with the Mhem phenotype, in addition to participating in erythrophagocytosis, suppress the development of oxidative stress, accumulation of lipid droplets, and formation of foam cells [80]. The role of M(Hb) macrophages in the pathogenesis of atherosclerosis is also associated with the induction of cholesterol efflux, leading to a sharp decrease in foam cell formation [81].

MMe macrophages are characterized by high activity of NADPH oxidase-2 and iNOS, which play important roles in inflammation and generation of reactive oxygen species [65]. In turn, the Mox macrophage phenotype, which is often found in already developed atherosclerotic plaques, activates the expression of Srnx-1 and Txnrd-1 [82].

PLAQUE DESTABILIZATION FACTORS

The main mechanism through which existing atherosclerotic lesions begin to shrink is through a decrease in circulating plasma lipid concentrations and stabilization of inflammatory cascades [83–85]. In animal models, this is often followed by an increase in cholesterol efflux from foam cells via the ATP-binding cassette transporter (ABCA)1 into apoA1/HDL (high-density lipoprotein) via the reverse cholesterol transport pathway.

When cholesterol efflux is induced in high-HDL environments, atherosclerotic plaque macrophages adopt a pro-resolving M2-like phenotype, producing anti-inflammatory cytokines, such as IL-10 and TGF-1β, supporting connective tissue cell proliferation and angiogenesis [86]. The pro-resolving phenotype also enhances phagocytosis of debris and efferocytosis of apoptotic cells, which contributes to the reduction of the necrotic core. Indeed, efferocytosis and apoptosis of atherogenic field cells enhance macrophage proliferation, increasing the number of macrophages available for efferocytosis and potentiating the plaque regression process [87].

Polyunsaturated fatty acids (PUFAs) have been shown to have pronounced atheroprotective properties, which are associated with their anti-inflammatory action [88]. Linoleic acid suppresses the expression of proinflammatory genes in macrophages and inactivates NF-κB, CCL2, and COX-2 through PPARγ receptors, thereby reducing the progression of atherosclerosis [89]. In addition, PUFAs can modulate the atherogenic effects of saturated fatty acids, such as palmitate-induced expression of the lectin-like receptor for oxidized LDL-1 (LOX1) [90].

The atheroprotective functions of HDL are associated with stimulation of cholesterol catabolism and efflux. The antioxidant and anti-inflammatory properties of HDL and its anti-apoptotic effects on endothelial cells and endothelial progenitor cells are worth noting [91]. HDL enhances the proliferation and migration of endothelial cells and endothelial progenitor cells, thereby contributing to the restoration of endothelial integrity [92].

At the same time, the atheroprotective effect of HDL is partly mediated by its anti-inflammatory effect. Studies using a mouse model of atherogenesis have shown that HDL promotes the polarization of macrophages from the M1 phenotype to the M2 phenotype and inhibits the reverse polarization of cells to the M1 phenotype [79].

The migration of monocytes through the endothelium into atherosclerotic plaques is mediated by chemokines (CCR2–CCL2 (or MCP-1), CX3CR1–CX3CRL1, and CCR5-CCL510) secreted by endothelial cells, intimal macrophages, and smooth muscle cells [93]. Vascular endothelial adhesion molecules CD31 (also known as von Willebrand factor) and VCAM1 are involved in monocyte transmigration [93].

It is worth noting that neural guidance signals are involved in the recruitment of monocytes in atherosclerosis; in particular, netrins, semaphorins, and ephrins are expressed by endothelial cells in the arterial wall [94]. Their effects depend on the vascular wall microenvironment. For example, ephrin B2 expression increases under proatherosclerotic conditions and enhances leukocyte recruitment to atherosclerosis-prone areas of the arterial wall, even in the absence of additional chemokines [95]. In contrast, netrin 1 and semaphorin 3A expression inhibits chemokine-directed migration of human and mouse monocytes *in vitro* [96].

The uptake of lipoproteins by monocyte-derived macrophages is considered as one of the earliest stages of atheroma development, leading to the formation of foam cells. Although macrophages can clear ApoB-containing lipoproteins via the LDL receptor, the expression of this receptor is reduced early in foam cell formation, owing to increased cholesterol levels in the cells [97]. These observations have led to the well-established hypothesis that lipoproteins must undergo modification of the arterial wall and be taken up by alternative mechanisms.

Macrophage-expressed scavenger receptors, a type of pathogen pattern recognition receptor (PRR), play a significant role in atherosclerosis and were initially described for their ability to recognize and process modified LDL. Numerous members of the scavenger receptor family include scavenger receptor A (SRA; encoded by MSR), MARCO, CD36, scavenger receptor class B member 1 (SRB1), lectin-type oxidized low-density lipoprotein receptor 1 (LOX1), scavenger receptor class 1 member 1 (SREC1), and scavenger receptors for phosphatidylserine and oxidized low-density lipoprotein (SRPSOX; also known as CXCL16). These receptors bind oxidized LDL and promote foam cell formation [98]. These receptors internalize lipoproteins; in lysosomes, lipoprotein-cholesterol esters are hydrolyzed to free cholesterol and fatty acids [99]. Free cholesterol from the endolysosomal apparatus is subsequently transported to the endoplasmic reticulum, where it is re-esterified by cholesterol ester acyl-CoA transferase to fatty acid esters, which constitute the «foam» of foam cells [77].

Modification of LDL by various proteases and lipases present in the intima can also mediate its aggregation. Glycoproteins of the extracellular matrix contribute to this process by retaining lipoproteins and modulating the activity of various lipolytic enzymes (secretory phospholipase A2 group IIA and secretory sphingomyelinase), which produce modified forms of LDL that are taken up by a scavenger receptor-independent pathway [100].

In understanding the concept of atherogenesis, the necrotic core is of great importance, playing a major role in the vulnerability of atherosclerotic plaques. It is essential to consider the role of primary and secondary inflammation, cell death, and debris removal as well as other factors that may be involved in the formation of the necrotic core, such as MMP activation and diapedetic hemorrhage [101]. The free cholesterol content in the necrotic cores of high-risk plaques is significantly higher than that in low-risk plaques [102]. Free cholesterol is deposited largely because of the extravasation of erythrocytes, which increases with intimal neovascularization, as new vessels are highly permeable, and erythrocyte membranes are rich in free cholesterol [103].

CONCLUSION

Inflammation plays a crucial role in all stages of atherogenesis. Elucidating and investigating intricate cellular and subcellular interactions in atherogenesis in greater detail will provide a foundation for the development of novel strategies for targeted anti-inflammatory therapy of atherosclerosis aimed at mitigating primary cardiovascular and residual cardiovascular risk.

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Authors' contribution

Avagimyan A.A., Kaktursky L.V., Urazova O.I., Trofimenko A.I., Sukiasyan L.M., Kogan E.A., Demura T.A., Pogosova N.V. – collection and analysis of literature data, drafting of the article. Pogosova N.V., Urazova O.I., Demura T.A. – editing of the article, critical revision of the manuscript for important intellectual content, final approval of the manuscript for publication.

Authors' information

Avagimyan Ashot A. – Cand. Sci. (Med.), Lecturer, Department of Propaedeutics of Internal Medicine, Mkhitar Heratsi Yerevan State Medical University, Yerevan, Armenia, avagimyan.cardiology@mail.ru, http://orcid.org/0000-0002-5383-835

Kaktursky Lev V. – Dr. Sci. (Med.), Professor, Corresponding Member of the RAS, Scientific Director of the Avtsyn Research Institute of Human Morphology of the Federal state budgetary scientific institution «Petrovsky National Research Center of Surgery", Moscow, levkaktur@mail.ru, https://orcid.org/0000-0001-7896-2080

Urazova Olga I. – Dr. Sci. (Med.), Professor, Corresponding member of the RAS, Head of the Pathophysiology Division, Siberian State Medical University, Tomsk, urazova.oi@ssmu.ru, http://orcid.org/0000-0002-9457-8879

Trofimenko Artem I. – Cand. Sci. (Med.), Associate Professor, Department of Pathophysiology, KubSMU, Krasnodar, artemtrofimenko@mail.ru, http://orcid.org/0000-0002-9457-8879

Sukiasyan Lilit M. – Cand. Sci. (Med.), Researcher, Central Research Laboratory, Mkhitar Heratsi Yerevan State Medical University, Yerevan, Armenia, lilit.sukiasyan@inbox.ru, https://orcid.org/0000-0001-7696-0639

Kogan Evgeniya A. – Dr. Sci. (Med.), Professor, Head of the Department of Pathological Anatomy named after Academician A. I. Strukov, Head of the Reference Center for Pathomorphological and Immunohistochemical Research Methods, I. M. Sechenov First Moscow State Medical University (Sechenov University), Moscow, kogan_e_a@staff.sechenov.ru, https://orcid.org/0000-0002-1107-3753

Demura Tatyana A. – Dr. Sci. (Med.), Professor, Director of the Institute of Clinical Morphology and Digital Pathology, Vice-Rector for Research, I. M. Sechenov First Moscow State Medical University (Sechenov University), Moscow, demura_t_a@staff.sechenov.ru, https://orcid.org/0000-0002-6946-6146

Pogosova Nana V. – Dr. Sci. (Med.), Professor, Deputy Director General for Science and Preventive Cardiology, E.I.Chazov National Medical Research Center for Cardiology, Moscow; Head of the Department of Evidence-Based Medicine, RUDN University, Moscow, nanapogosova@gmail.com, https://orcid.org/0000-0002-4165-804X

(✉) **Avagimyan Ashot A.,** avagimyan.cardiology@mail.ru

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Sleep disordered breathing and its impact on the course of chronic non-communicable lung diseases

Bespalova I.D., Mitrichenko U.M., Koshchavtseva Yu.I., Kapitanova D.V., Badmaev A.Z., Agaeva S., Zhukovskaja O.V., Kolmakova V.M., Belyakova T.V., Teteneva A.V., Bukreeva E.B., Boyarko V.V., Nesterovich S.V., Vinokurova D.A., Kalyuzhin V.V.

Siberian State Medical University
2, Moscow Trakt, Tomsk, 634050, Russian Federation

ABSTRACT

The lecture synthesizes and analyzes the findings of research concerning the impact of sleep disordered breathing (SDB) on the progression of the most prevalent chronic non-infectious lung diseases (CNLDs). SDB, including conditions, such as snoring, sleep hypoventilation syndrome, and obstructive and central sleep apnea syndrome, constitutes a significant medical concern due to its high prevalence and adverse health consequences. SDB is regarded as an independent risk factor for the development and progression of a range of CNLDs. Timely diagnosis and management of SDB may serve as an effective preventive measure against severe manifestations and complications associated with this group of diseases.

Keywords: sleep disordered breathing, snoring, apnea, hypoxia, chronic non-communicable lung diseases

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Нарушения дыхания во сне и их влияние на течение хронических неинфекционных заболеваний легких

Беспалова И.Д., Митриченко У.М., Кощавцева Ю.И., Капитанова Д.В., Бадмаев А.З., Агаева С., Жуковская О.В., Колмакова В.М., Белякова Т.В., Тетенева А.В., Букреева Е.Б., Боярко В.В., Нестерович С.В., Винокурова Д.А., Калюжин В.В.

Сибирский государственный медицинский университет (СибГМУ)
Россия, 634050, г. Томск, Московский тракт, 2

РЕЗЮМЕ

В лекции обобщены и проанализированы результаты исследований, касающихся изучения влияния нарушений дыхания во сне (НДС) на течение наиболее распространенных хронических неинфекционных забо-

✉ *Bespalova Inna D.,* innadave@mail2000.ru

леваний легких (ХНЗЛ). Нарушения дыхания во сне, такие как храп, синдром гиповентиляции во сне, синдром обструктивного и центрального апноэ сна, представляют собой актуальную медицинскую проблему ввиду их высокой распространенности и неблагоприятных последствий для здоровья. Нарушения дыхания во сне рассматриваются как независимый фактор риска развития и прогрессирования целого ряда ХНЗЛ. Своевременная диагностика и коррекция НДС может быть эффективной мерой профилактики тяжелого течения и осложнений этой группы заболеваний.

Ключевые слова: нарушения дыхания во сне, храп, апноэ, гипоксия, хронические неинфекционные заболевания 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.

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INTRODUCTION

The beginning of the twenty-first century has set new challenges for medical science and clinical medicine related to the epidemic level of prevalence of chronic non-communicable diseases and the need to search for mechanisms underlying their progression and effective methods of elimination. A special place in the profile of morbidity is occupied by chronic non-communicable lung diseases (CNLDs), which represent a serious problem for the health care systems of most countries. CNLDs are characterized by persistent inflammation, damage to the lung parenchyma, and progressive deterioration of the functional activity of external respiration, which underlies disability and high mortality and contributes to a significant decrease in the quality of life.

The accumulation of clinical and experimental knowledge, the development of modern medical technologies, and a new branch of practical medicine – somnology, have made it possible in recent years to identify new aspects of the pathogenesis of a number of pathological conditions and to consider sleep disordered breathing (SDB) as one of the mechanisms underlying the progressive course and complications of chronic non-communicable diseases. SDB is a heterogeneous group of syndromes characterized by periodic or persistent changes in the breathing pattern during sleep, which may include episodes of apnea (short-term pauses in breathing), hypopnea (decreased respiratory activity), and other disorders. These conditions lead to hypoventilation, hypoxemia and can be associated with various somatic symptom and mental disorders, which, along with the high prevalence, determines their medical significance. According to the mechanism of occurrence, obstructive apnea/hypopnea, caused by upper airway closure, and central apnea/hypopnea, associated with collapse of the respiratory center, are distinguished [1].

The most studied type of SDB is obstructive sleep apnea (OSA) – a condition in which episodes of a lack of pulmonary ventilation with pauses in breathing during sleep for more than 10 seconds (apnea/hypopnea) are recorded, accompanied by snoring, periodic pharyngeal collapse, hypoxemia, excessive daytime sleepiness, and gross fragmentation of sleep [2]. The prevalence of OSA in the Western population is 5–7% of the entire population over 30 years of age. Severe forms of the disease affect about 1–2% of this group. In people over 60 years of age, the frequency of OSA increases significantly and is about 30% in men and about 20% in women. The prevalence of clinically pronounced SDB reaches 15% in patients with internal diseases and increases with cooccurring comorbidities.

According to a number of studies, the incidence of OSA in overweight patients exceeds 30%, reaching 50–98% in patients with morbid obesity

[3]; the prevalence of OSA and other forms of SDB are observed in 35–80% of patients with arterial hypertension (AH) [4], 38–65% of patients with coronary heart disease (CHD) [5], 38–72% of patients with a previous stroke [6], 35–40% of patients with heart failure (HF), and 56–74% of patients with rhythm disturbances [4]. Given the current trend towards population aging and the obesity pandemic, a steady increase in the prevalence of SDB is expected, since obesity and old age are recognized as the main risk factors for OSA [7].

Unfortunately, the level of awareness of patients and primary care specialists about the problem of SDB is low; OSA often remains undiagnosed in a large part of the population due to the low specificity of complaints and the unavailability of instrumental diagnostic methods [8].

