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PECULIARITY OF POMORS OF ONEGA PENINSULA AND WINTER COAST IN THE GENETIC CONTEXT OF NORTHERN EUROPE

Okovantsev VS[1]✉, Ponomarev GY[1], Agdzhoyan AT[1], Agdzhoyan AT[1], Pylev VY[1,2], Balanovska EV[1,2]

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The peculiarity of the Russian North gene pool has long become scientific fact, but has yet to receive informative explanation. Genetic drift cannot be the only contributing factor in the striking genetic differences between not only northern Russian populations and the southern ones, but among individual northern populations as well. Studying Russian North gene pools previously underrepresented in scientific literature may help understand this phenomenon. The work aimed to perform a subtotal study of the gene pool of the Arkhangelsk Oblast Pomors (Onega Coast, Summer Coast, the western fragment of the Winter Coast; *n* = 130) using a panel of 60 Y-chromosome SNP markers through multidimensional scaling and mapping of genetic distances. The frequencies of 14 identified haplogroups differ drastically in Pomor populations: haplogroups I1, R1a, and N3 each comprise a quarter of the total Pomor gene pool, I2-P37.2, and R1b each comprise about 8%, and the rest of the haplogroups are rare. The Onega Coast Pomors showed genetic similarity to a wide range of North-Eastern Europe Finnic-speaking populations, as well as to Russian populations with a strong pre-Slavic substratum. The Summer Coast Pomors are close to the Scandinavian gene pools, and the Winter Coast Pomors are similar only to specific Finn and Swede populations. None of the Pomor populations demonstrate genetic similarity with the Novgorod Oblast Russian populations, with which the origin of the Pomors is traditionally associated. The genetic distances between Pomor populations are so great, they are comparable to the general range of variability between the Eastern Slavic, Baltic, and Finno-Ugric peoples of the region. The reasons for such pronounced originality of Pomor populations presumably include, along with genetic drift, the gene pool of each population being underlied by a different pre-Slavic substrate, with later gene flows as an additional factor.

Keywords: gene pool, gene geography, Y-chromosome, Y-SNP, Russian North, Pomors, Fennoscandia

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СВОЕОБРАЗИЕ ПОМОРОВ ОНЕЖСКОГО ПОЛУОСТРОВА И ЗИМНЕГО БЕРЕГА В ГЕНЕТИЧЕСКОМ КОНТЕКСТЕ СЕВЕРА ЕВРОПЫ

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Своеобразие генофонда Русского Севера, давно став научным фактом, так и не получило содержательного объяснения. Резкие генетические отличия северных русских популяций не только от южных, но и друг от друга, нельзя объяснить лишь дрейфом генов. Расширение спектра изученных генофондов Русского Севера и их соседей может дать ключ к разгадке этого феномена. Целью работы было субтотальное исследование генофонда поморов Архангельской области (Онежского берега, Летнего берега, западного фрагмента Зимнего берега; *n* = 130) по панели 60 SNP-маркеров Y-хромосомы методами многомерного шкалирования и картографирования генетических расстояний. Популяции поморов резко различаются по частотам 14 выявленных гаплогрупп: каждая из гаплогрупп I1, R1a, N3 составляет по четверти общего генофонда поморов, по 8% I2-P37.2 и R1b, остальные гаплогруппы редки. Поморы Онежского берега оказались генетически схожими с широким кругом финноязычных народов Северо-Восточной Европы и тех русских популяций, у которых есть мощный дославянский субстрат. Поморы Летнего берега близки к генофондам Скандинавии. Поморы Зимнего берега схожи лишь с единичными популяциями финнов и шведов. Ни одна из популяций поморов не имеет генетического сходства с населением Новгородчины, с которым традиционно связывают происхождение поморов. Генетические расстояния между популяциями поморов настолько велики, что сопоставимы с общим размахом изменчивости между восточными славянами, балтами и финно-уграми региона. Причинами столь ярко выраженного своеобразия популяций поморов наряду с дрейфом генов предположительно можно назвать разный дославянский субстрат, лежащий в основе генофонда каждой популяции, а также более поздние потоки генов.

Ключевые слова: генофонд, геногеография, Y-хромосома, Y-SNP, Русский Север, поморы, Фенноскандия

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The study of the genetic history of the Russian people develops to cover an ever expanding range of both Russian populations and their neighbors [1–18]. A clear “white spot” on the emerging panorama concerns the northernmost Russian populations of the White Sea region, the Pomors. Their importance is not only that of a model for the evolution of populations on the very periphery of the ethnic range. In fact, the White Sea periphery is in general extremely peculiar: the history of the Pomors, a society greatly influenced by the sea, led not only to an unusual way of life, but to similarly unusual ways of interacting with Northern and North-Eastern Europe communities.

The "White Sea Pomors'' moniker emerged as a name for Russians dwelling on the coast of the White Sea. They hunted sea animals, fished on the high seas, and traded by sea, and were distinguished by many peculiarities in their way of life and preservation of features of ancient Russian culture [19]. The first mentions of permanent Russian settlements on the White Sea coast at the turn of the 14th century are linked to the Novgorod colonization [20–22]. According to the chronicles, the settlers met a Finnic-speaking population on these lands - the tribes of the Zavolochskaya Chud, often associated with the Vepsians. But the settlement of the region began in the Mesolithic period, about 8 thousand years ago. In the Neolithic period, traces of the two closely related Kargopol and White Sea archaeological cultures are recorded. A new wave of newcomers in the Bronze Age (4–3 thousand years ago) is linked with the Finno-Ugric population (primarily with the Sámi [23–28]), and the last Slavic wave of migrants is associated with the Novgorod colonization of the North.

Existence on the very northern periphery of the Russian people (where toponymy has preserved traces of the pre-Slavic population to this day) coupled with the unique culture and economy of the Pomors suggests that their gene pool was likewise one of a kind; but only a small sample of Pomors (n = 28) was previously studied using extremely narrow panels of Y-chromosome and mtDNA markers [1, 2]. In the broad context of the population of Northern Eurasia, the researchers considered Pomors a part of the “Northern Russian” population [1]. The features of their gene pool are explained by possible assimilation of the Ural- or Baltic-speaking population by the Slavs [2]. Data on the autosomal genome of the Arkhangelsk Oblast Mezensky District Pomors (n = 96) indicates similarity with the Finnic-speaking population (more so with the Finno-Perm than with the Finno-Volga) [3].

A wider range of populations, namely the Russian North gene pool, was characterized by four systems of markers (Y chromosomes, mtDNA, autosomal DNA markers, and surname frequencies). The analysis includes the populations of the Pinezhsky, Leshukonsky, Krasnoborsky and Lensky Districts of the Arkhangelsk Oblast [4]; the scale of the genetic originality of those populations was shown in earlier research [5]. mtDNA and autosomal DNA markers indicate the similarity of these populations to the Northern European population. The diversity of "paternal" lines, linked to the heritage of the most ancient Paleo-European population, reveals the similarity of the Russian North gene pool with that of the population of a vast territory from the Baltic States to Pechora. Genomic data on these populations allowed to analyze the genetic history of the Balto-Slavic peoples. On maps of genetic distances, the Russian North populations form one of the main patterns of the European gene pool [7]. The search for genetic traces of Novgorodian colonization in the Russian North gene pool, carried out using a genome-wide panel of the autosomal genome [8], revealed the absence of the “Novgorod” ancestral component in the north of the Arkhangelsk Oblast, and in the southern Arkhangelsk Oblast Krasnoborsky and Lensky Districts, the contribution of the “Novgorod” component amounted to no more than one third of the gene pool.

Even this brief review of the study of the gene pool of the Russian North reveals a serious lack of data on its northernmost periphery: the focus is either on the “mainland” populations of the Arkhangelsk Oblast, or on a small sample of Pomors using a narrow panel that comprises only a small fraction of the modern DNA marker spectrum.

New data on the gene pool of Pomor populations, obtained using an extensive panel of markers, can provide clues to understanding the enormous genetic diversity and originality of the populations of the Russian North [4, 5, 7]. Due to the tradition of patrilocality among Pomors [23] and the high efficiency of studying “paternal lines” [1, 9–11], this work considers the polymorphism of Y-chromosome markers with the aim of solving two problems: creating “genetic portraits” of the three Pomor populations, all studied for the first time, and searching for genetic traces of Novgorodian colonization in their gene pool.

METHODS

Materials

The indigenous population of the White Sea coast was studied using a modern panel of Y-chromosome markers (Fig. 1). During the 2021 expedition, the settlements of the Onega Peninsula (Onega Coast and Summer Coast) and the western (Onega) fragment of the Winter Coast were surveyed (Fig. 1) (for brevity, hereinafter all three populations are referred to as “Onega Pomors”). The survey was conducted subtotally: blood samples were taken in settlements with dense Pomor communities from almost all men meeting the inclusion criteria — the sample included only unrelated individuals whose ancestors (up to the third generation) belonged to the studied population and considered themselves Russians (or Pomors). Literary information and unpublished data from the Biobank of Northern Eurasia were used to compare the collected samples with those of the indigenous populations of the European North.