The disappointing values for morbidity, disability, and mortality from respiratory diseases are largely determined by the epidemic level of prevalence of metabolic syndrome components in the general population and a high cardiovascular risk in different population groups [9, 10]. In view of the fact that SDB is an independent risk factor for cardiovascular diseases [1], there is a need to consider it as a significant mechanism for a severe course and development of complications in the most common CNLDs.

SLEEP DISORDERED BREATHING AND BRONCHIAL ASTHMA

Despite significant advances in the diagnosis and treatment of bronchial asthma (BA), high morbidity and low control levels in the presence of comorbid pathologies, including SDB, require studying the mechanisms underlying syntropy and finding effective treatment approaches. The incidence of OSA in patients with BA is higher than in the general population, regardless of body mass index (BMI), gender, age, or smoking status [11–13]. According to various data, the prevalence of SDB in patients with BA varies from 23 to 46% and depends on the severity of the disease. The prevalence of OSA in patients with BA ranges from 19 to 60% in mild BA and reaches up to 95% in severe asthma.

BA was shown as an independent risk factor for habitual snoring, which is the mildest form of SDB. The wide range of epidemiological parameters is associated with the peculiarity of the study design, with the use of different diagnostic criteria for pathological conditions, and with patient inclusion criteria. Researchers from Saudi Arabia revealed high prevalence of BA in patients with SDB (35.1%), which is also significantly higher than in the general population. Patients with OSA with BA had higher BMI and greater apnea / hypopnea index compared to patients with OSA without BA, while BMI > 35 kg / m^2 was a significant predictor of BA in patients with OSA [14]. The authors believe that such high prevalence of the association of two diseases (OSA and BA) cannot be a coincidence and is determined by the pathophysiology of the diseases [15].

OSA and BA have some common characteristics. Both diseases are obstructive respiratory diseases, but with different mechanisms and anatomy of obstruction. In patients with co-occurring OSA and BA, there is obstruction of both upper and lower airways during sleep. Both pathological processes have the same comorbidities, such as obesity, allergic rhinitis, and gastroesophageal reflux disease (GERD). It was also noted that smoking, obesity, GERD, and allergic rhinitis should be considered as important risk factors for OSA in patients with BA. A comparative analysis of parameters characterizing sleep quality in groups of patients with BA demonstrated that patients with OSA had higher BMI, higher incidence of allergic rhinitis, a more severe course, and worse predicted forced expiratory volume in the first second (FEV_1). This category of patients has poor sleep quality, which is often associated with high morbidity and mortality. Researchers agree that BA and OSA are characterized by a bidirectional interaction.

On the one hand, the severity and duration of BA affect the predisposition to OSA. The mechanisms of this phenomenon are considered to be systemic inflammation and neuroimmune interactions due to their involvement in the control of breathing, as well as the negative effects of inhaled glucocorticoids (ICS) on smooth muscles and fat content, changing the anatomy of the upper respiratory tract. On the other hand, OSA affects

airway inflammation, promotes their remodeling and dysfunction in such a way that it determines resistance to standard therapy, which explains the relationship between OSA and BA with worse clinical outcomes at all stages of medical care. Moreover, the prevalence of OSA correlates not only with the duration and severity of the disease, but also with the dosage of glucocorticoids taken [15]. Thus, the absence of OSA treatment can lead to increased ICS therapy, which, in turn, will accelerate this vicious circle and contribute to irreversible dysfunction of the lower respiratory tract [16, 17].

There is growing evidence of a relationship between SDB and BA based on common pathophysiological factors and mutual influence. The exact mechanisms by which these diseases interact are not fully understood. SDB is believed to stimulate inflammatory responses through hypoxia, hypercapnia, and sleep fragmentation, leading to a reversible increase in C-reactive protein (CRP) levels and TNFα production and is associated with airway collapse. At the same time, the level of both proinflammatory cytokines usually decreases after CPAP therapy, positively affecting the course of BA, pulmonary function parameters, and quality of life.

OSA, in turn, aggravates nocturnal manifestations of BA due to reflex bronchoconstriction associated with upper airway irritation during snoring. Inflammatory infiltration of the upper airways in BA, increased fat deposition in the pharyngeal walls due to steroid use [18], or the presence of comorbidities, such as obesity, lead to a decrease in the cross-sectional diameter of the upper airways. The frequent association of BA with allergic rhinitis, nasal polyps, and adenoid hypertrophy contributes to airflow resistance and creates high negative pressure during inspiration, which increases the risk of upper airway collapse [19]. BA is thought to affect pharyngeal muscle function either directly by affecting neural sensory pathways due to inflammation, or indirectly by muscle weakness due to ICS therapy [15]. Pharyngeal muscle myopathy increases the ability of the upper airways to collapse, increasing the risk of OSA. Japanese scientists established that the severity of OSA, estimated by the value of the apnea/hypopnea index in BA, is determined by the thickness of the mediastinal adipose tissue and the severity of bronchial hyperreactivity [20].

The practical significance of studying the mechanisms of mutual aggravation of the two diseases is determined by the fact that BA with co-occurring SDB is characterized by a low level of control, has a worse prognosis, is associated with a high risk of repeated hospitalizations due to exacerbation, and, as a result, is associated with high treatment costs [21, 22].

Currently, there is an obvious need to diagnose SDB in patients with BA, especially in cases with refractory BA, frequent night attacks, concomitant obesity, GERD, and atopic rhinitis. Timely diagnosis of SDB in patients with BA and appropriate treatment will stop the vicious circle of OSA and eliminate associated adverse effects of basic therapy, which will also help reduce cardiovascular risk in this category of patients, improve their quality of life, and naturally reduce the economic burden of medical care.

SLEEP DISORDERED BREATHING AND CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Chronic obstructive pulmonary disease (COPD) is a progressive disease characterized by persistent airflow limitation, which is a consequence of the chronic inflammatory response of the airways and lung tissue to the effects of inhaled harmful particles or gases. COPD is an urgent medical and social problem, being, according to experts from the World Health Organization, one of the leading causes of death in the world, ranking third.

SDB and sleep disorders are an extremely common and often underestimated problem in patients with COPD. It is assumed that each form of SDB in COPD is associated with adverse clinical outcomes, including an increased risk of exacerbations, hospitalizations, cardiovascular events, decreased survival, and deterioration in the quality of life [23]. Due to the high incidence of OSA, much attention has been paid by researchers to the study of co-occurring COPD and OSA, which is called overlap

syndrome. Given the high prevalence of COPD and OSA separately, researchers have suggested that the coexistence of both disorders may arise solely based on chance association [24]. The prevalence of overlap syndrome in the general population has been reported to range from 1 to 3.6% [25].

However, this figure increases significantly when the prevalence of overlap syndrome is assessed in patient populations from specialized clinics for the diagnosis and treatment of OSA or COPD. Studies including patients with diagnosed OSA have shown that the prevalence of overlap syndrome ranged from 7.6 to 55.7%. The presence of comorbid OSA in populations with established COPD has also been assessed, and again a wide prevalence range from 2.9 to 65.9% was observed [25]. However, smaller studies have reported significantly higher prevalence of overlap syndrome, which may indicate the possibility of incidental findings or higher-risk patient cohorts [26]. The reasons for these contradictory results are unclear, but they may reflect differences in the study populations, recording methods, and diagnostic methods for OSA and COPD.

Intermittent upper airway obstruction in OSA may worsen the course of COPD, leading to more pronounced hypoxemia and hypercapnia, which, in turn, accelerates the development of pulmonary hypertension and chronic respiratory failure [27]. Chronic inflammation and airway remodeling in COPD create the prerequisites for the development of OSA by reducing the tone of the upper airway muscles and increasing their collapsibility. There is a correlation between the severity of obstructive respiratory disorders and sleep disorders in patients with overlap syndrome [28]. Patients with COPD have a deterioration in sleep quality due to a decrease in its efficiency and reduction in the REM phase, which is an additional factor in the potential association with OSA [29].

The manifestations of systemic inflammation and oxidative stress observed in COPD and OSA indicate a deep pathogenetic relationship between these pathologies and their impact on the development of cardiovascular diseases [30]. Systemic inflammation is a major factor in the pathogenesis of atherosclerosis, and intermittent (periodic) hypoxemia associated with recurrent episodes of apnea/hypopnea in OSA significantly affects this inflammatory response. Hypoxemia, both intermittent and persistent, is more pronounced in co-occurring OSA and COPD compared to each disease separately and, therefore, is expected to increase the inflammatory response. Patients with overlap syndrome have higher sympathetic and lower parasympathetic activity compared to patients with OSA or COPD alone.

Thus, it can be expected that cardiovascular diseases will be more common in patients with a combination of OSA and COPD. Retrospective studies have shown higher prevalence of AH, diabetes mellitus, metabolic syndrome, and atrial fibrillation in patients with co-occurring OSA and COPD compared to patients with OSA alone [31, 32]. From a cardiovascular perspective, the importance of recognizing concomitant OSA in patients with COPD is supported by a study in a rodent model, which found that cardiovascular changes caused by chronic intermittent hypoxia can be reversible under normoxia [33].

The overlap syndrome is associated with a more severe disease course, a high risk of exacerbations, hospitalizations, and mortality compared to the isolated course of each of these diseases. In particular, a research team from Uzbekistan showed that patients with COPD in the presence of OSA are characterized by an increase in the intensity of dyspnea, the severity of obstructive disorders, and a decrease in exercise tolerance. In addition, the course of the disease was accompanied by an increase in the number of exacerbations requiring hospitalization [34].

According to the latest data, different phenotypes of COPD suggest the participation of diverse, different pathophysiological mechanisms in the formation of SDB [35]. In particular, patients with a predominantly bronchitis phenotype of COPD are most often diagnosed with OSA, which leads to an increase in mortality rates, the risk of cardiovascular complications, hospitalizations, and the frequency of exacerbations [36]. In addition, there is growing evidence that hyperinflation of the lungs associated with emphysema reduces the likelihood of developing OSA [37]. However, in practice, the vast majority of patients with COPD have a combination of emphysema and chronic bronchitis, and thus the likelihood of developing OSA is likely to depend on the balance of protective and contributing factors in individual patients [24].

Therefore, early diagnosis of SDB in patients with COPD allows for identifying concomitant pathologies,

which, in turn, contributes to the development of adequate treatment strategies and improvements in the patient's quality of life. In addition, a correct assessment and monitoring of sleep can contribute to more accurate control of clinical manifestations of COPD. Consequently, the diagnosis of SDB in this category of patients becomes an integral part of a comprehensive approach to treatment and health maintenance.

SLEEP DISORDERED BREATHING AND INTERSTITIAL LUNG DISEASES

Interstitial lung diseases (ILD) are parenchymatous lung diseases characterized by a chronic, sometimes rapidly progressive course and a high mortality rate. ILD includes about 130 nosological entities of known and unknown etiology. One of the most unfavorable diseases of this group is idiopathic pulmonary fibrosis (IPF). However, with the development of inflammation and fibrosis of the pulmonary interstitium and air spaces, other interstitial diseases can acquire a progressive course and be very close to IPF in severity, progression of respiratory failure, and mortality prognosis.

The patient's life expectancy from the moment of IPF diagnosis is 2 to 5 years. Hypoxic vasoconstriction, obliteration, and remodeling of the vascular endothelium contribute to the development of pulmonary hypertension, which is an unfavorable prognostic sign for the course of ILD. The most common cause of death in this group of patients is progressive respiratory failure [38]. Factors that determine the course and prognosis of patients with ILD include age, forced vital capacity (FVC), diffusing capacity of the lungs for carbon monoxide (DLCO), and exercise tolerance [39, 40].

It is known that due to ventilation dysfunction and gas exchange limitations, SDB is a very common concomitant pathology of ILD, varying from 45 to 90% depending on the diagnostic methods used. Most of the studies on co-occurring ILD and SDB are devoted to IPF and lung damage in systemic connective tissue diseases. Despite the small number of studies, it was shown that OSA and nocturnal hypoxemia are associated with progression and adverse outcomes of the disease. In the study by N.I. Laz et al., 69 patients with ILD identified by high-resolution chest computed tomography were divided into groups with and without OSA. Patients were assessed using the STOP-BANG questionnaire, Epworth Sleepiness Scale, and nocturnal polysomnography to diagnose and classify SDB. More than half of the patients (60.9%) had SDB, of which 57.1% had OSA, the incidence of mild OSA was only 21.7% [41].

In a prospective study of 46 patients with ILD that lasted for 18 years, a multivariate regression analysis showed that exercise desaturation (hazard ratio (HR) 8.2; 1.8–36.5 95% confidence interval (CI); $p = 0.006$) and apnea/hypopnea index ≥ 30, namely the threshold for severe OSA (HR 7.5; 1.8–30.6; $p = 0.005$), were the only independent variables associated with disease progression [42].

In another prospective observational study, 102 patients with ILD who did not have daytime hypoxemia underwent a home sleep study for 1 year. Nocturnal hypoxemia was defined as ≥ 10% of the total sleep time with $SpO_2 < 90\%$, and OSA was detected if the apnea/hypopnea index was ≥ 15 events / hour. Nocturnal hypoxemia was detected in 20 (19.6%) of them, and OSA in 32 (31.4%). Nocturnal hypoxemia was associated with a significant deterioration in the quality of life and a higher risk of death from all causes within one year (HR 8.21; 95% CI 2.4–28.1; $p < 0.001$). A similar association was not found for OSA [43].

According to some authors, treatment of SDB in patients with IPF can improve the quality of life and disease prognosis. In a prospective pilot study, 50 patients with IPF and SDB were systematically monitored and received CPAP therapy and/or nocturnal oxygen therapy depending on the type of SDB. Sleep studies revealed some type of SDB in 70% of patients: OSA – in 36% of cases, central sleep apnea – in 22% of cases, and nocturnal hypoxemia – in 12%. Over the course of one year of therapy, polysomnography revealed an improvement in the morphological parameters of IPF, while no significant changes in the functional parameters were noted. The authors conclude that episodes of apnea/hypopnea in patients with IPF contribute to recurrent traction lung injury and enhance fibrotic changes [44].

Future prospective randomized studies with a longer follow-up period will allow to study in detail the mechanisms of interaction between ILD and SDB, assess their impact on the quality of life of patients, and develop effective methods for treating this combined pathology.

SLEEP DISORDERED BREATHING AND RESPIRATORY CANCER

Recent studies have shown that there is a link between SDB and an increased risk of cancer development and progression [45]. According to modern scientific data, there are a number of mechanisms that contribute to this link. The key pathogenetic factor is the presence of intermittent hypoxia at night [46]. Hypoxia is an important component of carcinogenesis; it can enhance the malignant properties of tumor tissue: promote more aggressive tumor growth, active proliferation, invasion and metastasis, reduce the effectiveness of radiation therapy or chemotherapy; increase the frequency of cancer recurrence and mortality [47].

In 2012, according to the Wisconsin Cohort Study, which lasted more than 20 years, a link was shown between SDB and cancer mortality, and this link remained significant after adjusting for possible concomitant variables, including age, gender, smoking, BMI, physical activity, diabetes mellitus, waist circumference, and sleep duration [48]. A study by N. Marshall et al. published in 2014 showed that moderate or severe OSA (apnea/hypopnea index > 15) was associated with a relative risk of 2.5 for cancer incidence and 3.4 for cancer mortality [49]. A later Israeli cohort study of 5,243 patients found that patients under 45 years of age with severe OSA had significantly higher incidence of all types of cancer than the general population [50]. It was shown that the presence of severe OSA was associated with a 15% increased risk of developing cancer of various localizations compared to those who did not have OSA [51].

The association between SDB and lung malignancies deserves special attention. A meta-analysis published in 2022, which included seven large studies, showed that the presence of SDB was independently associated with higher incidence of lung cancer [adjusted odds ratio (OR): 1.28; 95% CI 1.11–1.47; $p < 0.001$; 12 = 37%] [52]. The results indicate not only high incidence of malignant neoplasms in the context of SDB, but also wide prevalence of SDB among cancer patients. It was found that the prevalence of OSA among this category of patients was 46% (95% CI, 27–67), and in patients with OSA, the incidence of cancer was 1.53 (95% CI 1.01–2.31) times higher than in patients without OSA, and it depended on the severity [53]. There are data confirming significant prevalence of SDB among patients with tumors of both upper and lower respiratory tract.

Thus, according to the results of a cross-sectional study conducted by Spanish researchers among 66 patients with a confirmed diagnosis of lung cancer, the overwhelming majority (80%) were diagnosed with OSA (apnea/hypopnea index > 5) during the examination, and 50% had moderate or severe OSA (apnea/hypopnea index > 15) [54]. A recent study by a team of scientists from New Delhi also demonstrated high prevalence of SDB in patients with lung cancer. The researchers set themselves the goal of establishing the prevalence of SDB in patients with newly diagnosed lung cancer. Among 30 such patients, SDB and OSA were confirmed in 66.6 and 56.6% of patients, respectively, using polysomnography [55].

SDB is quite common in patients with tumors of the head and neck (namely, the upper respiratory tract – nasopharynx, oropharynx, larynx). The high prevalence of OSA before treatment in patients with head and neck tumors can be explained, on the one hand, by structural abnormalities due to growing tumor tissue with airway obstruction; on the other hand, the development/worsening of OSA occurs during treatment due to structural changes in the upper respiratory tract due to surgery and/or radiation therapy [56]. The authors suggest that the main reason for the worsening of OSA in patients after radiation therapy is a decrease in the function and control of the pharyngeal dilator muscle, which can affect the compliance and resistance of the upper airways [57]. It was found that the clinical cancer outcome (recurrence of the disease or mortality) in patients with head and neck tumors was significantly associated with the apnea/hypopnea index [58].

Recent publications emphasize the importance of further research on the development of OSA in patients with respiratory cancer aimed at identifying the mechanisms and developing effective pathogenetically substantiated methods of correction [59]. Timely diagnosis and treatment of SDB will reduce the potential risk of developing cancer and help improve the prognosis and course of existing tumor processes.

CONCLUSION

Thus, SDB should be considered as a risk factor for a severe course and complications of socially sensitive chronic non-communicable lung diseases. The prevalence of SDB of varying severity in

respiratory diseases is extremely high, especially in the context of comorbid pathology. In the context of the obesity epidemic and the trend towards population aging, a widespread increase in the prevalence of SDB is expected in the coming years. Timely diagnosis and elimination of SDB can be an effective measure for preventing a severe course and complications of this group of diseases.

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Authors' information

Bespalova Inna D. – Dr. Sci. (Med.), Head of the Division of Propedeutics of Internal Diseases with a Course in Therapy, Siberian State Medical University, Tomsk, innadave@mail2000.ru, http://orcid.org/0000-0002-4513-6329

Mitrichenko Ulyana M. – Graduate Student, Division of Propedeutics of Internal Diseases with a Course in Therapy, Siberian State Medical University, Tomsk, strashkovaum@gmail.com, http://orcid.org/0000-0001-6091-4849

Koshchavtseva Yuliya I. – Teaching Assistant, Division of Propedeutics of Internal Diseases with a Course in Therapy, Siberian State Medical University, Tomsk, kossy09@mail.ru, http://orcid.org/0000-0001-5260-4832

Kapitanova Darya V. – Cand. Sci. (Med.), Associate Professor, Division of Propedeutics of Internal Diseases with a Course in Therapy, Siberian State Medical University, Tomsk, kapitanova.dv@ssmu.ru, http://orcid.org/0000-0001-9588-1637

Badmaev Agvan Z. – Teaching Assistant, Division of Propedeutics of Internal Diseases with a Course in Therapy, Siberian State Medical University, Tomsk, badmaev.az@ssmu.ru

Agaeva Sofia – Teaching Assistant, Division of Propedeutics of Internal Diseases with a Course in Therapy, Siberian State Medical University, Tomsk, agaeva.sa@ssmu.ru

Zhukovskaja Olga V. – Laboratory Researcher, Division of Propedeutics of Internal Diseases with a Course in Therapy, Siberian State Medical University, Tomsk, zhukovskaya.ov@ssmu.ru

Kolmakova Vera M. – Laboratory Researcher, Division of Propedeutics of Internal Diseases with a Course in Therapy, Siberian State Medical University, Tomsk, olmakova.vm@ssmu.ru

Belyakova Tatyana V. – Laboratory Researcher, Division of Propedeutics of Internal Diseases with a Course in Therapy, Siberian State Medical University, Tomsk, belyakova.tv@ssmu.ru

Teteneva Anna V. – Dr. Sci. (Med.), Professor, Division of Propedeutics of Internal Diseases with a Course in Therapy, Siberian State Medical University, Tomsk, anna.dubodelova@mail.ru, http://orcid.org/0000-0002-4323-2798

Bukreeva Ekaterina B., Dr. Sci. (Med.), Professor, Division of Propedeutics of Internal Diseases with a Course in Therapy, Siberian State Medical University, Tomsk, kbukreeva@mail.ru, http://orcid.org/0000-0002-7699-5492

Boyarko Valentina V. – Cand. Sci. (Med.), Associate Professor, Division of Propedeutics of Internal Diseases with a Course in Therapy, Siberian State Medical University, Tomsk, vvboyarko@mail.ru, http://orcid.org/0000-0002-5700-1640

Nesterovich Sof'ja V. – Cand. Sci. (Med.), Chief Physician of SSMU Clinics, Siberian State Medical University, Tomsk, nesterovich.sv@ssmu.ru, ORCID 0000-0003-2098-2964

Vinokurova Darya A. – Head of the Internal Medicine Clinic, Siberian State Medical University, Tomsk, vinokurovadarial@gmail.com, http://orcid.org/0000-0002-8422-8349

Kalyuzhin Vadim V. – Dr. Sci. (Med.), Professor, Head of the Advanced Therapy Division with a Course in Rehabilitation, Physiotherapy and Sports Medicine, Siberian State Medical University, Tomsk, kalyuzhinvv@mail.ru, http://orcid.org/0000-0001-9640-2028

(✉) **Bespalova Inna D.,** innadave@mail2000.ru

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Radionuclide GRPR imaging in malignant pathology of the mammary and prostate glands: clinical experience

Bragina O.D.[1, 2], Ivanova A.G.[1, 3], Usynin E.A.[1]

[1] Cancer Research Institute, Tomsk National Research Medical Center (NRMC), Russian Academy of Sciences
5, Kooperativny Lane, Tomsk, 634009, Russian Federation

[2] National Research Tomsk Polytechnic University (NR TPU)
30, Lenina Av., Tomsk, 634050, Russian Federation

[3] Siberian State Medical University
2, Moscow Trakt, Tomsk, 634050, Russian Federation

ABSTRACT

In this lecture, we presented current clinical studies on targeted radionuclide imaging of breast and prostate tumors with overexpression of the gastrin-releasing peptide receptor (GRPR). GRPR is a transmembrane receptor, the activation of which promotes the growth and proliferation of tumor cells. The highest level of GRPR expression is observed in malignant pathologies of breast and prostate, which is of particular interest for radionuclide diagnostics.

The conducted clinical studies assessed the safety, pharmacological properties, and effectiveness of imaging using radiopharmaceuticals based on peptide agonists and antagonists of GRPR labeled with technetium-99m and gallium-68 radionuclides. The results clearly demonstrate the advantage of GRPR antagonists over GRPR agonists, since they have optimal pharmacological properties, good tolerability, rapid elimination by organs with a physiological level of receptor expression, and high imaging efficiency of mammary and prostate tumors with overexpression of GRPR.

Keywords: GRPR, targeted radionuclide diagnosis, breast cancer, prostate cancer

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Радионуклидная визуализация GRPR при злокачественной патологии молочной и предстательной желез: опыт клинического применения

Брагина О.Д.[1, 2], Иванова А.Г.[1, 3], Усынин Е.А.[1]

Научно-исследовательский институт (НИИ) онкологии, Томский национальный исследовательский медицинский центр (НИМЦ) Российской академии наук
Россия, 634009, г. Томск, пер. Кооперативный, 5

✉ *Bragina Olga D.*, bragina_od@mail.ru

[2] *Национальный исследовательский Томский политехнический университет (НИ ТПУ)*
Россия, 634050, Томск, пр. Ленина, 30

[3] *Сибирский государственный медицинский университет (СибГМУ)*
Россия, 634050, Томск, Московский тракт, 2

РЕЗЮМЕ

В лекции представлены актуальные клинические исследования относительно таргетной радионуклидной визуализации опухолей молочной и предстательной желез с гиперэкспрессией рецептора гастрин-высвобождающего пептида (GRPR). Рецептор GRPR представляет собой трансмембранный рецептор, активация которого способствует росту и пролиферации опухолевых клеток. Наиболее высокий уровень экспрессии GRPR наблюдается при таких злокачественных патологиях, как рак молочной и предстательной желез, что представляет особый интерес для радионуклидной диагностики.

В проведенных клинических исследованиях оценивались безопасность, фармакологические свойства, эффективность визуализации радиофармпрепаратов на основе пептидов-агонистов и антагонистов GRPR, меченных радионуклидами технецием-99m и галлием-68. Результаты испытаний наглядно демонстрируют преимущество антагонистов GRPR перед агонистами GRPR, поскольку обладают оптимальными фармакологическими свойствами, хорошей переносимостью, быстрым выведением органами с физиологическим уровнем экспрессии рецептора, высокой эффективностью визуализации опухолей молочной и предстательной желез с гиперэкспрессией GRPR.

Ключевые слова: GRPR, таргетная радионуклидная диагностика, рак молочной железы, рак предстательной железы

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

Источник финансирования. Работа выполнена в рамках гранта Российского научного фонда № 22-15-00169 по теме «Фенотип BRCA-подобных опухолей в процессе канцерогенеза и лечения».

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INTRODUCTION

Breast cancer (BC) and prostate cancer (PC) are the most common malignancies among the female and male populations, respectively [1]. The rapidly developing fields of molecular biology and oncology continue to seek new promising targets to optimize the diagnosis and treatment of oncological diseases, including gastrin-releasing peptide receptors (GRPR, BB2, Gastrin Releasing Peptide Receptor) [2].

GRPR belongs to the bombesin receptor family (BB1, BB2, BB3) and is a 7-transmembrane receptor coupled to a G protein. Its endogenous ligand is gastrin-releasing peptide (GRP), a regulatory molecule involved in stimulating gastrin release from gastric G cells and a number of other processes by binding to GRPR and activating the phospholipase C signaling pathway. In the human body, GRPR is expressed in neuroendocrine cells of the gastrointestinal tract (GI tract), brain, lungs, prostate, exocrine cells of the pancreas and mammary glands providing exocrine and endocrine functions, contraction of smooth muscles of the GI tract and genitourinary system, effects on immune cells, thermoregulation, circadian rhythm, and the growth and proliferation of both normal and pathological cells [3].

Literature data show that GRP and other peptides analogs of bombesin act as a growth factor contributing to proliferation of various cell types [4]. The binding of GRP to GRPR stimulates the phosphorylation of tyrosine kinase receptors, causing cross-talk of G-protein-coupled receptors (GPCR) [5]. Similarly, faster activation of the epidermal growth factor receptor (EGFR) is provided, through which subsequent signaling occurs via the mitogen-activated protein kinase (MAPK) signaling pathway in cells of head and neck carcinoma and non-small cell lung cancer [6]. In addition, GRPR is capable

of increasing the expression of cyclins, such as D1 and E, while simultaneously reducing p27 (cyclin-dependent kinase inhibitor) and hyperphosphorylating retinoblastoma protein (pRb), resulting in the cell transition from the G1 phase of the cell cycle to the S phase. Another effect may be the effect of GRP on cell survival and the involvement of PI3K-Akt signaling pathways after GRPR activation. This assumption has not been fully studied yet and requires further research (Figure) [7].

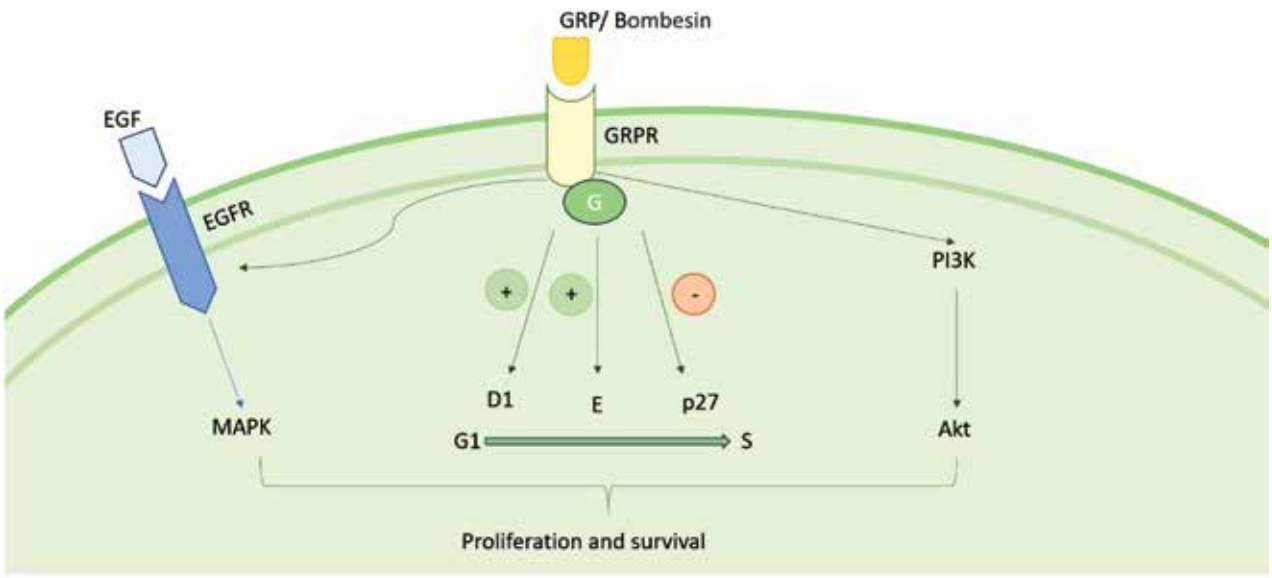

Figure. The role of GRPR in cell proliferation

Overexpression of GRPR has been found in a variety of malignant neoplasms, such as non-small cell lung cancer, kidney cancer, gastrinomas, gastrointestinal stromal tumors, head and neck cancers, and neuroblastomas. However, the highest expression of the GRPR is most often observed in prostate and breast cancers [4]. According to literature data, GRPR expression is found in 75.8% of malignant breast tumors and is largely associated with the expression of estrogen receptors (ER) [8, 9]. A greater number of GRPR expression cases are observed in luminal A – 86.2%, 70.5% – in luminal HER2-negative, 82.8% – in luminal HER2-positive, 21.3% – in HER2–positive (non-luminal), and 7.8% – in triple-negative (TNBC) subtypes of breast cancer [4]. According to the literature , the GRPR expression in prostate cancer is 63–100% [10].