Genotyping

Total DNA was isolated from venous blood samples using the magnetic particle method on an automated QIAsymphony facility (QIAGEN; The Netherlands). Genotyping was performed by real-time PCR using TaqMan probes and OpenArray technology on a QuantStudio 12 Flex amplifier (Thermo Fisher Scientific; USA) on the following panel of 60 Y-SNP markers: D-M174, E-M35, E-M78, C-M217, C-F3791, C-F5481, C-F3918, C-M48, C-SK1066, C-M407, G-M201, G1-M285, G2-P15, G2-FGC595, G2-M406, G2-P303, H -M69, I-M170, I-M253, I-P37.2, I-M223, J1-M267, J1-P58, J2-M172, J2-M12, J2-M67, J2-M9, L-M20, L -M317, T-M70, N-M231, N-M128, N-Y3205, N-M178, N-B211, N-M2118, N-CST10760, N-Z1936, N-F4205, N-B202, N-B479 , O-P186, O-M119, O-P31, O-M122, O-P201, O-M134, Q-M242, R1a-M198, R1a-PF6202, R1a-Y2395, R1a-CTS1211, R1a-Z92, R1a -Z93, R1b-M343, R1b-Y13887, R1b-M269, R1b-L51, R1b-Z2105, R2-M124.

Statistical and cartographic analysis

Nei’s matrix of pairwise genetic distances was calculated (in the original DJ program [5]) based on the data on the frequencies

A

B

Population	Sample size (N)	Settlements	Arkhangelsk Oblast district
Winter Coast	38	Verkhnyaya Zolotitsa, Nizhnyaya Zolotitsa, Patrakeyevka	Primorsky District
Summer Coast	45	Verkhnyaya Zolotitsa, Nizhnyaya Zolotitsa, Patrakeyevka	Primorsky District
Onega Coast	47	Lyamtsa, Purnema, Nizhmozero, Kyanda, Tamitsa, Pokrovskoe, Pushlakhta	Onezhsky and Primorsky Districts
Total sample	130	16 settlements	2 districts

Fig. 1. Location of the studied groups in the Pomor population system. **A**. Traditional Pomor settlements on the shores of the White Sea and the birthplaces of paternal ancestors of survey participants. **B**. Arkhangelsk Oblast settlements where the survey was carried out. (Map source in A: http://lexicon.dobrohot.org/images/c/c5/00133909.jpg)

of 14 Y-chromosome haplogroups identified in the three Pomor populations, with a multidimensional scaling plot constructed in the Statistica 7.0 package (StatSoft; USA). Cartographic analysis was performed using the original GeneGeo software package [29] using an extended spectrum of 26 Y-chromosome haplogroups characteristic of the region. Distribution maps of the 26 haplogroups were constructed according to frequencies from the Y-Base database (developed under the supervision of O.P. Balanovsky) using weighted average interpolation method with an influence radius of 800 km and a weight function value of 3 [29]. The algorithm for creating each map of genetic distances consisted of two stages. First, a map of genetic distances from a given Pomor population to interpolated values at each point of the map was created for each of the 26 haplogroups. Then the average genetic distances from a given population of Pomors to each point of the map were calculated based on the resulting 26 maps. As a result, a map was created for each Onega Pomor population, which shows the degree of genetic similarity of the studied Pomor population with each of the comparison populations.

RESULTS

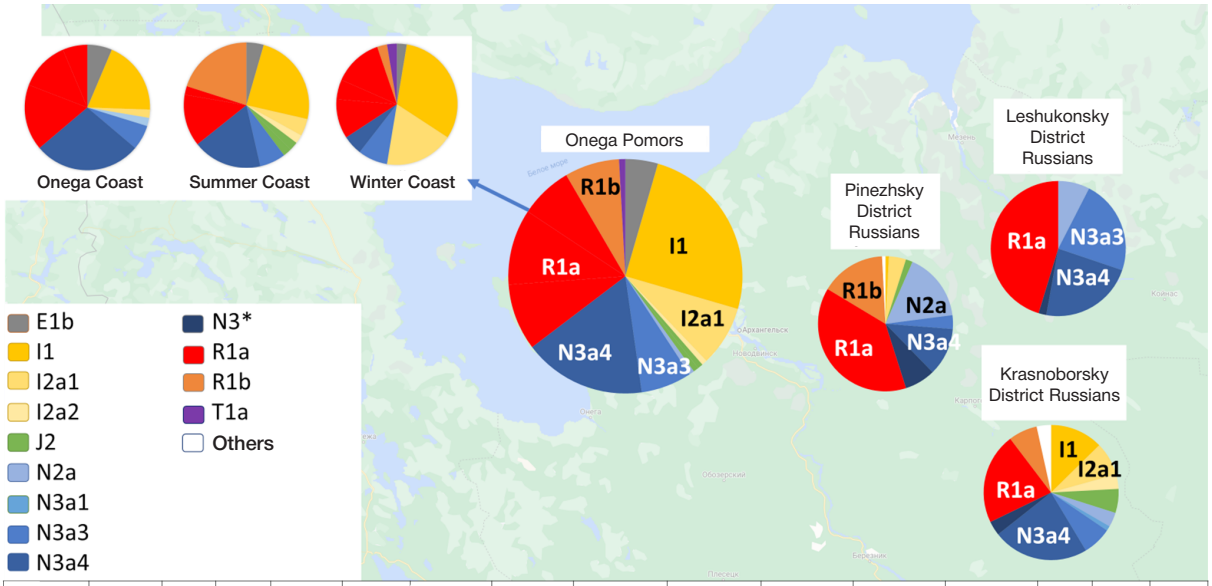
Y-chromosome haplogroup spectrum

14 Y-chromosome haplogroups were found in the gene pools of the three Onega Pomor populations (Fig. 2): E-M78, I1-M253, I2-P37.2, I2-M223, J2-M92, J2-M67, N2a-Y3205, N3a3- CST10760, N3a4-Z1936, R1a-PF6202, R1a-CTS1211, R1a-Z92, R1b-L51, T1a-M70 (haplogroups hereafter referred to by their short names). Haplogroups I1, N3, and R1a were the most frequent; each constitutes circa 25% of the total Onega Pomor gene pool (Fig. 2). Haplogroup R1a is represented by three branches (PF6202, CTS1211, Z92), haplogroup N3 by two (CST10760, Z1936). Haplogroups I2-P37.2 and R1b are next in frequency (each constitutes 8% of the gene pool), the rest are rare.

Despite the geographical proximity of the three Pomor populations (80–170 km; table), their genetic portraits differ noticeably, most significantly in four haplogroups: I2, N3a4, R1a, and R1b. Although each of the three Pomor populations has at least nine “general portrait” haplogroups, the haplogroup spectrums of the populations are markedly different from each other. The Winter Coast Pomors have a reduced frequency of the N3a4 haplogroup and an increased frequency of I2; in the Summer Coast Pomors, the haplogroup R1a-PF6202, characteristic of the other two populations, was absent, but the frequency of R1b was increased; in the Onega Coast Pomors, the frequency of N3a4 is high, but that of I2 is low (Fig. 2).

There is a decrease in the share of haplogroups I1 and I2a from the east (Winter Coast) to the west (Onega Coast), while the opposite trend is true for haplogroups N3a3 and N3a4; this “longitudinal” trend is absent altogether in haplogroups R1a (increased frequency in the populations of the Winter and

A



B

	Sample size	E1	I1	I2a1	I2a2a	J2	J2a1b	N2a	N3a3	N3a4	R1a	R1a	R1a	R1b	T1a
		M78	M253	P37.2	M223	M92	M67	Y3205	CST10760	Z1936	PF6202	CTS1211	Z92	L51	M70
Winter Coast	38	0.03	**0.32**	**0.18**	0	0	0	0	**0.08**	**0.05**	**0.11**	**0.05**	**0.13**	0.03	0.03
Summer Coast	45	0.04	**0.24**	0.04	0.02	0.02	0.02	0	**0.07**	**0.18**	0	**0.13**	0.02	**0.20**	0
Onega Coast	47	**0.06**	**0.19**	0.02	0	0	0	0.02	**0.06**	**0.28**	**0.17**	**0.13**	**0.06**	0	0
Onega Pomors	130	0.04	**0.25**	**0.08**	0.01	0.01	0.01	0.01	**0.07**	**0.17**	**0.09**	**0.10**	**0.07**	**0.08**	0.01

Fig. 2. Spectrum of Y-chromosome haplogroups in the studied Arkhangelsk Oblast populations. **A**. Main haplogroup shares in the Russian North gene pool. The Krasnoborsky District group (n = 81) also included samples from the neighboring Lensky District (n = 8), as their gene pool proximity was previously shown [5]. **B**. Y-chromosome haplogroup frequencies (main ones subdivided into branches) in Onega Pomors. The top row of the table shows the abbreviated names of the Y-chromosome haplogroups, the second row shows the corresponding SNP markers. Haplogroup frequencies in bold correspond to the 5% polymorphism level criterion

Onega Coasts) and R1b (maximum frequency on the Summer Coast). The frequency of R1a is high in the Winter Coast (29%) and Onega Coast (36%) gene pools, with all three branches of R1a found with a frequency of ≥ 5%. However, on the Summer Coast, the R1a frequency is two times lower and only the R1a-CTS1211 (13%) and R1a-Z92 (2%) branches were found. The decreased frequency of R1a and the sharp increase in the frequency of R1b (20%), observed only in the Summer Coast population, may result from either gene drift or migration flow. The frequency of R1b is also high in Arkhangelsk Oblast Pinezhsky District Russians (Fig. 2), but a different branch of R1b is common there. In Onega Pomors, the L51 branch was found, which is characteristic of the peoples of North-Western Europe, rather than North-Eastern Europe. Phylogenetic approaches are necessary to link it to either migration or preservation of the ancient genetic landscape of the region.