Currently, several techniques are used to determine the level of GRPR expression in tumors, such as autoradiography of frozen sections, quantitative reverse transcription polymerase chain reaction (RT-qPCR); however, immunohistochemistry (IHC) of formalin-fixed and paraffin-embedded material, or matrix RNA (mRNA) are used more often [11]. As a rule, rabbit or mouse recombinant human polyclonal antibodies are used for IHC, as they form an antigen – antibody complex with the desired GRPR receptor. The expression of this receptor is evaluated in the form of an immunoreactivity index (II), which takes into account the intensity of staining (0 – no detectable staining, 1 – weak staining, 2 – moderate staining and 3 – strong staining) and the percentage of stained tumor cells (0 – no positive cells, 1 – ≤ 10% of positive cells, 2 – 11–50% positive cells, 3 – 51–80% positive cells and 4 – > 80% positive cells). Thus, the final assessment of II (staining intensity × percentage of positive cells) varies from 0 to 12: 0–1 – no GRPR expression, 2–3 – weak GRPR expression, 4–8 – moderate GRPR expression, 9–12 – strong GRPR expression [12].

Despite its accessibility, high sensitivity and specificity, IHC has a number of disadvantages. They include the necessity of an invasive procedure, the failure to obtain tumor samples due to tumor localizationn, violation of the methodology, for example, the molecular characteristics of the antigen may change during the fixation of histological material under the influence of fixing agents and different factors (such as long delivery of the material to the laboratory, the choice of a fixing agent,

failure to observe the fixation time), as a result of which the antigen – antibody reaction will be disrupted [13, 14].

Targeted radionuclide diagnosis. Currently, one of the areas in the diagnosis of malignant tumors is targeted radionuclide imaging, where synthetic proteins are increasingly used as a targeting module. Proteins are characterized by their small size, structure stability, affinity for antigen, optimal pharmacological and pharmacodynamic properties, and low cost of production due to expression in the bacterial system. Intravenous administration of this type of radiopharmaceuticals makes it possible to detect not only the primary tumor, but also the possible metastatic sites in in regional lymph nodes and distant organs and tissues. It also allows to detect the molecular biological characteristics of the identified tumor sites [15–18].

There are two main methods of radionuclide diagnostics – single-photon emission computed tomography (SPECT) and positron emission tomography (PET), which allow to detect areas of pathological hyperfixation of radiopharmaceuticals in metabolically active neoplasms *in vivo* [16]. Due to the high level of GRPR in mammary and prostate tumors compared with normal tissues (in particular in the pancreas and neuroendocrine cells of the GI tract), SPECT and PET are considered to be promising methods to detect GRPR expression [19, 20].

RADIONUCLIDE DIAGNOSIS OF PROSTATE CANCER WITH GRPR OVEREXPRESSION

Labeled peptides – bombesin analogues. Over the past two decades, studies with bombesin receptor agonists have been actively conducted as it was assumed that targeting GRPR using bombesin receptor agonists in radionuclide diagnosis of prostate cancer would allow for visualization of a primary tumor with high specificity due to its high affinity for this receptor. One of the first compounds which underwent clinical trials was the protein *RP527*, labeled with technetium-99m *([^{99m}Tc]Tc-RP527*). The study conducted in 2001 involved 10 patients: six patients had breast cancer, and four patients had prostate cancer. After the administration of *[^{99m}Tc]Tc-RP527*, its pathological accumulation in the tumor was observed in 4 out of 6 cases of breast carcinomas and in one out of four cases of prostate carcinomas [21]. This analysis was the starting point for further GRPR-targeted radionuclide imaging and allowed for further studies in that direction.

A clinical trial with the N-terminal modified BBN protein (1-14) labeled with ^{99m}Tc (*[^{99m}Tc]Tc-BN*) conducted in 2003 involved ten patients: eight patients had prostate cancer, two – benign prostate adenoma. According to the results of SPECT, there was high tumor uptake of radiopharmaceuticals in all 8 patients with prostate cancer after the administration of *[^{99m}Tc]Tc-BN* [22].

The *DB4* protein labeled with ^{99m}Tc (*[^{99m}Tc]Tc-DB4*) at the preclinical stage demonstrated high accumulation in PC3 xenografts of human prostate cancer in combination with its rapid excretion by the kidneys [23]. In the subsequent phase I of the clinical trial involving eight patients with prostate cancer, two individuals had a primary tumor and did not receive hormone therapy, and six patients had metastatic prostate cancer and received hormone therapy. After intravenous administration of *[^{99m}Tc]Tc-DB4*, the primary tumor node in the prostate was visualized on SPECT in all patients who did not receive systemic treatment, while in patients with metastatic prostate cancer, the accumulation was extremely low [24].

Another bombesin receptor agonist that was studied in a clinical trial was the molecule *AMBA* (*DOTA-Gly-4-aminobenzoyl-BBN (7-14*)), which is a BBN protein modified at the side ends of amino acids. The analysis of ten patients with IHC confirmed tumors of various localizations (prostate cancer, breast cancer, medullary thyroid cancer, uterine and colon tumors) and yielded visualization of the primary tumor, regional and distant metastases after the administration of the [^{68}*Ga]Ga- AMBA* to patients with prostate and breast cancer. At the same time, the results in patients with thyroid cancer and tumors of the colon and uterus were unsatisfactory (Table 1) [25].

Labeled peptides – bombesin antagonists. Despite the satisfactory data from clinical research (Table 1), it was noted that the use of bombesin analogs as a targeting module has a number of disadvantages, which include receptor activation and subsequent cascade reaction, mitogenic effect on tumor cells, significant side effects in the form of nausea and vomiting, abdominal cramps (which is apparently associated with the activation of GRPR not only in tumor cells, but also in neuroendocrine cells in the GI tract and in the pancreas), and rapid desensitization of receptors to the ligand. The identified disadvantages

allowed to consider alternative compounds that exhibit antagonism towards GRPR [19].

The results of preclinical trials performed by R. Cescato et al. demonstrated a greater advantage of GRPR antagonists compared to agonists in aiming at a target due to the neutralization of the receptor activation effects, including side effects [26].

SB3 molecule was developed for diagnostic and therapeutic purposes. Radiolabeling was carried out using isotopes ^{68}Ga and ^{111}In, ^{177}Lu was used for radiotherapy. According to the results of *in vivo* studies, *[^{111}In]In-SB3* and *[^{177}Lu]Lu-SB3* were rapidly catabolized and subsequently were not admitted to clinical trials. A clinical trial with the *[^{68}Ga]Ga-SB3* involved 17 patients with a disseminated process (eight patients with breast cancer and nine – with prostate cancer) and allowed to visualize breast tumors in four out of eight patients and prostate cancer in five out of nine patients. [27].

Another GRPR antagonist *[^{68}Ga]Ga-RM2* was studied in tumor models *in vivo* and demonstrated good tolerability, specificity and sensitivity to GRP receptors, optimal pharmacological properties, and a high degree of accumulation in tumor tissue. The subsequent clinical study involved 32 patients with IHC-confirmed prostate cancer recurrence with elevated levels of prostate-specific antigen, in whom standard diagnostic methods (computed tomography and magnetic resonance imaging) did not prove to be effective. According to the study, recurrence of tumors in the prostate gland was detected in 71.8% of cases (23 out of 32 cases) [28]. *[^{68}Ga]Ga-RM2* was also studied in 2022 in 41 patients with moderate and high-risk prostate cancer. The PET data after *[^{68}Ga]Ga-RM2* administration were comparable with the results of histologic examination of subsequent surgical material after prostatectomy performed in 32 patients and with the results of multivariate magnetic resonance imaging of 36 patients [29].

Another GRPR antagonist, the *RM26* molecule labeled with ^{68}Ga and ^{99m}Tc, demonstrated similar results. The first RM26 clinical trial was conducted in 2018 by J. Zhang et al., and included five healthy individuals and 28 patients diagnosed with prostate cancer (17 patients with diagnosed prostate tumors who did not receive treatment and 11 patients who underwent treatment). The *[^{68}Ga]Ga-RM26* administration did not have any side effects and was well tolerated. Visualization of the primary tumor was noted in 15 out of 17 patients, metastatic lymph nodes were observed in three out of eleven patients with previous treatment, and distant bone metastases were detected in eight out of eleven cases [30].

Phase I clinical trial of the *[^{99m}Tc]Tc-maSSS-PEG$_2$-RM26* was carried out at the Department of Radionuclide Therapy and Diagnostics of Cancer Research Institute of Tomsk NRMC in 2023. The study included six patients with prostate cancer and seven patients with breast cancer who did not receive specialized treatment. Images of the primary tumor were obtained in four out of six cases of prostate cancer, a correlation was noted with the prostate-specific antigen (PSA) level (optimal visualization was achieved in the patient with the highest PSA value) and the size of the tumor. In breast cancer patients, the effectiveness of primary tumor imaging was observed in all seven participants. Additionally, accumulation of the *[^{99m}Tc]Tc-maSSS-PEG$_2$-RM26* in metastatic regional lymph nodes was noted in three out of seven cases [31].

The antagonist of the gastrin-releasing peptide receptor *NeoBOMB1* is one of the solutions developed over the last decade. The first results of the study of the *[^{68}Ga]Ga -NeoBOMB1* including four prostate cancer patients demonstrated good tolerability and high levels of accumulation of radiopharmaceuticals in tumors, regional lymph nodes, and metastatic lesions of the liver and bones [32]. Another study focusing on *[^{68}Ga]Ga-NeoBOMB1* involved 19 patients with solid tumors of various localizations with overexpression of GRPR (tumors of the mammary and prostate glands, colorectal cancer, and lung cancer). The overexpression of GRPR was confirmed by the data of the IHC in all cases. The results of this clinical study demonstrated satisfactory tolerability of the *[^{68}Ga]Ga-NeoBOMB1*, as well as visualization of primary and metastatic tumors based on PET data [33].

Table 1

Clinical trials of bombesin analogs and antagonist peptides for radionuclide diagnosis of prostate cancer with GRPR overexpression

Analog GRPR	Radionuclide	Visualization method	Researcher, year
Bombesin analog peptides			
RP527	^{99m}Tc	SPECT	Van de Wiele, 2000
BBN	^{99m}Tc	SPECT	Scopinaro, 2003
AMBA	^{68}Ga	PET	Baum, 2007
DB4	^{99m}Tc	SPECT	Mather, 2014
Bombesin antagonist peptides			
SB3	^{68}Ga	PET	Maina, 2016

Table 1 (continued)

Analog GRPR	Radionuclide	Visualization method	Researcher, year
NeoBOMB1	^{68}Ga	PET	Nock, 2017 Djaileb, 2020
RM2	^{68}Ga	PET	Minamimoto, 2018
RM26	^{68}Ga ^{99m}Tc	PET SPECT	Zhang, 2018 Chernov, 2023

RADIONUCLIDE DIAGNOSIS OF BREAST CANCER WITH GRPR OVEREXPRESSION

Due to successful applications of labeled peptides that are bombesin analogs and its antagonists for prostate cancer, researchers concluded that this area should be studied in terms of targeted radionuclide imaging of breast tumors (Table 2).

Labeled peptides – bombesin analogs. In 2008, C. Van de Wiele et al. studied the *[^{99m}Tc]Tc-RP527* in 14 breast cancer patients, five of whom had negative expression of estrogen and progesterone receptors. The tumor process was visualized in all patients who had not previously received tamoxifen hormone therapy. In addition, metastasis to regional lymph nodes was also detected in all ER-positive patients. Imaging of tumors with a negative hormonal status was negative in all five patients [34].

Table 2

Clinical trials with bombesin analog peptides and antagonist peptides for radionuclide diagnosis of breast cancer with GRPR overexpression

Analog BBN	Radionuclide	Visualization method	Researcher, year
Bombesin analog peptides			
RP527	^{99m}Tc	SPECT	Van de Wiele, 2000 Van de Wiele, 2008
BBN	^{99m}Tc	SPECT	Scopinaro, 2002
AMBA	^{68}Ga	PET	Baum, 2007
Sestamibi	^{99m}Tc	SPECT	Urbano, 2020
Bombesin antagonist peptides			
SB3	^{68}Ga	PET	Maina, 2016
RM2	^{68}Ga	PET	Stoykow, 2016
RM26	^{68}Ga ^{99m}Tc	PET SPECT	Zhang, 2018 Chernov, 2023
NeoBOMB1	^{68}Ga	PET	Djaileb, 2020
DB15	^{99m}Tc	SPECT	Nock, 2021

A clinical study of *[^{99m}Tc]Tc-BBN* and *[^{99m}Tc]Tc-Sestamibi* with three breast cancer patients showed the high specificity of *[^{99m}Tc]Tc-BBN* and the possibility of using it to detect metastatic lymph nodes due to selective uptake by tumor cells and no uptake by nonspecific inflammatory cells [35, 36].

Labeled peptides – bombesin antagonists. Clinical trials focused on studying antagonists that have shown promising results in prostate cancer trials, as well as new molecules, are presented in Table 2.

A study of the *[^{68}Ga]^{68}Ga-RM2* conducted by C. Stoykow et al. in 2016 and included 15 patients with unilateral (n = 12) and bilateral (n = 3) breast cancer. Results demonstrated high accumulation of *[^{68}Ga]^{68}Ga-RM2* in the pathological sites in 73% of cases (in 13 out of 15 patients). In all patients, the diagnosis was confirmed by histologic examination of the biopsy material: 14 tumors were classified as invasive ductal carcinoma, three – as invasive lobular carcinoma, and one – as mucinous carcinoma. At the same time, lobular carcinoma was also detected during the study, but was not visualized using standard PET with 18F-fluorodeoxyglucose. In addition, the use of the *[^{68}Ga]^{68}Ga-RM2* showed its accumulation in axillary lymph node metastases with a diameter of < 5 mm. Visualization of lymphatic metastases using ^{18}F -fluorodeoxyglucose may be difficult due to the metabolic activity of nonspecific cells (macrophages, adipocytes, etc.) [37, 38].