Haplogroups R1a and N3a4 are frequent in Onega Pomors and other Arkhangelsk Oblast Russian populations alike (although N3a4 is rare in the Winter Coast population). The frequency of haplogroup I1, on the other hand, constitutes a major difference between the “coastal” Pomors and the “mainland” Arkhangelsk Oblast populations: on average, it makes up a quarter of the Onega Pomor gene pool (25%) despite not being typical for other northern Russians (12% in the Krasnoborsky District population, 1% in the Pinezhsky District population, and absent in the Leshukonsky District population).

Onega Pomors in the Northern Europe genetic spectrum

An obvious initial observation on the degrees of genetic similarity (table) is the surprising magnitude of genetic distances between Pomor populations (d = 0.28) despite their geographical and cultural proximity. Furthermore, the distance between Onega Pomors and other Russian populations is almost 3 times greater (d = 0.76), with some significant exceptions (table). The closest to the gene pool of the Onega Pomors (d = 0.29) was a geographically remote (about 500 km) Russian population in the Arkhangelsk Oblast Krasnoborsky and Lensky Districts; and even then, it is extremely close only to the Onega Coast Pomors (d = 0.15), but genetically distant from the Summer (d = 0.33) and Winter (d = 0.38) Coasts populations.

Russian populations outside of the Arkhangelsk Oblast generally show no genetic similarity with the Onega Pomors, except for Kostroma Oblast Russians (d = 0.50) and the Yaroslavl Oblast Mologa population (d = 0.63). Only Onega Coast Pomors are close to the Mologa gene pool (d = 0.17), while the Summer Coast and Winter Coast gene pools are extremely distant (d = 0.85). Previously, it was shown that among the Yaroslavl populations, Mologa specifically retained a clear genetic trace of a pre-Slavic population (presumably Meryans) [9].

The Vepsians (d = 0.43) and the Northern Karelians (d = 0.46) are genetically the closest to the Onega Pomors when compared to other Russian peoples, and again primarily to the Onega Coast Pomors (d = 0.23 and d = 0.12, respectively). The Onega Coast Pomors are genetically closer to their Finnic-speaking neighbors than to other Pomor populations (d = 0.28). But the representatives of the eastern wing of the Finnic-speaking peoples, the Udmurts, turned out to be the most genetically distant from the Pomors (d = 2.50), which contradicts the conclusion [3] about the similarity of the gene pool of the Pomors and the Finno-Perm peoples.

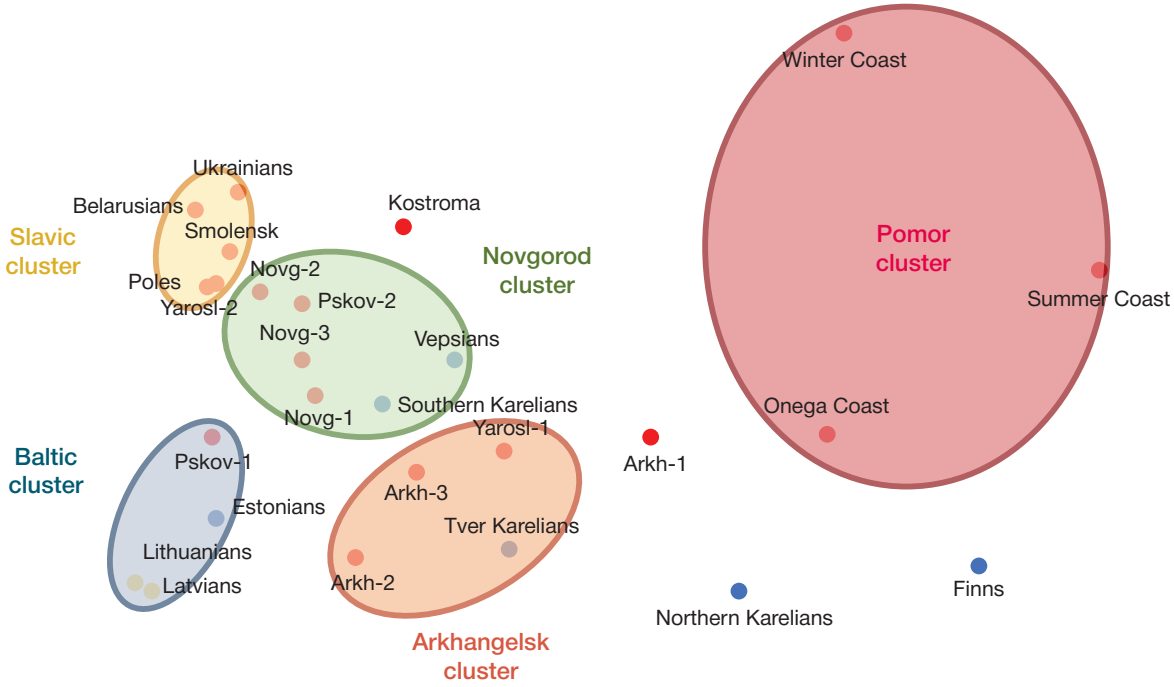


Fig. 3. Pomor gene pool in the genetic space of North-Eastern Europe. The multidimensional scaling plot was drawn using the frequencies of 15 Y-chromosome haplogroups, alienation index 0.13, stress index 0.11. The colors indicate linguistic affiliation of the populations: Slavic peoples (red), Baltic peoples (yellow), Finno-Ugric peoples (*blue*). The names of the Russian populations are deciphered in the table

However, the Onega Pomors show the greatest genetic similarity with the peoples of foreign Northern Europe (table): the genetic distance from the Pomors to the Swedes and Finns (d = 0.28) is the same as the average distance between Pomor populations, and the distance to the Sámi gene pool is two times less (d = 0.14). Curiously, the distance to the gene pools of the Finns and the Sámi decreases four times as you move west from the Winter Coast to the Onega Coast. The genetic distances to the Scandinavians (Danes, Norwegians, Swedes), however, follow the opposite trend: the distance to the Onega Coast is twice as big as the distance to the Summer Coast and the Winter Coast populations, equally close to the Scandinavians. While the Winter Coast Pomors are close only to the Scandinavians, the Summer Coast Pomors also show genetic similarity with a wide range of European populations, from the Germans (d = 0.36) to the Irish (d = 0.65).

Five tentatively named clusters are distinguished in the genetic space of multidimensional scaling (Fig. 3) (the plot is based on 14 “Pomor” Y-chromosome haplogroups); average distances between populations ($\bar{d}$) were calculated for each cluster. The "Slavic" cluster ($\bar{d}$ = 0.05) included Belarusians, Ukrainians, Poles, Smolensk Oblast Russians, and Yaroslavl Oblasts Russians. The related “Novgorod” cluster ($\bar{d}$ = 0.06) unites all three Novgorod Oblast populations and the Pskov Oblast Porkhov population (Porkhov having once been part of the Novgorod lands), as well as the Finnic-speaking Vepsians and the Southern Karelians. The "Baltic" cluster ($\bar{d}$ = 0.04) included all Baltics (Latvians, Lithuanians and Estonians) as well as the Pskov Oblast population (Ostrov *group*). The “Arkhangelsk” cluster ($\bar{d}$ = 0.09) united the populations of the Arkhangelsk Oblast Pinezhsky and Leshukonsky Districts with the Yaroslavl Oblast Mologa population and the Tver Karelians.

The Pomors formed their own large cluster — the distances between the Pomor populations ($\bar{d}$ = 0.28) are almost five times greater than the average distance within other clusters ($\bar{d}$ = 0.06), and the area of the "Pomor" cluster is only slightly less than the sum of all four comparison group clusters, which included Finnic-speaking, Baltic-speaking and Slavic populations. But we emphasize that although the differences between the Pomor populations are great, they all took their own “Pomor” place in the genetic space of Northeastern Europe.

Maps of genetic distances (Fig. 4) calculated from 26 Y-chromosome haplogroups typical for the entire region help determine the regions with which the Pomor gene pools are similar with more accuracy and significantly expand the range of comparison populations.

Total Pomor gene pool (Fig. 4A) is genetically close to the southern part of Finland, as opposed to its north, represented by the Sámi.

Onega Coast gene pool (Fig. 4B) reveals a vast area of genetic similarity: it covers almost all of Finland in the west, is clearly delineated by the Northern Dvina and Sukhona from the east, and reaches the Yaroslavl and Leningrad Oblasts in the south and southwest of Russia. This area of similarity also includes Finnic-speaking peoples (Vepsians, Izhoras, Ingrians, Karelians, Finns), and those Russian populations in whose gene pool a significant contribution of the pre-Slavic population can be traced.

Summer Coast gene pool (Fig. 4C) showed the greatest similarity with the distant Swedes and Norwegians, and a less pronounced one with the Sámi (representing the very north of Scandinavia).

Winter Coast gene pool (Fig. 4D) is relatively genetically close to only a few populations of Finns and Swedes. This is the only Pomor population for which one may assume that its genetic portrait has largely been shaped by genetic drift. However, the population of the Winter Coast is still represented only by its “Prionega” part (Fig. 1). The study of the gene pool of the entire Winter Coast is currently underway, which will soon make it possible to draw a reasonable conclusion about its genetic history.