Another molecule with antagonist properties was assessed in a prospective clinical trial involving 35 breast cancer patients. The administration of *[^{68}Ga]Ga-NOTA-RM26* demonstrated a positive correlation between its accumulation in tumor tissue expressing GRPR and estrogen receptors (estrogen-independent tumors were visualized worse). The authors considered the dependence of the accumulation of *[^{68}Ga]Ga-NOTA-RM26* on the phase of the menstrual cycle as another important result of the study. In this case the maximum value of SUV was observed in the secretory phase of the menstrual cycle, which can lead to distortion of the results and should be taken into account when planning further studies [39].

The *DB15* peptide labeled with technetium-99m is one of the latest advances. According to the results of preclinical *in vivo* studies, the *[^{99m}Tc]Tc-DB15* made it possible to accurately visualize primary tumors and metastatic cancer and had optimal pharmacological characteristics [40]. Two patients with advanced breast cancer participated in the first clinical trial of the *[^{99m}Tc]Tc-DB15*. No adverse effects were observed after *[^{99m}Tc]Tc-DB15* administration. According to the SPECT results, distant metastases were visualized in bones, lungs, and pleura. However, accumulation was not observed in intra-abdominal metastatic sites, which were later confirmed using

standard diagnostic methods (PET with FDG and histologic examination) [41].

CONCLUSION

The rapid development of radionuclide diagnosis demonstrates its unquestionable advantages over standard diagnostic procedures, significantly increasing the diagnostic value and reducing the cost of research. The requirements for optimizing the diagnosis of malignancies (in particular, breast cancer) contribute to expanding the scope of research to seek additional molecular targets, one of which is gastrin-releasing peptide receptors.

The results of preclinical and clinical trials have demonstrated the advantage of radiopharmaceuticals based on bombesin antagonist peptides compared with agonist peptides in the visualization of primary malignant breast tumors, as well as regional and distant metastases. At the same time, radioactively labeled GRPR antagonists showed a higher cumulative effect directly in the tumor tissue expressing this target, rapid elimination from the pancreas and other tissues with physiologically normal GRPR levels. The positive characteristics of bombesin receptor antagonists may contribute to the introduction of this method into clinical practice and consider GRPR not only as a diagnostic, but also as a therapeutic target.

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Authors' information

Bragina Olga D. – Dr. Sci. (Med.), Oncologist, Senior Researcher, Department of Radionuclide Therapy and Diagnostics, Cancer Research Institute, Tomsk NRMC; Senior Researcher, Oncotheranostics Research Center, NR TPU, Tomsk, bragina_od@mail.ru, http://orcid.org/0000-0001-5281-7758

Ivanova Anastasia G. – Resident Physician, Siberian State Medical University; Cancer Research Institute, Tomsk NRMC, Tomsk, anfioran@yandex.ru, http://orcid.org/0009-0009-3775-9989

Usynin Evgeny A. – Dr. Sci. (Med.), Head of the General Oncology Department, Cancer Research Institute, Tomsk NRMC, Tomsk, gusi70@list.ru, http://orcid.org/0000-0001-7127-0188

(✉) **Bragina Olga D.,** bragina_od@mail.ru

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Opisthorchiasis and pancreatic cancer

Ivanov V.V.[1], Komkova T.B.[1], Lyzko I.A.[1], Perina E.A.[1], Popov I.A.[2], Udut E.V.[1], Khmelevskaya E.S.[1]

[1] Siberian State Medical University
2, Moscow Trakt, Tomsk, 634050, Russian Federation

[2] Moscow Institute of Physics and Technology
9, Institutsky Lane, Dolgoprduny, 141701, Russian Federation

ABSTRACT

Chronic opisthorchiasis is recognized as a precancerous condition that can present similarly to other diseases of the hepatopancreatoduodenal zone. Statistically, there is a proven correlation between the duration and intensity of parasitic invasion and the development of carcinogenesis, with the manifestations of opisthorchiasis often obscuring the early symptoms of cancer. Many researchers are working to find methods for the early diagnosis of pancreatic cancer against the background of chronic opisthorchiasis, which may enable timely treatment of the disease in the early stages.

The authors of this lecture present a literary review of the data on the incidence of pancreatic cancer in patients with chronic opisthorchiasis. Additionally, some factors contributing to cholangiocarcinoma carcinogenesis are discussed, since the exact mechanisms leading from the introduction of a trematode to the formation of a malignant process are multifunctional. Certain phenomena regarding the effect of opisthorchis on the human body currently lack explanation and require further study and clarification.

Keywords: cholangiocarcinoma, pancreatic cancer, cholangiogenic cancer, opisthorchiasis invasion

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Описторхоз и рак поджелудочной железы

Иванов В.В.[1], Комкова Т.Б.[1], Лызко И.А.[1], Перина Е.А.[1], Попов И.А.[2], Удут Е.В.[1], Хмелевская Е.С.[1]

[1] Сибирский государственный медицинский университет (СибГМУ)
Россия, 634050, г. Томск, Московский тракт, 2

[2] Московский физико-технический институт (МФТИ)
Россия, 141701, г. Долгопрудный, Институтский пер., 9

✉ *Lyzko Ilya A.,* ilya50@yandex.ru

РЕЗЮМЕ

Известно, что хронический описторхоз рассматривается как предраковое заболевание, протекающее под маской других заболеваний органов гепатопанкреатодуоденальной зоны, и статистически имеется доказанная зависимость канцерогенеза от длительности и интенсивности паразитарной инвазии, а проявления описторхоза маскируют собой ранние симптомы онкологического заболевания. Многие авторы работают над поиском методов ранней диагностики рака поджелудочной железы на фоне хронического описторхоза, что может дать возможность своевременного лечения заболевания на ранних стадиях.

Авторы лекции представили литературный обзор данных заболеваемостью раком поджелудочной железы у пациентов с хроническим описторхозом. Кроме того, приведены некоторые факторы канцерогенеза холангиокарциномы, поскольку точные механизмы, ведущие от внедрения трематоды до формирования злокачественного процесса, остаются не до конца ясными. Некоторые феномены действия описторхисов на организм человека в настоящее время не имеют объяснения и требуют дальнейшего изучения и детализации.

Ключевые слова: описторхоз, холангиокарцинома, рак поджелудочной железы, холангиогенный рак, описторхозная инвазия

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

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INTRODUCTION

The increase in the incidence of pancreatic and bile duct cancer is one of the most pressing issues of modern abdominal surgery. At the same time, according to a number of authors, the 5-year survival rate in this category of patients is about 10% [1, 2]. Research in the field of surgical oncology determines a clear correlation of the development of malignant processes with a long-term parasitic infection of the biliary system. The aim of this lecture is to provide a literature overview of the data on the incidence of pancreatic cancer in patients with chronic opisthorchiasis. In addition, the lecture presents some factors in the carcinogenesis of cholangiocarcinoma, since the exact mechanisms leading from the introduction of a trematode to the formation of a malignant process remain unclear.

As is known, human infection with *Opisthorchis felineus* occurs when eating raw or undercooked freshwater fish from the carp family, which contain metacercarial cysts in the muscles [3]. After metacercariae excyst in the jejunum or duodenum, they migrate to the bile ducts, where they mature into adult worms that remain viable for more than 10 years [4]. The clinical pattern of the disease caused by the invasion of *Opisthorchis felineus* is usually characterized by signs of damage to the hepatobiliary system, but it is often not limited to symptoms reflecting the pathology of the parasite host organs, which allows us to consider opisthorchiasis as a systemic disease [5, 6].

According to the World Health Organization, trematodoses are estimated to cause 2 million life years lost to disability and death worldwide every year, and opisthorchiasis is one of the most common helminthiases transmitted through infected fish [7]. About 40 million people in the world are infected with opisthorchiasis. At the same time, most of them are in Russia, and the incidence rate varies by region and often correlates with the level of consumption of freshwater fish, which is traditionally higher in rural areas [8].

According to the Federal Service for the Oversight of Consumer Rights and Welfare of the Russian Federation, parasitic infections in fish have been proven in 26 regions of the Russian Federation and cases of human infection are regularly recorded. The most endemic regions are located near large rivers where infected fish is caught for commercial sale. Thus, in the Ob-Irtysh river basin with the world's largest opisthorchiasis case number, up to 1,000 cases per 100,000 people are recorded, with 95% of the rural population infected [8].

In Russia, the highest incidence rate is recorded in the Tyumen and Tomsk regions, while the incidence rate is average in the rest of the regions. In her research, E.N. Ilyinskikh has established a trend toward an increase in the incidence of opisthorchiasis in Russia as a whole. During the analyzed period, opisthorchiasis was not registered only in the Pskov and Lipetsk regions. As a result of the analysis of territorial trends in the changes of morbidity growth, the authors concluded that in 22 territories there was a trend toward an increase in the number of cases, and only in two regions morbidity growth decreased. At the same time, the discussed indicators have remained at the average level for many years. Presumably, such dynamics could be explained by the fact that people in these regions do not usually eat raw or pickled fish [9].

O.S. Fedorova et al. believe that the increase in the incidence of opisthorchiasis in Russia is also influenced by the active migration of the population from Western Siberia to other territories. And if we take into account that in some areas of the Ob-Irtysh river basin, almost 100% of the population is diagnosed with opisthorchiasis, then there is a real threat of an increase in the number of patients throughout Russia. The authors draw attention to an increase in the incidence in areas where this helminthiasis was not previously detected, and in some territories of the Ob-Irtysh river basin, on the contrary, there is a decrease in the incidence rate [10].

In 2009, the International Agency for Research on Cancer (IARC) of the World Health Organization has identified *O. viverrini* among the definitive causes of bile duct cancer or cholangiocarcinoma, classifying this helminthiasis as a group 1 carcinogen [11].

A group of scientists including J.M. Banales, J.J.G. Marin, A. Lamarca, P.M. Rodrigues, S.A. Khan, L.R. Roberts and others in their study note that about 3% of all cases of malignant lesions of the gastrointestinal tract are cholangiocarcinoma, and among primary liver cancers, the incidence of this form reaches 15% [12]. Clinically, this pathology is also characterized by high mortality, which accounts for 2% of all deaths from cancer. Such statistics are explained, first of all, by the ineffectiveness of early, including non-invasive, diagnosis, and the need for histological confirmation of the diagnosis. In addition, this form of malignant liver damage is highly resistant to various treatment methods due to its properties at the genomic, epigenetic, and molecular levels. A number of authors draw attention to the fact that in inoperable forms of the disease, the median survival is only 3–13 months. The prognosis is usually poor, after treatment there is a high risk of relapse, and the five-year survival rate does not exceed 25–43% [12, 13]. All of the above determines the need to find methods for early diagnosis of cholangiocarcinoma, which may be one of the ways to improve treatment outcomes in patients with this serious pathology.

It is believed that the combination of mechanical damage caused by the attachment and nutrition of fluke and exposure to excretory/secretory products of flukes occurs over a long period of time and ultimately leads to the development of inflammation and chronic hepatobiliary disorders [14]. These processes cause oxidative stress, lead to DNA damage and gene mutation, as well as impaired regulation of cell growth in the form of goblet cell metaplasia, adenomatous dysplasia and epithelial hyperplasia, all of which creates the basis for carcinogenesis [10, 15, 16].

G.A. Maksimova et al. studied the effect of *O. felineus* on carcinogenesis. As a result of the conducted research, the authors concluded that the effect of *O. felineus* on the development of liver pathology is comparable with that of *O. viverrini* [17]. The results obtained may indicate the need to change the group of carcinogens to which *O. felineus* belongs according to the IARC classification. M.N Lvova et al. note that despite the similarity of these two parasites in morphology, significant differences in the timing of the histopathological profile and the very nature of these changes allow us to conclude that opisthorchiasis invasion caused by the European liver fluke *O. felineus* is more pathogenic than that caused by the Asian fluke *O. viverrini* [18].

According to the group of authors – O.A. Baykova, N.N. Nikolaeva, E.G. Grishchenko, L.V. Nikolaeva – similarity of pathogens of opisthorchiasis and the morphological identity of their damaging effects on the hepatobiliary system suggest that the model of cancer development by representatives of the Opisthorchidae family is similar [19]. The multifactorial nature of cancer development in opisthorchiasis is determined by three main components of carcinogenesis. These components include mechanical damage to the mucous membrane by parasites, toxic, anti-apoptotic, and hyperproliferative effects of secretory parasitic proteins, immunopathological processes (oxidative

stress). Mechanical damage to the epithelium of the bile ducts occurs as a result of the action of suckers, which allow the parasite to attach to the mucous membrane of the biliary system. The constant inflammatory process is accompanied by a regenerative reaction of the wound, which leads to cell proliferation and, ultimately, DNA damage, followed by the manifestation of carcinogenesis [17].

Currently, *O. felineus* is not recognized as a group 1 biological carcinogen due to insufficient evidence [20]. However, there are reports in the available literature on the results of some studies proving the role of *O. felineus* in the development of cholangiocarcinoma and carcinogenic potential in laboratory animals. A recent case-control study conducted in Western Siberia showed that people with a confirmed diagnosis (according to microscopy of fecal eggs and/or serum IgM or IgG ELISA) were at a significantly higher risk of developing cholangiocarcinoma than healthy people [21]. An increased risk of malignancy was also found in patients who were diagnosed with current or past *O. felineus* invasion [21].

O.S. Fedorova et al. analyzed the relationship between the incidence of opisthorchiasis and malignant neoplasms of the hepatobiliary system in residents of the Russian Federation using official medical reports [10]. According to the authors, the incidence of liver and intrahepatic bile duct cancer in 2011–2013 was 4.8±0.2 cases per 100,000 people in the population. The highest rates were recorded in the regions of the Far East, Siberia, and the Volgograd region. The authors obtained statistically significant data on the presence of a direct correlation between the incidence of opisthorchiasis (*O.felineus* invasion) in residents of endemic areas and malignant neoplasms of the duodenum, pancreas, and liver.

In their study [22], V.G. Bychkov , E.D. Khadieva, V.P.Zuevskiy, S.D. Lazarev, A.P. Baryshnikov, A.V. Simonov distinguished the following patterns of carcinogenesis in superinvasive opisthorchiasis:

1. superinvasive opisthorchiasis is a strong promoter of carcinogenesis in the parasite econiches and stomach;

2. the development of cholangiocarcinomas and adenocarcinomas in the pancreas is formed on the territory of proliferation of its own stem cells, committed cells, i.e., outside the ductal organ systems;

3. superinvasions significantly increase the mitogenic activity of tumor cells in the liver, pancreas, and stomach.

Opisthorchiasis is characterized by both local morphological changes in the parasitic organs and general, systemic, pathological processes. T.A. Khabelova et al. define acute opisthorchiasis as a hyperergic reaction to the antigens of the parasite [16]. The authors consider chronic opisthorchiasis as a systemic disease, which is accompanied by damage to the organs infected by parasites and involvement of intact organs and systems in the process. The authors note that the immunosuppressive, mutagenic effect of opisthorchis, as well as epithelial metaplasia, can collectively contribute to the development of cholangiogenic cancer.