DISCUSSION

The three considered Pomors populations are close not only in the purely geographical sense (Fig. 1): their economy and

Table. Genetic and geographical distances between the Onega Pomors and the comparison populations

Популяция	Onega Pomors			
	Average	Winter Coast	Summer Coast	Onega Coast
Onega Pomors, total	0.09	0.09	0.08	0.10
Onega Pomors, Winter Coast	0.19	0.00	0.25	0.32
Onega Pomors, Summer Coast	0.17	0.25	0.00	0.27
Onega Pomors, Onega Coast	0.20	0.32	0.27	0.00
Russians, Arkhangelsk Oblast № 1: Krasnoborsky and Lensky Districts	0.29	0.38	0.35	0.15
Russians, Arkhangelsk Oblast № 2: Leshukonsky Distric	0.97	1.10	1.36	0.44
Russians, Arkhangelsk Oblast № 3: Pinezhsky District	0.86	1.07	1.02	0.49
Russians, Novgorod Oblast № 1: Antsiferovo	0.73	0.72	1.06	0.40
Russians, Novgorod Oblast № 2: Kabozha	0.88	0.88	1.15	0.61
Russians, Novgorod Oblast № 3: Lyubytino	0.72	0.65	1.08	0.42
Russians, Pskov Oblast № 1: Ostrov	0.90	0.71	1.36	0.64
Russians, Pskov Oblast № 2: Porkhov	0.82	0.74	1.26	0.47
Russians, Yaroslavl Oblast № 1: Mologa	0.63	0.86	0.85	0.17
Russians, Yaroslavl Oblast № 2: Various districts	1.02	0.82	1.63	0.61
Russians, Kostroma Oblast	0.50	0.47	0.64	0.40
Russians, Smolensk Oblast	0.84	0.72	1.18	0.62
Karelians, northern	0.46	0.72	0.53	0.12
Karelians, southern	0.80	0.92	1.16	0.33
Karelians, Tver	0.64	0.86	0.88	0.17
Vepsians	0.43	0.41	0.65	0.23
Estonians	0.71	0.57	1.00	0.56
Latvians	0.99	0.89	1.25	0.82
Lithuanians	1.06	0.89	1.47	0.82
Udmurts, northern	1.71	1.73	2.01	1.38
Udmurts, central	2.93	3.23	2.42	3.14
Udmurts, southern	2.88	3.06	2.93	2.65
Belarusians	1.07	0.76	1.64	0.80
Ukrainians	0.93	0.65	1.35	0.78
Poles	1.05	0.85	1.53	0.76
Finns	0.28	0.45	0.25	0.14
Sámi	0.17	0.19	0.19	0.13
Swedes	0.28	0.20	0.19	0.45
Norwegians	0.50	0.40	0.39	0.71
Danes	0.52	0.46	0.23	0.86
Germans	0.75	0.77	0.36	1.13
English	1.30	1.37	0.47	2.05
Irish	1.63	1.73	0.58	2.57
French	1.45	1.65	0.58	2.11
Geographic distances between populations (km)		Winter Coast	Summer Coast	Onega Coast
Winter Coast Pomors	–	0	120	170
Summer Coast Pomors	–	120	0	80
Onega Coast Pomors	–	170	80	0

Note: *green* tones indicate maximum genetic similarity, *red* tones indicate genetic dissimilarity; numbers (#) of populations correspond to # on the multidimensional scaling plot; * — distance to populations was measured by frequencies of 11 haplogroups.

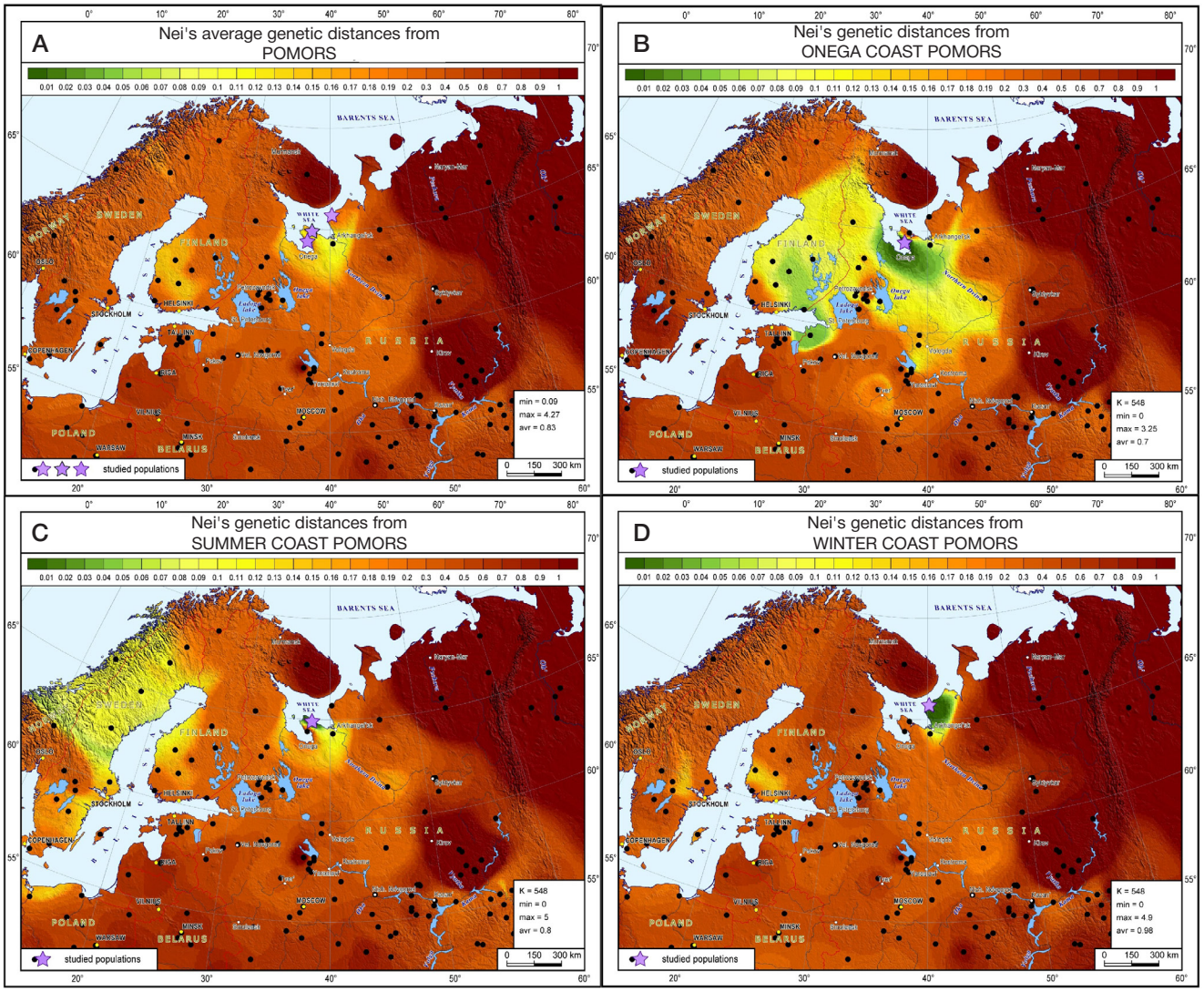


Fig. 4. Maps of Nei's genetic distances from the three Onega Pomor populations. **A**. General (average of three maps of individual populations). **B**. Onega Coast. **C**. Summer Coast. **D**. Winter Coast. The population from which distances are calculated is marked with an asterisk. *Green* and *yellow* tones reflect the minimum distances to the population, *red-brown* — maximum

culture, in contrast to settled farmers, involves movement by sea over long distances. Therefore, it was assumed that the differences between their gene pools would be extremely small. During the expeditionary survey, the task was to form a subtotal sample in order to capture even minor genetic differences between the three Pomor populations: all settlements with dense Onega Pomor communities were surveyed (Fig. 1). Although the analyzed samples are small (37–48 people for each population), they are reliable, as they represent the general population due to the subtotal nature of the survey and reflect reality, not sampling error. Even with such a small sample size, the differences in four haplogroups (I2, N3a4, R1a, R1b) out of 14 identified are significant, even though this type of analysis is based on the assumption that the samples were taken from an infinite general population of individuals. Therefore, the analysis of the significance of differences is not applicable to subtotal studies of small populations: subtotal samples provide the most accurate portrait of the population and do not require additional assessment of significance of differences.

Contrary to the initial hypothesis, it turned out that each of the three Pomor populations has a pronouncedly unique genetic portrait. The Onega Coast Pomors are genetically close both to the Finnic-speaking communities of Russia and Finland, and to the Arkhangelsk Oblast Russians. They are in general more genetically close to their Finnic-speaking neighbors than to other Onega Pomors. The Summer Coast Pomors are genetically similar only to the population of Scandinavia. Finally, the Winter Coast Pomors have practically no similar gene pools, save for some proximity to the Finns and Swedes. The great differences between the three Pomor populations are only slightly inferior in magnitude to the differences between the considered populations of Western and Eastern Slavs, Balts, and Finnic-speaking populations (Fig. 3). At the same time, all three Pomor populations occupy their own “Pomor” place in the genetic space despite the wide range of comparison populations.

It is impossible to attribute such originality of the Pomor gene pools to genetic drift only. Genetic drift acts independently on different haplogroups. Therefore, a “drifting” population may appear similar to a comparison population according to one marker, to a completely different population according to another, etc. Then when analysis of genetic distances for the entire set of genetic markers is conducted, such a “drifting” population, regardless of its real origin, turns out to be unlike any comparison group.

This model can, to some extent, explain the peculiarity of the gene pool of the Winter Coast Pomors. But the final conclusion can only be drawn after analyzing genetic portraits of other populations of the vast Winter Coast (Fig. 1). Genetic drift has doubtlessly been an important factor in the genetic history of all

Pomor populations, which have declined in numbers over the past generations. However, it failed to erase the genetic memory of the fact that their gene pools were based on different substrates. Onega Coast Pomors have common roots with a wide range of Finnic-speaking North European populations, while the Summer Coast Pomors are similar only to Scandinavian populations. Whole-genome studies will allow to verify the hypothesis of their different origins, as genes that formed their gene pools engaged in ancient migration flows. However, study of Y-chromosome polymorphism (Y-chromosome being the most stable part of the Pomor gene pool due to their patrilocality) directly indicates that the genetic identity of the Onega Pomor populations is linked to different genetic substrates underlying the populations, although these differences were covered by powerful gene drift.

The second important question concerns genetic similarity between Pomors and Novgorodians. The average genetic distance between these populations (d = 0.77) turned out to be the same (d = 0.76) as the distance between the gene pools of the Pomors and the other examined Russian populations (table). Novgorodians show great genetic difference from the Onega Coast Pomors (d = 0.48), and differ even more strikingly from the other Pomor populations (Winter Coast d = 0.75, Summer Coast d = 1.09). We previously concluded that the autosomal genome of Novgorodians differs from Russians in the north of the Arkhangelsk Oblast [8]. Now Y-chromosome markers reveal further pronounced differences between the gene pools of Novgorodians and Pomors. Both results contradict the view that the Russian North gene pool was shaped by the Novgorodian expansion. However, this is far from the only case in world history when internal colonization manifested through expansion of power and economic influence, but did not lead to decisive changes in the gene pool.

These and other results of studying the indigenous European population [4–5, 8–12, 30] provide a convincing argument against interpolating ideas about the region's history developed solely on the basis of the humanities data to the gene pool without additional research.

CONCLUSIONS

Y-chromosome polymorphism was studied in three White Sea Pomor populations: those of Onega, Summer and Winter Coasts. An analysis of subtotal samples of unrelated individuals from all locations with dense Pomor communities made it possible to create reliable genetic portraits of the three Pomor populations.

The study of the Pomor gene pool using a wide panel of Y-chromosome markers revealed 14 haplogroups, of which four (I2, N3a4, R1a, R1b) differed significantly in distribution by population despite small sample sizes (37–48 people). Differences in the genetic portraits of the Pomors are formed due to the gene pool originality of each of the populations: the Pomors of Winter Coast have a reduced frequency of the haplogroup N3a4 and an increased frequency of I2; in the Summer Coast population, there were no branches of the haplogroup R1a, characteristic of the other two populations, with an increased frequency of R1b; in the Onega Coast population, the frequency of N3a4 is high, but that of I2 is low. Spectrum features and haplogroup frequencies make the genetic portrait of each Pomor population unique.

Each of the three Pomor populations has its own range of genetically close populations. The Onega Coast Pomors are genetically similar to a wide range of Finnic-speaking peoples of North Europe, as well as to some Russian populations that express the contribution of the pre-Slavic population in their gene pool. The Summer Coast Pomors exhibited similarity only with the population of Scandinavia, which can be explained by a common Paleo-European substrate or later interactions between Scandinavians and Pomors. For the Winter Coast Pomors, some genetic affinity was recorded with only two populations of Finland and Sweden.

The genetic distances between the Pomor populations turned out to be comparable with the general range of variability between the Eastern Slavs, Balts, and Finno-Ugric peoples of the region. Aside from the genetic drift, the reason for this may be a different substrate underlying the gene pool of each population, since it is difficult to assume such different migration relationships in such geographically, ethnically and culturally close populations.

None of the three populations of the Onega Pomors show noticeable genetic similarity with the indigenous population of the Novgorod Oblast, indicating the absence of genetic traces of demic expansion during the Novgorod colonization of the Russian North.

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THE NATURE OF GENOTYPIC RESISTANCE TO FLUOROQUINOLONES IN *MYCOBACTERIUM TUBERCULOSIS* CIRCULATING IN RUSSIAN FEDERATION

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Fluoroquinolones are the main group of drugs used for treatment of multidrug resistant tuberculosis (MDR-TB). The study was aimed to assess the diversity of mutation in the *gyrA* gene and to evaluate the association of *gyrA* mutations with the phenotypic resistance to levofloxacin and the general drug resistance profile of the pathogen. The study involved assessment of diagnostic materials obtained from 2836 patients with pulmonary tuberculosis. TB-BIOCHIP-2 and Amplitube-FQ-RV kits were used for identification of the *gyrA* mutations. Phenotypic drug susceptibility of *M. tuberculosis* (MTB) was defined using the BACTEC MGIT 960 test system. It was shown that mutations D94G (41.63%; 95% CI: 38.03–45.32%) and A90V (21.32%; 95% CI: 18.44–24.50%) prevailed in MTB, although some isolates carrying these mutations were obtained from the newly diagnosed patients with pulmonary tuberculosis. It was found that mutation D94A was not strongly associated with the phenotypic resistance to fluoroquinolones. Fluoroquinolone resistance was usually associated with multiple drug resistance (93.52%; 95% CI 91.43–95.12%). In 2.31% (95% CI 1.78–3.00%) of cases, genotypic heteroresistance to fluoroquinolones was detected: mixed populations included 2–4 MTB pools with various structure of the *gyrA* QRDR. The results obtained lead to the conclusion that resistance to fluoroquinolones that is usually associated with the existing MDR arises in the modern MTB population. MTB carrying *gyrA* mutations D94G and A90V seems to be the most promising in evolutionary terms.

Keywords: *M. tuberculosis*, fluoroquinolones, resistance, *gyrA*, mutations, preXDR tuberculosis

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ОСОБЕННОСТИ ГЕНОТИПИЧЕСКОЙ РЕЗИСТЕНТНОСТИ К ФТОРХИНОЛОНАМ У *MYCOBACTERIUM TUBERCULOSIS*, ЦИРКУЛИРУЮЩИХ В РОССИЙСКОЙ ФЕДЕРАЦИИ

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Фторхинолоны — основная группа препаратов, применяемых для лечения туберкулеза с множественной лекарственной устойчивостью (МЛУ-ТБ). Целью исследования было оценить разнообразие мутаций в гене *gyrA*, а также установить ассоциацию мутаций в *gyrA* с фенотипической устойчивостью к левофлоксацину и общим профилем лекарственной устойчивости возбудителя. Исследование проведено на диагностическом материале от 2836 больных туберкулезом легких. Для определения мутаций в *gyrA* использовали наборы «ТБ-БИОЧИП-2» или «Амплитуб-FQ-РВ». Фенотипическую лекарственную чувствительность *M. tuberculosis* (МБТ) определяли в системе BACTEC MGIT 960. Показано, что у МБТ доминировали мутации D94G (41,63%; 95%ДИ: 38,03–45,32%) и A90V (21,32%; 95%ДИ: 18,44–24,50%), причем изоляты с этими мутациями были получены в том числе и от впервые выявленных больных туберкулезом легких. Установлено, что мутация D94A не являлась строго ассоциированной с фенотипической устойчивостью к фторхинолонам. Устойчивость к фторхинолонам, как правило, была ассоциирована с множественной лекарственной устойчивостью (93,52%; 95%ДИ 91,43–95,12%). В 2,31% (95%ДИ 1,78–3,00%) случаев выявлена генотипическая гетерорезистентность к фторхинолонам: смешанные популяции включали 2–4 пула МБТ с разной структурой QRDR *gyrA*. На основании полученных результатов можно заключить, что в современной популяции МБТ происходит формирование устойчивости к фторхинолонам, как правило, на фоне уже имеющейся МЛУ. Наиболее перспективными в эволюционном плане представляются МБТ с мутациями в *gyrA* D94G и A90V.

Ключевые слова: *M. tuberculosis*, фторхинолоны, устойчивость, *gyrA*, мутации, преШЛУ туберкулез

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The spread of tuberculosis caused by drug resistant pathogen is a major public health concern. Particularly worrisome is the wide spread of multidrug-resistant tuberculosis (MDR-TB), i.e. resistant to both of two most effective antituberculosis drugs, rifampicin and isoniazid. According to the WHO, the effectiveness of MDR-TB therapy is only 59% [1]. Russia is among the countries with a high burden of MDR-TB. Despite the fact that the prevalence of MDR-TB in the country has started to decline in recent years (from 20.6 per 100,000 population in 2020 to 18.1 per 100,000 population in 2021), the rate is still high [1, 2].

Regimens for treatment of MDR-TB necessarily include fluoroquinolones (group A drugs according to the WHO classification reflecting priorities of the drug inclusion in the treatment regimens) [3]. DNA gyrase, the enzyme essential for replication and transcription in the *M. tuberculosis* (MTB)

cell, is a target of fluoroquinolones [4, 5]. In 60–90% of cases, fluoroquinolone resistance is associated with mutations in the quinolone resistance-determining region (QRDR) of the gyrA gene encoding the DNA gyrase α-subunit [6, 7]. The TBDreamDB database (http://www.tbdreamdb.com) contains information about 17 variants of QRDR mutations associated with fluoroquinolone resistance, among which 10 variants show high reliability in terms of resistance to this group of medications [8].