O. felineus can parasitize the bile ducts of the liver for decades. In 20–40% of cases, parasites are also found in the ducts of the pancreas. The result of the presence of opisthorchis in the pancreatic ducts are papillitis, dactylitis, cholangitis, pancreatitis, and a number of other inflammatory processes of the duodenum, liver, and pancreas. The presence of parasites themselves, and obstruction of the ducts by opisthorchis detritus lead to the formation of cholangiectasis, strictures of the bile and pancreatic ducts. The mechanisms of development of these pathological processes are different. Thus, V.Yu. Rayn., V.P. Ionin, N.A. Kolmachevskiy distinguish four main components of the damaging effect of opisthorchis [23] including:

1. irritating effect of waste products of living opisthorchis and lysis of dead parasite bodies;

2. mechanical obstruction of the bile ducts by mature parasites and during egg deposition;

3. stimulation of lithogenesis;

4. translocation of bacteria during migration of opisthorchis from the duodenum to the bile ducts.

According to B.I. Alperovich et al., chronic proliferative cholangitis, stenosis of the large duodenal papilla, and extended strictures of the biliary tract contribute to the development of biliary hypertension and impaired outflow of pancreatic juice [24]. With massive invasion, the pancreatic duct can be obstructed by parasites and opisthorchiasis detritus, which leads to the development of inflammation in the pancreas, and is also often the cause of complications in the postoperative period following pancreatic cancer surgery [25].

The presence of opisthorchis invasion significantly increases the likelihood of developing ductal adenocarcinoma of the pancreas to low tumor differentiation [10]. The authors note that

a correlation has been established between the association of opisthorchiasis and the formation of foci of pancreatic intraepithelial neoplasia. The development of ductal pancreatic carcinoma is due to the progression of these processes. Interestingly, in the case of the development of highly differentiated neoplasia in opisthorchiasis, the life expectancy of patients is on average 2 months longer than without opisthorchiasis. But since low-grade forms of malignant lesions often develop in the presence of opisthorchiasis, the prognosis is less favorable.

According to the publication by N.A. Brazhnikova and M.V. Tolkaeva opisthorchiasis is a precancerous condition, which is confirmed by a number of clinical, pathomorphological, and epidemiological studies [25]. It has been statistically proven that in hyperendemic areas, the incidence of liver cancer is 2–3 times higher than average, the incidence of pancreatic cancer is 2 times higher, and that of extrahepatic bile duct cancer is 13 times higher. The urgency of the problem of earlier detection and surgical treatment of liver and pancreatic cancer in the context of chronic opisthorchiasis invasion is due to both the high incidence and the peculiarities of the clinical course of this pathology – a long asymptomatic period. Early manifestations of the malignant process mimic exacerbation of chronic opisthorchiasis, hepatocholecystitis, pancreatitis, and infectious hepatitis. Patients are admitted for surgical treatment already in the presence of complications, including jaundice more than three weeks.

When taking the medical history, in some cases, it is possible to identify changes in the nature of pain even before the clinical signs of jaundice, namely, an increase in the intensity of pain in the right hypochondrium and epigastrium, its constant nature, especially at night, the appearance of pronounced dyspeptic disorders, decreased appetite, weight loss for no particular reason, flatulence, unstable stool. In pancreatic cancer, weakness, progressive weight loss, and vomiting are more often detected, which in some patients is associated with impaired gastric emptying as a result of compression by a tumor or invasion into the wall of the duodenum. The nature of jaundice also changes, which is determined by etiopathogenetic factors.

Jaundice syndrome becomes persistent with a trend toward an increase in intensity, cholangitis may develop, which is accompanied by hyperthermia and chills, debilitating itching. All this is accompanied by apathy, adynamia in the lack of effect from therapy. The long-term asymptomatic course of the disease is one of the most important reasons for late hospitalization of patients and, as a result, unsatisfactory outcomes of surgical treatment. The presence of opisthorchis invasion disguises the clinical picture of the malignant process, which significantly complicates timely diagnosis and radical surgical intervention. According to the authors, only 10.6% of the surgeries were radical. Palliative treatment was provided to 57.7% of patients, exploratory surgeries were performed in 13% of cases, and in 19% of cases, surgical treatment was impossible due to the prevalence of the pathological process.

O.V. Reshetnikov, T.G. Openko, and S.A. Kurilovich provide data from the pancreatic cancer registry, which is one of the deadliest types of cancer. The authors note that morbidity and mortality in this form of malignant neoplasm are almost equal. In recent years, there has been a trend towards an increase in morbidity, which leads, respectively, to an increase in mortality rates in some European countries, the Baltic States, Japan, and the USA [15].

CONCLUSION

All of the above once again proves the need and importance of early diagnosis and prevention of opisthorchiasis, as well as deworming and medical examination of patients in order to detect opisthorchiasis and its complications earlier, including malignant progression.

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Author information

Ivanov Vladimir V. – Cand. Sci. (Biol.), Associate Professor, Head of the Preclinical Research Center, Siberian State Medical University, Tomsk, ivanovvv1953@gmail.com, https://orcid.org/0000-0003-3326-729X

Komkova Tatyana B. – Dr. Sci. (Med.), Professor, Head of the Surgical Conditions Division with a Traumatology and Orthopedics Course, Siberian State Medical University, Tomsk, tatyana.bkomkova@gmail.com; http://orcid.org/0000-0003-1622-2356

Lyzko Ilya A. – Cand. Sci. (Med.), Associate Professor, the Surgical Conditions Division with a Traumatology and Orthopedics Course, Siberian State Medical University, Tomsk, ilya50@yandex.ru, https://orcid.org/0009-0000-0151-8029

Perina Ekaterina A. – Junior Researcher, Preclinical Research Center, SibMed, Tomsk, catherineperina@gmail.com, https://orcid.org/0000-0002-4273-8228

Popov Igor A. – Cand. Sci. (Phys. and Math), Head of the Laboratory for Molecular Medical Diagnostics, Moscow Institute of Physics and Technology Dolgoprudny, hexapole@gmail.com, https://orcid.org/0000-0002-5904-2470

Udut Elena V. – Dr. Sci. (Med.), Head of the Central Research Laboratory, Siberian State Medical University, Tomsk, udut.ev@ssmu.ru, https://orcid.org/0000-0002-6104-4782

Khmelevskaya Ekaterina S. – Cand. Sci. (Med.), Researcher at the Center for Biological Research and Bioengineering of the Central Research Laboratory, Siberian State Medical University, Tomsk, catherineperina@gmail.com, https://orcid.org/0000-0003-1776-4149

(✉) **Lyzko Ilya A.,** ilya50@yandex.ru

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Epidemiologic basis for the comorbidity of aortic aneurysm and atherosclerosis

Kucher A. N., Koroleva Iu.A., Nazarenko M. S.

Research Institute of Medical Genetics, Tomsk National Research Medical Center of the Russian Academy of Sciences
10, Ushaika Embankment, Tomsk, 634050, Russian Federation

ABSTRACT

Aortic aneurysm and atherosclerosis are characterized by high clinical heterogeneity. The uncertainty in their comorbidity evaluations may be related to polyetiology of these diseases and the presence of not only common but also specific risk factors, as well as the complex pathogenesis of these conditions.

The aim of this review is to summarize information on the prevalence and risk factors of aortic aneurysm and atherosclerosis, explaining the possible mechanisms underlying the comorbidity of these pathologies. We conducted a search for scientific publications in Russian (eLIBRARY.RU) and international (PubMed) electronic libraries, prioritizing works published in the last 10 years.

Aortic aneurysm and atherosclerosis exhibit an age-dependent pattern of prevalence. The high prevalence of atherosclerosis compared to aortic aneurysm, along with the approximately similar age ranges for the manifestation of these pathologies,is related to their comorbidity. Conversely, these diseases share some common risk factors, albeit with varying contributions to atherosclerosis and aortic aneurysm of different localizations. Type 2 diabetes mellitus and lipid metabolism profiles are examples of risk factors with multidirectional influences. To understand the reasons for the discordant estimates of comorbidity between aortic aneurysm and atherosclerosis from an epidemiological perspective, a comprehensive approach to patient characterization, including a detailed analysis of risk factors recorded in the analyzed groups, is essential.

Keywords: aortic aneurysm, atherosclerosis, comorbidity, risk factors

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Эпидемиологическая основа коморбидности аневризмы аорты и атеросклероза сосудов

Кучер А.Н., Королёва Ю.А., Назаренко М.С.

Научно-исследовательский институт (НИИ) медицинской генетики, Томский национальный исследовательский медицинский центр (НИМЦ) Российской академии наук
634050, г. Томск, ул. Набережная Ушайки, 10

✉ *Nazarenko Maria S.,* maria.nazarenko@medgenetics.ru

РЕЗЮМЕ

Аневризма аорты и атеросклероз сосудов характеризуются высокой клинической гетерогенностью. Неоднозначность в оценках коморбидности данных заболеваний может быть связана с их полиэтиологичностью и наличием не только общих, но и специфичных факторов риска, сложным патогенезом данных заболеваний.

Цель настоящего обзора заключается в обобщении информации о распространенности и факторах риска аневризмы аорты и атеросклероза сосудов с точки зрения объяснения возможных механизмов формирования коморбидности данных патологий. При проведении поиска научных публикаций в отечественной (Научная электронная библиотека – eLIBRARY.RU) и зарубежной (PubMed) электронных библиотеках в качестве приоритетных рассматривались работы, опубликованные за последние 10 лет.

Аневризма аорты и атеросклероз сосудов имеют возраст-зависимый характер распространения. Высокая распространенность атеросклероза сосудов по сравнению с аневризмой аорты и примерно равные возрастные границы манифестации данных патологий связаны с их коморбидностью. С другой стороны, данные заболевания характеризуются некоторой общностью факторов риска, но с разным их вкладом в развитие атеросклероза сосудов и аневризмы аорты разной локализации. Сахарный диабет 2-го типа и показатели липидного обмена относятся к категории факторов с разнонаправленным влиянием на риск развития данных патологий. Для понимания причин противоречивых оценок коморбидности аневризмы аорты и атеросклероза сосудов с точки зрения эпидемиологии важен комплексный подход к характеристике пациентов с детальным анализом факторов риска, регистрируемых в анализируемых выборках.

Ключевые слова: аневризма аорты, атеросклероз сосудов, коморбидность, факторы риска

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

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INTRODUCTION

Aortic aneurysm (AA) and atherosclerosis (AS) are two vascular pathologies that are characterized by polyetiology and clinical heterogeneity. There are thoracic aortic aneurysms (TAA), abdominal aortic aneurysms (AAA) and mixed, thoracoabdominal aortic aneurysms, when the pathological process affects both sections of the aorta [1, 2]. TAA can develop in the aortic root, ascending thoracic aorta or descending thoracic aorta, as well as in several segments of thoracic aorta simultaneously. Atherosclerosis can also affect various arteries, and in most cases, the pathological process observed in an individual patient affects multiple vascular territories [3–6]. Both AA and AS are asymptomatic for long periods of time, but they are life-threatening conditions that can lead to disabling complications and represent a major issue for society and the healthcare system [7–11].

Both AA and AS are characterized by a number of common risk factors and some similarity in the development of the pathological process, which is accompanied by disruption of the structure of blood vessels [1, 2, 12–18]. At the same time, there are different, often completely opposite, evaluations of AA and AS comorbidity, irrespective of the location of pathological changes in the aorta. In some studies, AS is considered as a significant risk factor for AA development [19, 20], in others its contribution to the risk of AA is estimated as insignificant [21]. Some authors have proposed that AS may be associated with slower AA growth [22] and may even serve as a protective factor against complications of

AA, such as dissections [23]. It is frequently postulated that AA may have a protective effect on the development of atherosclerotic vascular lesions [24–28]. There are some opinions about the mutual influence of AS and AA on each other [29]. Additionally, there are publications that do not find a correlation between these pathologies [30].

The uncertainty in the evaluations of the comorbidity of AS and AA may be related to their polyetiology and the presence of not only common but also specific risk factors, and the complex pathogenesis of these diseases [1, 2, 31, 32]. In general, despite decades of research into the comorbidity of AA and AS [24, 30, 33], many issues remain unresolved. A more thorough analysis of the relationship between AA and AS is of interest since it would clarify the pathogenesis of these diseases, the mechanisms of comorbidity formation (direct or inverse), and the clinical heterogeneity of individual pathological conditions, which is important for the formulation of criteria for identifying risk groups, determining the conditions for a severe disease course, and optimizing patient management based on the presence of a single pathology or comorbid pathologies [23, 26]. To do this, it is necessary to consider the data from epidemiological and clinical studies devoted to the analysis of AA and AS risk factors and assessment of their comorbidity, as well as ideas about the specific features of the pathogenesis of these diseases at the cellular and molecular levels.

The aim of this review is to summarize information on the prevalence and risk factors of AA and AS in terms of explaining the possible mechanisms of comorbidity formation of these pathologies. We conducted a search for information in Russian (Scientific Electronic Library – eLIBRARY.RU) and international (PubMed) online libraries. During the search for scientific publications, we gave priority to works published in 2013–2024. However, in certain cases, we also considered studies from earlier periods, which are important for understanding the development of concepts regarding the comorbidity of AA and AS.

PREVALENCE OF AORTIC ANEURYSMS AND ATHEROSCLEROSIS

There is a notable contrast in the prevalence of AA and AS. A meta-analysis of population-based studies indicates that the average incidence of TAA globally is 5.3 per 100,000 individuals/year, and the prevalence is 0.16% [10]. Aneurysms of the aortic root, ascending aorta, or of both these segments are most frequently documented in TAA, whereas aneurysms of the descending aorta, aortic arch, and mixed forms are less prevalent [1, 10, 34]. In a study of 844 patients with TAA, isolated ascending thoracic aortic aneurysms were found in 74.4% of cases, isolated descending thoracic aortic aneurysms were detected in 15.4% of cases, and combined ascending and descending aortic aneurysms – in 10.2% of cases [34]. Other studies provide slightly different estimates for the location of AA in the thoracic segment. In the study, S. Ito et al. noted that 15% of TAA cases occurred in the ascending aorta, 60% – in the aortic arch, and 25% – in the descending thoracic aorta [35]. In the study conducted by L.K. Bickerstaff et al., the corresponding indicators were 51.3, 11.1, and 37.5%, respectively [36]. These data demonstrate the clinical heterogeneity of the studied samples of patients with TAA. The meta-analysis revealed that aneurysms of the ascending aorta, aortic arch, and descending thoracic aorta are present in 45.5, 21.3, and 34.6% of patients with TAA, respectively [10]. At the same time, the authors of the cited publication [10] highlighted the lack of well-designed population-based studies to assess the prevalence of TAA and the necessity to continue epidemiologic studies in the future.

Patients with TAA display a moderately elevated prevalence of AAA and cerebral aneurysms [1, 36]. For example, 15% of patients with TAA of nonhereditary (Marfan syndrome and other monogenic connective tissue disorders were excluded) and non-inflammatory etiology were found to have aneurysms of other locations – in abdominal aorta, brachiocephalic arteries, etc. [37]. In another study, AAA was registered in 25% of patients with TAA [36]. On the other hand,

among patients with AAA, 15.2% of men and 30.7% of women (on average, every fifth patient) had synchronous or metachronous TAA [38].