The development of additional resistance to fluoroquinolones by the MDR MTB results in pre-extensively drug resistant tuberculosis (pre-XDR TB), the treatment of which requires expensive long-term chemotherapy. The WHO estimates that up to 20% of tuberculosis cases in 105 countries fall in this category. To improve the effectiveness of pre-XDR TB treatment it is necessary to adjust the course of chemotherapy based on the fluoroquinolone susceptibility defined by molecular genetic methods as early as possible. However, according to the WHO, the global coverage of the fluoroquinolone susceptibility testing is still low: it accounts for 50% of the identified worldwide cases of tuberculosis [1]. Two domestic test systems for rapid detection of MTB susceptibility to fluoroquinolones are used in Russia. The first one, TB-BIOCHIP-2 (Biochip-IMB; Russia), includes biochips that detect 10 variants of the QRDR spot mutation. The second one, Amplitube-FQ-RV (Syntol; Russia) based on the allele-specific PCR, detects six mutations in the *gyrA* QRDR.

The study was aimed to assess the diversity of mutation in the *gyrA* gene and to evaluate the association of *gyrA* mutations with phenotypic resistance to levofloxacin and the general drug resistance profile of the pathogen.

METHODS

Research object

Diagnostic materials obtained from patients of all age groups admitted to the diagnostic and clinical departments of the Central Tuberculosis Research Institute in 2011–2019 were assessed.

Study design

Retrospective analysis of the data on mutations in the *gyrA* QRDR and MTB phenotypic drug resistance obtained within 9 years (2011–2019) from patients with pulmonary tuberculosis, who were treated in the Central Tuberculosis Research Institute, was conducted. Diagnostic materials were assessed in accordance with the standard algorithm accepted by Microbiology Department of the Central Tuberculosis Research Institute: each sample of diagnostic material was simultaneously assessed by the culture-based and molecular genetic methods. Diagnostic materials were decontaminated using the standard procedure, then the MGIT tubes were inoculated for further growth in the BACTEC MGIT 960 system [9]. DNA was isolated from the amount of diagnostic material remaining after inoculation, and PCR was conducted to detect the MTB DNA. Upon receipt of a positive PCR result, mutations in the genes associated with resistance to rifampicin, isoniazid, and fluoroquinolones were identified using biochips or allele-specific PCR. The MTB culture obtained was tested for susceptibility to eight antituberculosis drugs.

DNA extraction

DNA was extracted from diagnostic materials using the Amplitube-RV reagent kit for isolation, detection and quantification of the *Mycobacterium tuberculosis* complex DNA by real-time PCR, kit № 1 (Syntol; Russia) according to the instructions.

Identification of MTB DNA

To detect the MTB DNA, PCR was conducted using the Amplitube-RV reagent kit for isolation, detection and quantification of the *Mycobacterium tuberculosis* complex DNA by real-time PCR, kit № 2 (Syntol; Russia) according to the instructions. Amplification was performed in the CFX96 thermal cycler with optical module (Bio-Rad; USA).

Genotypic resistance to rifampicin and isoniazid

The test was performed either by the microchip technique using the TB-BIOCHIP-2 kit (Biochip-IMB; Russia), or using the Amplitube-MDR-RV kit (Syntol; Russia). Both procedures were conducted in accordance with the manufacturers' instructions.

Genotypic resistance to fluoroquinolones

The samples obtained in 2011–2015 were tested by the microchip technique using the TB-BIOCHIP-2 kit (Biochip-IMB; Russia), while the samples obtained in 2015–2019 were tested using the Amplitube-FQ-RV kit (Syntol; Russia). Both procedures were conducted in accordance with the manufacturers' instructions. In case the tests conducted during various stages of therapy were available for one patient or different diagnostic materials (for example, sputum and excision material) obtained from one patient were tested, the results of testing each of the samples for *gyrA* mutations were compared.

Culture-based diagnosis

MTB was detected in the Middlebrook 7H9 liquid growth medium with the BACTEC MGIT 960 system (BD; USA) in accordance with the standard protocol developed by the manufacturer [9].

Phenotypic drug resistance

The test for susceptibility to eight antituberculosis drugs (rifampicin, isoniazid, ethambutol, pyrazinamide, ethionamide, amikacin, capreomycin, and levofloxacin) was performed by the modified proportion testing method in the BACTEC MGIT 960 system (BD; USA) in accordance with the manufacturer's guidelines [9, 10].

Methods of statistical analysis

Descriptive statistics was used to assess the results: number of observations, frequency, share (percentage), and 95% confidence interval (95% CI) were taken into account. Chi-squared test (χ^2) was used for intergroup comparison. The differences were considered significant at $p < 0.05$. Analysis was performed using Microsoft Excel (Microsoft; USA).

RESULTS

In 2011–2019, microbiological diagnosis of tuberculosis was performed in 4451 patients. The study involved materials obtained from 2836 patients with positive MTB DNA PCR test results. Of them in 2082 cases (73.41%, 95% CI: 71.76–75.01%) no *gyrA* mutations were detected by molecular genetic methods (hereinafter, the wild type *gyrA*).

Table 1. Frequency of single *gyrA* mutations in the total number of strains with mutations in *gyrA* (*n* = 699)

Codon of the *gyrA* QRDR	Amino acid substitution	Frequency, No. (%)	95% CI
88	G → C	1 (0.14)	0.03–0.81
90	A → V	149 (21.32)	18.44–24.50
91	S → P	53 (7.58)	5.84–9.78
94	D → A	102 (14.59)	12.17–17.40
	D → N	54 (7.73)	5.97–9.94
	D → G	291 (41.63)	38.03–45.32
	D → H	11 (1.57)	0.88–2.80
	D → Y	38 (5.44)	3.99–7.37

MTB with single *gyrA* mutations were isolated from 699 patients (24.65%, 95% CI: 23.10–26.27%). In MTB isolated from 55 patients (2.31%, 95% CI: 1.78–3.00%), the results of determining genotypic resistance to fluoroquinolones changed over time or according to the diagnostic material type. These cases designated as heteroresistance will be described in detail below.

Frequency of single mutations in the *gyrA* QRDR

Single mutations detected in the *gyrA* QRDR were located in codons 88, 90, 91 or 94 (Table 1). Mutations were most often found in codon 94 of the gene (496/699, 70.96%; 95% CI: 67.49–74.20%). These were represented by five single-nucleotide variants, among which a D94G substitution was the most common (291/496, 58.67%; 95% CI: 54.29–62.92% among mutations in codon 94 and 291/699, 41.63%; 95% CI: 38.03–45.32% among MTB with single *gyrA* mutations). The A90V substitution was the second most frequent mutation (149/699, 21.32%; 95% CI: 18.44–24.50%). In total, MTB carrying mutations D94G and A90V accounted for more than half of all cases of MTB carrying single *gyrA* mutations (440/699, 62.95%; 95% CI: 59.31–66.45%).

Phenotypic susceptibility to levofloxacin

Phenotypic susceptibility to levofloxacin was defined for MTB isolated from diagnostic materials obtained from 1326 patients by the culture-based method. Testing of these MTB isolates for *gyrA* mutations revealed MTB with the wild type *gyrA* in 846 cases and MTB carrying single *gyrA* mutations in 480 cases. MTB with the wild type *gyrA* were largely susceptible to levofloxacin (814/846, 96.22%, 95% CI: 94.71–97.31%), while MTB carrying mutations usually were levofloxacin-resistant (448/480, 93.33%, 95% CI: 90.74–95.24%) (Table 2).

Polymorphism of MTB variants with mutant *gyrA* isolated in the new cases and previously treated patients with tuberculosis

Among 2836 patients with pulmonary tuberculosis, whose diagnostic materials were included in the study, there were 1253 new cases and 767 previously treated ones. No information about the status of another 816 patients was available.

MTB with the wild type *gyrA* were most often isolated in the new cases than in the previously treated ones: among 1475 isolates of MTB with the wild type *gyrA* obtained from patients with known status, 1012 (68.61%) were obtained in the new cases and 463 (31.39%) were isolated in the previously treated ones (p-value ≤ 0.001). The *gyrA* mutant variant A90V was significantly more common in the group of MTB isolated from the new cases; no significant differences were revealed for other mutant variants (Table 3).

gyrA mutations in MTB showing resistance of different types

MTB isolates with known genotypic susceptibility to fluoroquinolones were divided into five categories based on the resistance type (cases of heteroresistance were not included in the analysis): MDR MTB fell into the first category, polyresistant MTB (resistant to all combinations of antituberculosis drugs except the combination of rifampicin and isoniazid) fell into the

Table 2. MTB isolates with various *gyrA* QRDR structure showing phenotypic resistance to levofloxacin (*n* = 1326)

Mutation in QRDR *gyrA*	Strains with phenotypic resistance to levofloxacine	
	No. (%)	95% CI
G88C (*n* = 1)	1 (100)	20.65–100.00
A90V (*n* = 97)	95 (97.94)	92.79–99.43
S91P (*n* = 33)	32 (96.97)	84.68–99.46
D94A (*n* = 73)	49 (67.12)	55.73–76.81
D94N (*n* = 39)	39 (100)	91.03–100.00
D94G (*n* = 204)	200 (98.04)	95.07–99.23
D94H (*n* = 6)	6 (100)	60.97–100.00
D94Y (*n* = 27)	26 (96.30)	81.72–99.34
Total number of mutants (*n* = 480)*	448 (93.33)	90.74–95.24
WT (*n* = 846)	32 (3.78)	2.69–5.29

Note: WT — wild type *gyrA*; * — only MTB isolates with known phenotypic resistance to fluoroquinolones are taken into account.

Table 3. Abundance of the *gyrA* mutant MTB variants in the groups of the new cases and retreatment cases with tuberculosis

Patient category	Number of isolates carrying mutations, No. (%)							
	G88C	A90V	S91P	D94A	D94N	D94G	D94H	D94Y
NC (*n* = 239)	0 (0.00)	60 (25.10)	12 (5.02)	30 (12.55)	21 (8.79)	110 (46.03)	0 (0.00)	6 (2.51)
RC (*n* = 264)	1 (0.38)	44 (16.67)	25 (9.47)	41 (15.53)	16 (6.06)	117 (44.32)	6 (2.27)	14 (5.30)
P-value	0.341	0.038	0.066	0.375	0.26	0.776	0.02	0.117

Note: NC — new cases; RC — retreatment cases.

second, fluoroquinolone-monoresistant MTB fell into the third, MTB monoresistant to other antituberculosis drugs fell into the fourth, and MTB susceptible to all antituberculosis drugs fell into the fifth one (Table 4). MTB carrying *gyrA* mutations usually showed MDR or polyresistance (in total, 689/694, 99.28%, 95% CI: 98.32–99.69%). Fluoroquinolone-monoresistance was extremely rare (5/694, 0.72%, 95% CI: 0.31–1.68); in four cases, such MTB carried *gyrA* mutation D94A, and in one case mutation D94G was found. MTB with the wild type *gyrA* were almost equally divided between the categories of MDR MTB and MTB susceptible to all antituberculosis drugs.

Heteroresistance and multiple mutations

The listed above MTB isolates showed the same structure of the *gyrA* QRDR (wild type or single mutation) in all samples obtained from the same patient over time. However, the data on the *gyrA* QRDR structure obtained by dynamic monitoring of 55 patients differed (Table 5).

Thus, when assessing diagnostic materials during chemotherapy, MTB with varying structure of the *gyrA* QRDR were isolated from 35 patients. Of them in 22 cases both MTB with the wild type *gyrA* and MTB carrying mutations were revealed in the samples obtained from one patient. The wild type *gyrA* and *gyrA* with single mutations (mostly D94G) were found in MTB isolated from 15 patients (Table 5, item 1.1.1). MTB with the wild type *gyrA* and *gyrA* carrying multiple mutations were isolated from seven patients (Table 5, item 1.1.2). In three out of these seven cases, co-existence of two pools of MTB carrying single mutations instead of the existence of one pool with the double *gyrA* mutation was proven, since the samples with single mutations were also isolated over time. In four out of seven cases, the existence of two pools with single mutations or one pool with the double *gyrA* mutation was not proven.

On different dates samples were obtained from 13 patients, from which MTB carrying various single mutations were isolated (eight out of 13), or among which samples carrying double or single mutations alternated (five out of 13). This could indicate that there were several MTB pools with mutant *gyrA* in the patient's body (Table 5, item 1.2).

In five cases, heteroresistance was revealed when checking exceptions of the tests for phenotypic and genotypic resistance: MTB DNA carrying *gyrA* mutations was obtained from the diagnostic sample, while MTB culture obtained from the sample showed phenotypic susceptibility to levofloxacin, or vice versa. In such a case the available DNA samples were repeatedly (up to eight times) tested for *gyrA* mutations, and fresh DNA was isolated from the diagnostic sample and tested for *gyrA* mutations. The data both consistent with the initial results and different from these results were obtained in each of these five cases in a series of tests for *gyrA* mutations. This could somehow prove the presence of the MTB mixed population in one diagnostic sample (Table 5, item 2).

In another 15 cases, we detected double mutations in one sample (only one sample per patient was available for testing) (Table 5, item 3). It was usually one of the most common mutations (D94G or A90V) combined with one rare mutation (nine cases out of 15). However, in five cases out of 15, two rare mutations were simultaneously detected: it was the S91P + D94A combination only. MTB carrying two most common mutations (D94G and A90V) were isolated from only one patient. In all 15 cases, another PCR test of DNA isolated from the diagnostic sample revealed two mutations again. This indicated that either one MTB pool with the genomes containing double *gyrA* mutations, or two MTB pools carrying single *gyrA* mutations and represented in equal proportions were isolated from one patient. In these cases, diagnostic materials were collected from patients only once, that is why

Table 4. MTB isolates with various resistance profiles and various structure of *gyrA**

Mutation	Resistance profile, No. (%; 95% CI)				
	MDR	Poly	Mono to FQ	Mono to other ATBD	sens
G88C (*n* = 1)	1 (100; 20.65–100)	–	–	–	–
A90V (*n* = 149)	140 (93.96; 88.92–96.79)	9 (6.04; 3.21–11.08)	–	–	–
S91P (*n* = 53)	52 (98.11; 90.06–99.67)	1 (1.89; 0.33–9.94)	–	–	–
D94A (*n* = 102)	92 (90.20; 82.89–94.59)	6 (5.88; 2.72–12.24)	4 (3.92; 1.54–9.65)	–	–
D94N (*n* = 54)	49 (90.74; 80.09–95.98)	5 (9.26; 4.02–19.91)	–	–	–
D94G (*n* = 287)	270 (94.08; 90.72–96.27)	16 (5.57; 3.46–8.86)	1 (0.35; 0.06–1.95)	–	–
D94H (*n* = 10)	10 (100.00; 72.25–100)	–	–	–	–
D94Y (*n* = 38)	35 (92.11; 79.20–97.28)	3 (7.89; 2.72–20.80)	–	–	–
Total, with single mutations (*n* = 694)	649 (93.52; 91.43–95.12)	40 (5.76; 4.26–7.75)	5 (0.72; 0.31–1.68)	–	–
WT (*n* = 1412)	779 (55.17; 52.57–57.75)	–	–	54 (3.82; 2.94–4.96)	579 (41.01; 38.47–43.59)
Total (*n* = 2106)	1428 (67.81; 65.78–69.77)	40 (1.90; 1.40–2.58)	5 (0.24; 0.10–0.55)	54 (2.56; 1.97–3.33)	579 (27.49; 25.63–29.44)

Note: MDR — multiple drug resistance; Poly — polyresistance; Mono — monoresistance; FQ — fluoroquinolones; ATBD — antituberculosis drugs; sens — sensitive to ATBD; WT — wild type *gyrA*; * — only samples with known resistance type were included in the analysis.

Table 5. Heteroresistance to fluoroquinolones

Description	Number (abs)	Patient category			Nature of resistance to ATBD			
		NC	RC	Undefined	MDR	Poly	Mono FQ	N/D
1. Different structure of the *gyrA* QRDR in various samples obtained from one patient, such as:	35	1	27	7	31	1	2	1
1.1 Sequential isolation of MTB with the WT *gyrA* and *gyrA* mutations from various samples, including	22	1	18	3	18	1	2	1
1.1.1 WT + single mutations (2 pools):	15	1	11	3	12	1	1	1
WT + D94G	9	–	8	1	7	1	–	1
WT + A90V	4	1	2	1	3	–	1	–
WT + D94N	1	–	–	1	1	–	–	–
WT + D94Y	1	–	1	–	1	–	–	–
1.1.2 WT + multiple mutations	7	–	7		6	–	1	–
1.1.2.1 (3 pools)	5	–	5		4	–	1	–
WT + D94G + S91P	1	–	1			–	1	–
WT + D94G + A90V	1	–	1		1	–	–	–
WT+D94G+D94N, then D94N only	1	–	1		1	–	–	–
WT+D94G+D94N, then D94N only	1	–	1		1	–	–	–
WT+D94G+A90V, then D94N only	1	–	1		1	–	–	–
1.1.2.2 (4 pools)	2	–	2		2	–	–	—
WT + A90V + S91P + D94N	1	–	1		1	–	–	–
WT + A90V + D94G + D94N	1	–	1		1	–	–	–
1.2 Sequential isolation of MTB with various *gyrA* mutations from various samples	13	–	9	4	13	–	–	–
1.2.1 Various single mutations (2 pools)	8	–	6	2	8	–	–	–
A90V or D94G	4	–	2	2	4	–	–	–
A90V or D94A	1	–	1		1	–	–	–
D94H or D94Y	1	–	1		1	–	–	–
D94G or D94N	1	–	1		1	–	–	–
D94G or D94H	1	–	1		1	–	–	–
1.2.2 Alternating double and single mutations	5	–	3	2	5		–	–
1.2.2.1 (2 pools)	4	–	2	2	4	–	–	–
D94G + A90V or A90V	1	–	1	–	1	–	–	–
D94G + A90V or D94G	1	–	1	–	1	–	–	–
A90V + S91P or A90V	1	–	–	1	1	–	–	–
D94N + D94G or D94G	1	–	–	1	1	–	–	–
1.2.2.2 (3 pools)	1	–	1	–	1	–	–	–
A90V + D94N + D94Y or A90V	1	–	1	–	1	–	–	–
2 Different variants of QRDR in one sample	5	1	4	–	3	1	1	–
2.1 (2 pools)	4	1	3	–	2	1	1	–
WT + D94G	1	–	1	–	1	–	–	–
WT + A90V	1	–	1	–	–	1	–	–
WT + S91P	1	–	1	–	1	–	–	–
WT + D94N	1	1	–	–	–	–	1	–
2.2 (3 pools)	1	–	1	–	1	–	–	–
WT + D94Y + A90V + (A90V и D94Y)	1	–	1	–	1	–	–	–
3. Double mutation	15	–	9	6	11	4	–	–
A90V + D94N	3	–	–	2	2	–	–	–
S91P + D94A	5	–	4	1	3	2	–	–
S91P + D94G	1	–	–	1	1	–	–	–
A90V + D94A	2	–	1	1	2	–	–	–
A90V + D94H	3	–	3	–	1	2	–	–
A90V + D94G	1	–	–	1	1	–	–	–
S91P + D94N	–	–	1	–	1	–	–	–
Total	55	2	40	13	45	6	3	1
Of those:	–	–	–	–	–	–	–	–
2 pools*	46	2	31	13	37	6	2	1
3 pools	7	–	7	–	6	–	1	–
4 pools	2	–	2	–	2	–	–	–

Note: NC — new cases; RC — retreatment cases; ATBD — antituberculosis drugs; FQ — fluoroquinolones; MDR — multiple drug resistance; Poly — polyresistance; Mono — monoresistance; N/d — no data available; WT — wild type *gyrA*; * — cases of the detected double *gyrA* mutation are included.

no ongoing monitoring allowing us to clarify the data obtained was performed.