The prevalence of AAA in the age cohort of 64–83 years varies between different populations, with a reported range of 1.4 to 8% [39–41]. However, regional variations exist, with a higher prevalence of AAA in developed countries compared to developing ones [42]. The lowest estimates of AAA annual incidence rate per 100,000 were documented in Central Asia (105.92 in 1990, 114.7 in 2005, and 113.43 in 2010), and the highest estimates were reported in Australia (382.65, 318.83, and 310.27 in the years indicated, respectively) [42]. Despite a decrease in the global prevalence of AAA between 1990 and 2010, certain regions observed an increase in the incidence of this condition (Oceania, tropical Latin America, the Asia-Pacific with high income, Southern, Central and Western Sub-Saharan Africa, South, West and Central Asia) [42]. Temporal dynamics in the prevalence of TAA were also observed [36].

The abdominal aorta is also characterized by differences in the incidence of aneurysms across different segments, with the infrarenal segment being most often affected [35, 43]. In the study by S. Ito et al., up to 96% of AAAs were located in the infrarenal, 2% – in the juxtarenal, and 1% – in the suprarenal segments of the abdominal aorta [35]. At this time, no data are available on the prevalence of AAA in Russia, which can be explained by the long-term asymptomatic course of AAA, as well as the absence of mandatory screening and population-based studies designed to detect this pathology [44].

In contrast to AA, AS is a more prevalent condition across various populations, but estimates of its prevalence also vary between studies, potentially due to different approaches to diagnosing AS. One of the approaches to estimating the prevalence of AS is the analysis of the prevalence of cardiovascular diseases associated with atherosclerosis, which include coronary heart disease (CHD), atherothrombotic ischemic stroke, transient ischemic attacks, peripheral atherosclerosis with atherosclerotic plaques causing > 50% stenosis [45], previous acute myocardial infarction, acute coronary syndrome, coronary revascularization and other arterial revascularization procedures, as well as aortic aneurysm [44]. In recent years, estimates of AS prevalence in populations (including subclinical forms) have been made on the basis of histologic analysis and data from instrumental examination of arteries [3, 6, 46].

A cross-sectional, population-based study was conducted in five cities of the Vladimir region of Russia between May 2018 and March 2020. The study included 1,350 men and women aged 30–69 years, and cardiovascular diseases associated with atherosclerosis were found in 17% of individuals [47]. A population-based prospective cohort study conducted in China (including more than 3,000 people aged 50 to 75 years, of whom 53.5% were women) revealed atherosclerotic plaques in at least one vascular territory in 93.6% of cases. Atherosclerotic plaques in more than one blood vessel were found in 82.8%, and in four or more vascular territories – in 46.8% of cases. Atherosclerotic plaques were mostly (79.6%) detected in the aorta [6]. A random sample of the middle-aged population (25,182 individuals without CHD, of whom 50.6% are women) was examined using coronary computed tomography angiography, and AS was found in 42.1% of individuals [46].

As indicated by various researchers, the prevalence of subclinical AS in various groups ranges from 36 to 63% (cited according to [48]). A total of 318 individuals (51% women), aged between 36 and 78 years (mean age 60 years), were examined as part of the Framingham Heart Study. In patients who were free of overt cardiovascular disease, the presence of AS was observed in 38% of women and 41% of men, as evidenced by the results of cardiac magnetic resonance (CMR) imaging [3]. Atherosclerosis of variable degree in one or more vessel segments was observed in 95.6% of Korean women who died from external causes. A total of 90 aortic samples were analyzed, each divided into 7 segments. AS was common in the distal infrarenal, proximal thoracic, and

proximal ascending segments of the aorta [4]. At the same time, for both men and women across all age cohorts, atherosclerotic plaques were more prevalent in the abdominal aorta than in the thoracic aorta, particularly in its ascending segment [3]. These data suggest that atherosclerosis-related diseases, particularly CHD, are not always detected in patients with atherosclerotic vascular lesions.

Thus, the estimates of the prevalence of AS and AA differ, but both pathologies are characterized by unequal lesions of different vascular territories, heterogeneity of prevalence estimates in different populations and age cohorts. The reasons for such heterogeneity of the prevalence of these pathologies may be attributed to inter-population (geographical) differences in the profile of AA and AS susceptibility in different sections of the aorta, differences in the significance of risk factors, peculiarities of sample formation, diagnostic methods, as well as the temporal dynamics of prevalence (since the studies were performed in different years).

RISK FACTORS FOR THE DEVELOPMENT OF AORTIC ANEURYSM AND ATHEROSCLEROSIS

Despite differences in prevalence, a number of common risk factors for AS and AA are known (Table). These factors include: old age, smoking, male sex, as well as arterial hypertension, hyperlipidemia, vascular wall injury, and inflammation [1, 49, 50]. Genetic factors, represented by both monogenic and polygenic components, also contribute to the risk of developing both AS and AA [1, 31, 51–57]. In general, risk factors can be divided into non-modifiable (genetic factors, malformations, sex, age, ethnicity) and modifiable ones. At the same time, the relative importance of common modifiable and non-modifiable factors may differ for AS and AA, for pathological conditions of vessels of different locations and in representatives of different sexes [1, 31, 34] (Table).

Table

Common and specific risk factors for the development of atherosclerosis and aortic aneurysm of different locations*

Risk factors	Significance of risk factors for AA and AS
Age	Both AA and AS are age-dependent diseases. In the case of AA, the age of diagnosis increases based on the type: hereditary – familial – sporadic. Women tend to develop both AA and AS later in life.
Sex	The risk is increased in men.
Genetic factors	In 20–30% of cases, pathogenic variants are found in genes that cause syndromes (Marfan, Loeys–Dietz, Ehlers-Danlos, etc.), the Mendelian forms of TAA (*ACTA2*, *MYH11*, *PRKG1*, *MYLK*, etc.). These variants are more often registered in aneurysms of the aortic root and ascending aorta, less often in aneurysm of the descending aorta. Pathogenic variants in some genes leading to the development of TAA can be detected in AAA. For AS, the risk increases in monogenic forms of hypercholesterolemia (pathogenic variants in the *LDLR*, *APOB*, *PCSK9*, *LDLRAP1* genes, etc.). AS and AA are also characterized by a polygenic predisposition. Family history increases the risk of developing AA and AS.
Congenital defects	Congenital anomalies (bicuspid aortic valve, coarctation of the aorta, complex congenital heart defects) are the cause of TAA development, in some cases the defects are genetically determined.
Ethnicity	It contributes significantly to the risk of AAA development (the risk of developing pathology is higher in Caucasians), but it is less significant for AS development.
Arterial hypertension	It is characteristic of both AS and AA. It is less common in sporadic aneurysms of the aortic root and ascending aorta compared to the descending aorta.
Hyperlipidemia	It is more significant for AS and AAA and is also associated with TAA of the descending aorta.
Type 2 Diabetes Mellitus	It is not specific for AA and acts as a protective factor; it is sometimes considered to be a risk factor for AAA as well as TAA of the descending aorta. It is a significant risk factor for AS.
Obesity	It is characteristic of AAA and AS, but not of TAA.
Inflammatory diseases	Giant cell arteritis (Horton disease), Takayasu arteritis, Behcet's disease, sarcoidosis, etc. increase the risk of TAA, but these diseases are rarely reported in AAA. The inflammatory component is significant in the development of AS.

Table (continued)

Risk factors	Significance of risk factors for AA and AS
Infectious diseases	Bacterial, fungal infections, and syphilis increase the risk of TAA development. The pathogenesis of AS is affected by viral and bacterial infections.
Traumatic aortic injury	It is registered in AA and AS.
Atherosclerosis	Atherosclerosis of various locations is registered more often in AAA than in TAA. AS with an atypical clinical pattern has been described in individuals with TAA.

* compiled from sources [1, 3, 33, 43, 46, 50, 58–65].

Among the non-modifiable risk factors of AA and AS, genetic factors are of particular interest. Monogenic forms account for 20–30% of all TAA cases, and a family history without an established genetic cause is found in another 20% [1, 66, 67]. Pathogenic variants in genes of syndromic and monogenic forms of TAA are more often registered in aneurysms of the aortic root and ascending aorta, and less often – in aneurysms of the descending aorta [1, 68].

Some studies have shown that family history is less important in relation to the risk of TAA development. For example, according to O. Leone et al. [69], in ascending TAA family history of aortic disease was rare, accounting for about 6% of cases [69]. Among the patients with a degenerative histotype of TAA, 3.9% had TAA-associated syndromes (Turner syndrome in 0.5% of cases, Marfan syndrome in 2.9%, Loeys–Dietz syndrome in 0.5%), and more than 30% of patients had bicuspid aortic valve (BAV), which is also highly heritable [1, 30, 70]. The association of genetically determined TAAs with certain histotypes has also been observed by other researchers [71].

BAV is considered as an independent risk factor for the development of TAA [1]. In some populations, this pathology is registered in 30% or more of TAA patients [69, 71–73]. BAV has also been identified in samples of non-TAA patients undergoing diagnostic tests (for example, computed tomography) for one reason or another, with a frequency comparable to that of TAA patients [73]. Such samples can potentially be used as control groups, including in the studies of AA and AS comorbidity. BAV is also characterized by genetic determination, and the monogenic component of BAV partially overlaps with that of TAA [1, 30, 70].

For AAA, genetic risk factors are not considered to be the most important, but pathogenic variants characteristic of TAA are sometimes registered in patients with this pathology [10, 74]. In addition, family history is noted in AAA [58, 75, 76]. It has been shown that the chance of developing AAA increases significantly with a family history (odds ratio OR = 1.9; 95% CI: 1.9–2.2; $p < 0.0001$) [75]. According to N. Sakalihasan et al. [58], a family history (taking into account ultrasound screening data) was recorded in 13% of AAA patients, and the highest (25%) prevalence of this pathology was found in brothers. In another study, more than 20% of patients with AAA had a family history [76].

A polygenic component is also known in the profile of the genetic predisposition to TAA and AAA [77]. According to the data of A. Gyftopoulos et al. [57], genes related to AS, lipid metabolism, and tumor development are associated specifically with sporadic AAA, while genes controlling extracellular matrix structure (remodeling), and tumor growth factor β function are associated with both AAA and TAA. Contractile element genes uniquely predispose to ascending TAA [57]. In other words, both common (but with different significance for the risk of AA development) and specific (and they are the majority) genetic factors are known for AAA and TAA [57, 77].

Dyslipidemia plays a leading role in the development of the atherosclerotic process, and the risk profile of both hypercholesterolemia and AS (as in the case of AA) also includes polygenic and monogenic components [52, 56, 77]. Familial hypercholesterolemia, which is a risk factor for AS development, is registered in various populations with a frequency of 1 per 200–500 people [51, 78]. At the same time, the prevalence of familial hypercholesterolemia in individuals with cardiovascular diseases associated with AS is 10–20 times higher than in populations [78]. In some regions, the family history of atherosclerosis-related diseases reaches 30% [47], and patients with atherosclerosis-related diseases

in most cases have four or more chronic diseases at the same time [79]. Despite the presence of a monogenic component in the determination of both AS and AA, the spectrum of causative genes for these diseases is specific [61].

AS and AA are age-dependent diseases, but they can occur at any age. A younger age of manifestation is typical of hereditary and familial TAAs, an older age is typical of sporadic AAs [1, 68, 76, 80]. At the same time, even genetically determined TAAs manifest in patients aged from 17 to 89 years [68], indicating the presence of additional factors contributing to the clinical manifestation of pathogenic variants of monogenic and syndromic TAA genes.

The prevalence of AAA also increases with age: the incidence per 100, 000 population was 55 in men aged 65 to 74 years, increased to 112 at 75 to 85, and to 298 at 85 years and older [59]. Estimates of AAA prevalence (per 100,000 people) in 1990 varied from 8.43 cases among individuals in the age group of 40–44 years to 2,422.53 in the age group of 75–79 years, and in 2010 they were 7.88 and 2,274.82, respectively [42]. For familial AAA cases, the age at diagnosis is younger than in the case of sporadic forms (67.8 and 70.2, respectively); 38.8% of patients with familial forms and 28.8% of patients with sporadic AAA were diagnosed before the age of 65 years [76].

The age at which AS is diagnosed also varies greatly. As in the case of monogenic AA, in hereditary hypercholesterolemia, signs of lipid metabolism disorders – and, consequently, AS – are seen at a young age (and even in children) [81]. In addition, the use of modern diagnostic techniques has shown that the first signs of AS can be detected in the population at a young age [65], but in general, the prevalence of AS increases with age [3].

The incidence of AA (TAA and AAA) and AS differs between the sexes [30, 42, 46, 69, 71, 76]. According to M.H.C.Pham et al., while the overall prevalence of AA among participants in the Copenhagen General Population Study was 2.1%, the incidence of this pathology was 4.0% in men and 0.7% in women [82]. The incidence of AAA is 4.1–14.2% in men and 0.35–6.2% in women [49]. Among patients with TAA, men account for 70% or more in some studies [34, 69, 71, 83]. AS manifests in women later than in men (10 years later on average in some populations) [46]. At the same time, earlier reports have shown no differences in the incidence of TAA between the sexes, but differences were recorded in the mean age of men and women (the age of patients ranged from 47 to 93 years, with a mean age of 65 years for men and 77 years for women) [36].

It should be noted that the combination of various risk factors in patients with AA may vary between different age cohorts, geographic regions, between sexes, between groups with familial and isolated forms of AA, with the presence or absence of syndromic or monogenic forms of AA, and even between AA of different locations. For example, compared with isolated ascending TAA, descending and mixed types of TAA are more often registered in men, in older individuals who smoke more often, have hypertension, type 2 diabetes mellitus (T2DM), and AAA [34].

The analysis of the published data showed a strong positive correlation between ascending TAA and genetic causes and a negative correlation with dyslipidemia, atherosclerosis, and diabetes, whereas the last three pathologies as well as hypertension are risk factors for descending AA [60]. According to the authors of the cited study, the data presented in the review support the hypothesis that ascending TAA is genetically mediated, and descending TAA is predominantly an acquired pathology [60]. The samples of patients with and without syndromic forms of TAA differ in the frequency of registration of risk factors, such as arterial hypertension, hypercholesterolemia, and diabetes mellitus [69]. Among patients with thoracic aortic dissection, the carriers of pathogenic variants in the genes of Mendelian forms of TAA had significantly lower rates of hypertension and smoking [68]. Despite the high heritability of BAV (from 47 to 89%), its incidence is higher in men than in women (9.2 vs. 3.5%, respectively) (cited in [84]). In the study by S. Ito et al., risk factors such as AS (intima-media thickness of carotid

arteries), smoking, hypertension, and type 2 diabetes mellitus were less significant for familial forms of AAA compared to sporadic forms [35]. Aortitis was more common in Asia, whereas in Western countries, inflammatory AAAs were commonly associated with AS [85]. At the same time, ethnicity is more significant as a risk factor in the AAA development (the risk of pathology is higher among Caucasians) than in the AS development [31].

TAA and AAA are also characterized by varying degrees of significance of different risk factors. CHD, chronic obstructive pulmonary disease, and diabetes mellitus are associated with the risk of AAA, while body mass index, arterial hypertension, and cerebral infarction are associated with TAA [35]. A recent population-based study [82] showed that common risk factors for TAA and AAA were sex, age, and body surface area; the specific risk factor for TAA was hypertension, and specific risk factors for AAA were hypercholesterolemia and smoking.

Additionally, there are also known risk factors not only with different significance for AA and AS, but also with multidirectional effects on the risk of their development, which some researchers find debatable. For example, diabetes mellitus, hypercholesterolemia, and obesity are highly significant for the development of AS, but their effects are not as expressed in AAA, whereas smoking, sex, and ethnicity are highly significant risk factors for AAA, but have a lesser effect on the risk of AS (cited in [31]).

In a number of studies, type 2 diabetes mellitus, which is a risk factor for the development of AS [31, 32], has been considered as a protective factor for AA [32, 64, 73, 86]. In patients with ascending aortic aneurysm (both with BAV and TAV), diabetes mellitus was documented at a frequency that was more than twofold lower than in individuals with a normal aorta [37]. Additionally, T2DM was less often identified in individuals with isolated ascending TAA (5%) compared to those with descending TAA (12%) and mixed type (13%) [34]. It is interesting to note that the presence of diabetes mellitus in patients with AAA was associated with a 43% reduction in the risk of developing both synchronous and metachronous TAA [38]. According to I.Y.Cho et al., this endocrine pathology led to a decreased risk of AAA development [64]. During dynamic follow-up (mean follow-up period was 23.1 years) of 5,381 individuals from Malmö Diet and Cancer Study cardiovascular cohort, no participant with diabetes mellitus at baseline developed isolated AAA [32]. At the same time, in some publications, type 2 diabetes is classified as a risk factor for both TAA and AAA [35].

Dyslipidemias may be regarded as one of debatable risk factors for AA and AS. A number of dyslipidemia-specific lipid parameters (higher levels of low-density lipoprotein (LDL) cholesterol and lipoprotein (a), lower levels of high-density lipoprotein cholesterol), have been demonstrated to correlate with the presence of such a risk factor for TAA as BAV [87]. In turn, hyperlipidemia is usually considered as a risk factor for AS and AAA [88]. However, according to different researchers, the lipid profile of patients with AAA is highly variable, particularly the levels of lipoprotein A [88]. Moreover, a higher probability of developing ascending TAA was identified in patients with relatively low LDL levels (at 75 mg/dl, OR = 1.21; 95%CI: 1.05–1.38), and a lower risk of aneurysm development was observed at high LDL levels (at 150 mg/dl, OR = 0.62; 95%CI: 0.46–0.84; at 200 mg/dl, OR = 0.29, 95%CI: 0.14–0.65) [89]. In an earlier study [21], the atherogenic lipid profile was found to be negatively associated with the diameter of the ascending aorta. Specifically, higher levels of high-density lipoprotein cholesterol and apolipoprotein A-I were associated with larger diameters, while higher levels of triglycerides and apolipoprotein B-100 were associated with smaller diameters of the ascending aorta.

The list of risk factors for AA and AS, which is presented in Table, is continually expanding. Atrial fibrillation, degenerative scoliosis, type 1 diabetes mellitus, chronic kidney disease, physical inactivity, and other factors have been considered as risk factors [2, 63, 90]. For example, adult degenerative scoliosis may act as a risk factor for aortic dilation and aortic

atherosclerosis [90]. The altered composition of the gut microbiota, as well as air pollutants, increases the risk of AAA, especially in individuals with a genetic predisposition [91, 92]. Sleep disorders, microbiome alteration, air pollution, environmental stress, etc. are considered as risk factors for the development of AS [93, 94]. At the same time, in some patients with AS, vascular risk factors are not detected at all [6]. Thus, a population-based study in China revealed no traditional risk factors for AS in 16% of patients, 1 risk factor – in 41.4%, 2 risk factors – in 21.3%, 3 and more risk factors – in 21.3% [6].

The study of risk factors for both AS and AA of different locations has recently undergone a significant shift towards the search for biochemical and molecular markers [89, 95–100]. However, even on the basis of the analysis of classical risk factors we can conclude the presence of both common and specific factors for AS and AA, as well as for AA of different locations, and, in addition, some relationships between different risk factors.

Thus, AA and AS differ in prevalence, but in both cases the age-dependent nature of the manifestation is found. Given the higher prevalence of AS compared to AA and the approximately equal age limits of manifestation of these pathologies, a high level of comorbidity of these diseases could be expected. On the other hand, despite the differences in AA and AS prevalence, they are both characterized by some common risk factors for their development, but with their different contribution to the risk structure of AS and AA development, as well as AA of different locations. Type 2 diabetes mellitus and, potentially, lipid parameters can be attributed to the category of factors with a multidirectional influence.

CONCLUSION

Different combinations of risk factors in patients may determine the features of the clinical patterns of AS, AA and their comorbidity [69, 101]. O. Leone et al. [69] demonstrated that BAV was more often registered in patients with TAA, in the case of a degenerative histological type of aorta; the group with atherosclerosis was older and exhibited higher incidence of hypertension, hypercholesterolemia, diabetes mellitus, current smoking, and CHD; the group with aortitis was the oldest, predominantly comprised of women, and exhibited high prevalence of classic cardiovascular risk factors, such as hypertension, hypercholesterolemia, and diabetes mellitus. Therefore, the inconsistency of comorbidity estimates across different studies may be related to the specific characteristics of the patient groups used in each study. In view of the above, in order to understand the causes for conflicting estimates of AA and AS comorbidity, it is important to apply an integrated approach to the characterization of patients with these aortopathies (both isolated and combined) with a detailed analysis of risk factors registered in the analyzed groups.

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Authors' information

Kucher Aksana N. – Dr. Sci. (Biol.), Professor, Leading Researcher, Laboratory of Population Genetics, Research Institute of Medical Genetics, Tomsk NRMC, Tomsk, aksana.kucher@medgenetics.ru, https://orcid.org/0000-0003-3824-3641

Koroleva Iuliia A. – Junior Researcher, Laboratory of Population Genetics, Research Institute of Medical Genetics, Tomsk NRMC, Tomsk, yuliya.koroleva@medgenetics.ru, https://orcid.org/0000-0003-1498-6934

Nazarenko Maria S. – Dr. Sci. (Med.), Professor, Head of the Laboratory of Population Genetics, Research Institute of Medical Genetic, Tomsk NRMC, Tomsk, maria.nazarenko@medgenetics.ru, https://orcid.org/0000-0002-0673-4094

(✉) **Nazarenko Maria S.,** maria.nazarenko@medgenetics.ru

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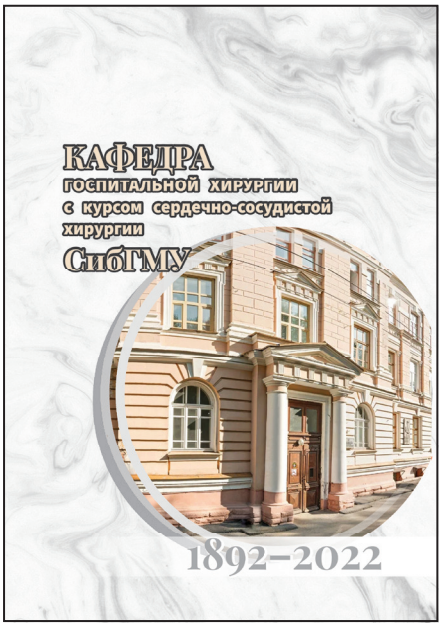
Издательский дом Сибирского государственного медицинского университета представляет серию книг

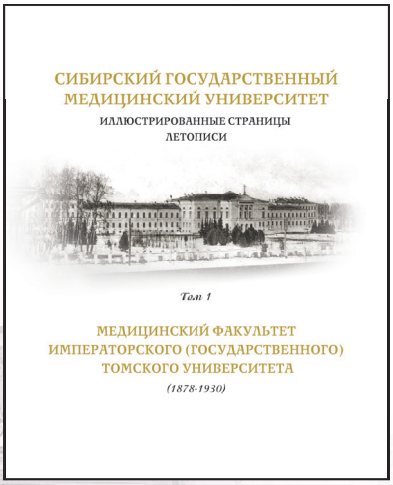
«Наследие томской медицины»



Книга посвящена 130-летнему юбилею кафедры госпитальной хирургии СибГМУ. Приведены биографические данные 79 сотрудников клиники и кафедры госпитальной хирургии в период с 1892 по 2022 г. Им предшествует подробная статья, характеризующая основные научно-практические достижения коллектива на каждом историческом отрезке. В издании упомянуты не только выдающиеся хирурги, звезды мировой величины, но и рядовые профессора, доценты, ассистенты, врачи-ординаторы, многие из которых связали с кафедрой и клиникой всю свою трудовую биографию. При изложении материала наряду с традиционными источниками информации использованы автобиографические документы, данные из семейных архивов, производственные характеристики нередко с сохранением авторского стиля.

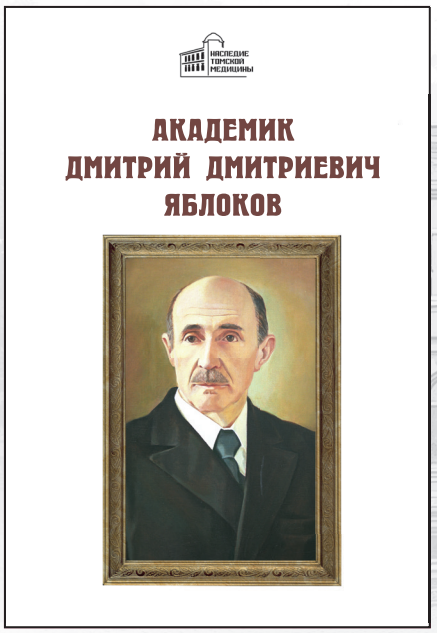
Это позволяет полнее ощутить атмосферу в обществе и рабочем коллективе в разные годы существования клиники. Текстовая информация сопровождена богатым иллюстративным материалом, многие фотографии опубликованы впервые.

Издание предназначено для хирургов, студентов старших курсов врачебных факультетов, специалистов по истории медицины.



Трёхтомная иллюстрированная летопись одного из старейших и наиболее авторитетных медицинских вузов России – Сибирского (Томского) государственного медицинского университета является по сути первой серьёзной попыткой осветить более чем 140-летнюю историю этого прославленного университета.Особенностью издания является его богатейший иллюстративный материал, включающий более четырёх тысяч фотографий (в том числе ранее практически неизвестных), и никогда не публиковавшиеся до этого крайне любопытные и интересные факты о жизни университета, его студентов и профессоров, воспоминания и рассказы выпускников и преподавателей вуза.

Для самого широкого круга читателей, интересующихся историей российских университетов, отечественного высшего медицинского образования и науки, развитием клинических и научно-медицинских школ, здравоохранения, историей Томска, Сибири, России…



В книге представлены биография и обзор научной, педагогической и общественной деятельности выдающегося ученого, терапевта, клинициста, академика АМН СССР, Героя Социалистического труда, лауреата Сталинской премии Дмитрия Дмитриевича Яблокова (1896-1993).

Для врачей, студентов, всех интересующихся историей медицины.

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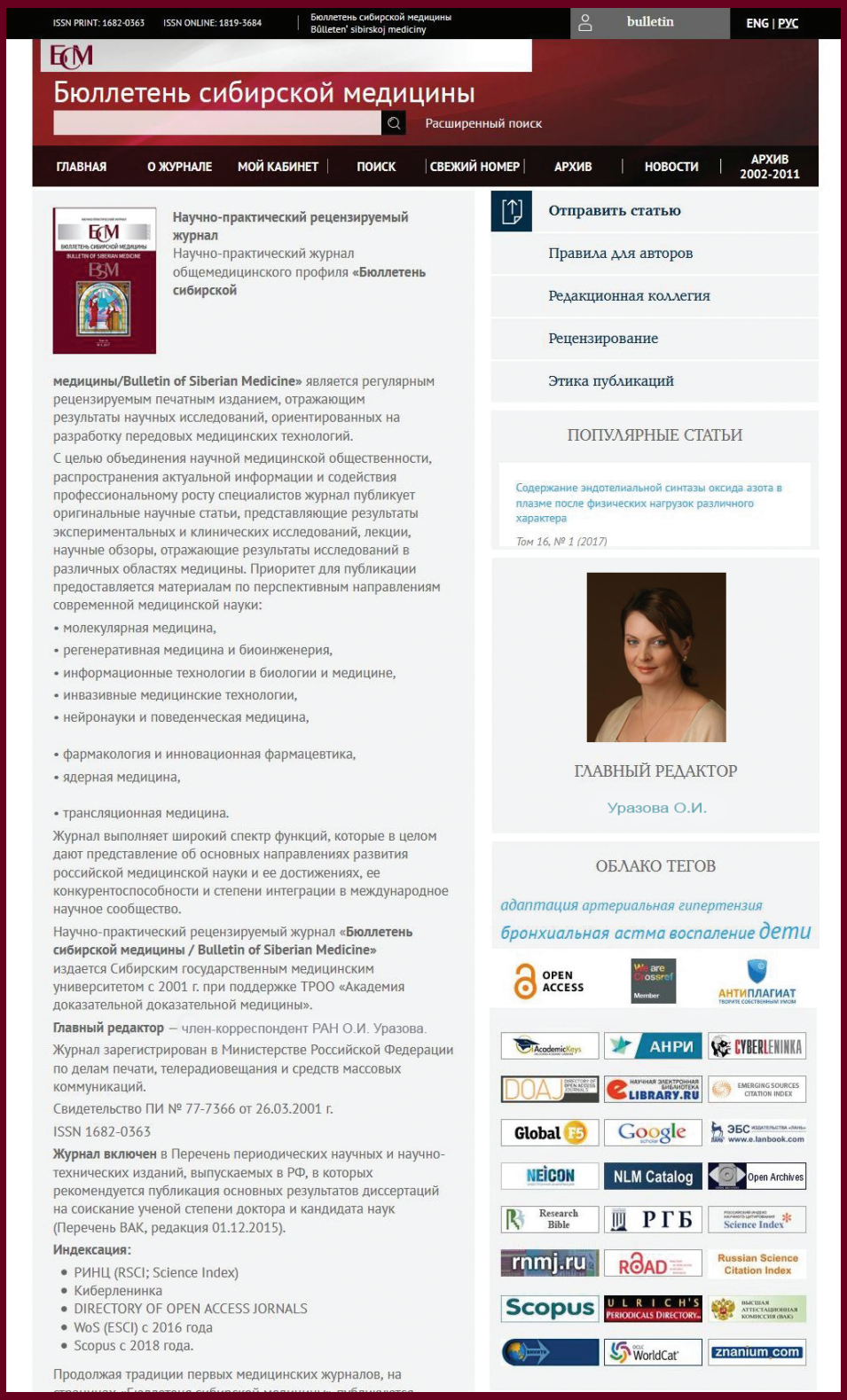

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