Thus, we have shown that the patient could be infected with 2–4 MTB pools showing different structure of the *gyrA* QRDR. Mixed populations were most often represented by two MTB pools (46/55, 83.64%, when 15 cases of double mutations with unproven membership in two different pools were included in the analysis). Of those in 19 cases the population consisted of the MTB pool with the wild type *gyrA* and MTB pool carrying single *gyrA* mutations. In all other cases, (27, when 15 cases of double mutations for which no data of the ongoing monitoring were available were included in the analysis) MTB population was represented by two MTB pools with various *gyrA* mutations

In seven cases, MTB population found in one patient was represented with three pools showing different *gyrA* structure. In six cases, one MTB pool contained the wild type *gyrA* and two pools carried different *gyrA* mutations, while in one case all three MTB pools carried various *gyrA* mutations. Co-existence of three MTB pools showing different *gyrA* structure in one patient was proven by ongoing monitoring in five cases out of seven.

The presence of four MTB pools showing different *gyrA* QRDR structure could be suspected, since in one case susceptible MTB were isolated, and in another case tree *gyrA* mutations were identified in two samples of diagnostic material. It is hard to imagine that independent sequential processes of spontaneous mutagenesis could result in the emergence of three mutations at once within the same gene region, that is why it is reasonable to assume that there are three independent MTB pools carrying different mutations.

Mixed populations of MTB were usually isolated from the previously treated patients (40/55, 72.73%, 95% CI: 59.77–82.72). These populations were represented mostly by MDR MTB (45/55, 81.82%, 95% CI: 69.67–89.81). However, in two cases out of 55, mixed populations of MTB characterized by levofloxacin monoresistance were isolated from the new cases.

DISCUSSION

A retrospective study that covered a significant number of tuberculosis cases diagnosed in 2011–2019 was carried out to assess the diversity of mutation in the *gyrA* gene QRDR of MTB.

Of the eight identified nutant variants, seven were highly credible in terms of developing resistance to fluoroquinolones [8]. Mutations D94G and A90V were the most common, which was typical for the entire world's population [6, 7]. Our data on the frequency of these mutations (40.42% for D94G and 21.26% for A90V) showed that it was slightly higher compared to the global population (21–32% and 13–20%, respectively) [6].

The featured study shoes that mutations in the gyrA QRDR were most often associated with phenotypic resistance to fluoroquinolones, however, in rare cases, testing of MTB for *gyrA* mutations by the culture-based method revealed no fluoroquinolone resistance. This was explained by the MTB population heteroresistance after conducting additional studies. It is known that in case the share of one strain in the mixture is less than 5% when assessing phenotypic and genotypic resistance to fluoroquinolones, it is impossible to define its genotype and phenotype, and the results reflect the characteristics of the strain that dominates in the mixture [11]. This is important to consider when interpreting the mismatching results of testing for fluoroquinolone resistance by the culture-based and molecular genetic methods, since the initial low concentration of MTB with certain genotype in the cell mix and the likelihood of uneven distribution of MTB cells with various genotypes among samples collected for further molecular genetic and culture-based tests cannot be excluded.

When D94A mutation was detected in *gyrA* in 24 cases out of 73 (32.88%), MTB showed phenotypic resistance to levofloxacin. It is hard to explain such a high percentage by the undetected heteroresistance or errors in the culture-based or genotypic testing. Furthermore, other papers also report cases of the gyrA_D94A genotype corresponding to the susceptible phenotype: phenotypic resistance to fluoroquinolones was detected in one out of seven and four out of 12 MTB strains carrying this mutation, depending on the studied population [12, 13]. Therefore, it can be concluded that regardless of the fact that mutation D94A is highly credible in terms of developing resistance, mutation is not strongly associated with the fluoroquinolone resistance.

There is a theory that the widespread use of fluoroquinolones for treatment of nontuberculous infections may result in fluoroquinolone resistance developed by patients with undiagnosed tuberculosis [14]. In this regard it was important to evaluate the nature of resistance in the fluoroquinolone-resistant MTB: association of this parameter with multiple drug resistance of the pathogen can indicate that resistance to fluoroquinolones develops during treatment of MDT-TB, while identification of the cases of fluoroquinolone monoresistance, especially in the new cases, may be an indicator of fluoroquinolone resistance developed by MTB during treatment of other infectious diseases. We showed that the MTB genotypic resistance to fluoroquinolones was most often associated with MDR: 649 isolates carrying single *gyrA* mutations out of 694 (93.52%) and 45 cases of mixed populations out of 55 (81.00%) fell into the MDR category.

The findings prove that fluoroquinolone resistance is developed by MTB during treatment of MDR-TB. However, we cannot rule out the fluoroquinolone resistance developing during treatment of nontuberculous infections, since MTB showing fluoroquinolone monoresistance have been also found. Unfortunately, we do not know, whether these patients were previously treated with fluoroquinolones.

The study of the MTB genome structure, that involved MTB circulating in Samara region, performed by whole genome sequencing, allowed the authors to conclude that MTB were more likely to acquire fluoroquinolone resistance during therapy, and the cases of human infections caused by the fluoroquinolone-resistant MTB clones were rare. Based on this observation, it was assumed that the development of fluoroquinolone resistance resulted in the reduced MTB fitness [13]. The population-based study of the MTB primary fluoroquinolone resistance distribution across the Novosibirsk region also showed that resistance to fluoroquinolones most often resulted from the use of drugs of this group in chemotherapy of MDR-TB [15].

The findings presented here also confirm that the development of fluoroquinolone resistance during treatment is more frequent: MTB carrying *gyrA* mutations were more often isolated from the previously treated patients than from the newly diagnosed ones. However, we have found that fluoroquinolone-resistant MTB could be also isolated from the newly diagnosed patients with tuberculosis. In these cases *gyrA* mutations D94G and A90V were the most common, although the frequency of A90V mutation in MTB isolated from the newly diagnosed patients was significantly higher than in that isolated from the previously treated ones. Therefore, it can be concluded that MTB carrying this mutation are transmitted rather actively between humans.

The described possibility of the co-existence of several MTB population varying in *gyrA* mutations in one patient refers to the development of fluoroquinolone resistance in the population. A number of studies also showed that there could be several MTB clones with different structure of *gyrA* in one diagnostic sample; such samples accounted for 1–3% of the total number, which was consistent with our results [16–18].

Thus, we have shown that fluoroquinolone resistance is currently developing in the population of MTB circulating in the RF, which is usually associated with the pre-existing MDR. MTB resistance to fluoroquinolones has good prospects in terms of evolution, since it is developed in favorable genetic conditions during treatment of tuberculosis, caused by pathogen showing MDR, that results from the combination of mutations, that do not reduce MTB fitness [19]. The fact, that MTB carrying *gyrA* mutations D94G and A90V were also rather frequent in the new cases, allows us to conclude that it is these mutants that would play a key role in the spread of pre-XDR tuberculosis across the RF.

CONCLUSIONS

Retrospective analysis of the range of mutations in the QRDR of the *gyrA* gene of MTB isolated in 2011–2019 showed that genotypic resistance to fluoroquinolones was detected in 26.96% of the MTB clinical isolates, including cases of heteroresistance. Mutations D94G and A90V were the most common, their frequency totaled 62.95% of the number of MTB carrying a single *gyrA* mutation. It was also shown that these two mutations were rather common in MTB isolated in the new cases (D94G was found in 46.03%, and A90V was found in 25.10% MTB in this group), thus confirming successful spread of these MTB mutant variants in the modern population. The presence of *gyrA* mutations was usually associated with phenotypic resistance to levofloxacin, except for mutation D94A that was associated with phenotypic resistance to levofloxacin in only 67% of cases. The *gyrA* mutations were found mostly in MDR MTB: 93.52% of the strains carrying *gyrA* mutation were also resistant to rifampicin and isoniazid. The findings proved that resistance to fluoroquinolones was developed during treatment of MDR-TB. Furthermore, in 2.31% of cases heteroresistant MTB populations were found that included 2–4 MTB pools with different structure of the *gyrA* QRDR. Fluoroquinolone heteroresistance indicates active development of resistance to this group of medications in the current conditions.

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COMBINED EFFECTS OF BACTERIOPHAGE VB_SAUM-515A1 AND ANTIBIOTICS ON THE *STAPHYLOCOCCUS AUREUS* CLINICAL ISOLATES

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Currently, the search for new therapy options for infectious diseases caused by multidrug-resistant *Staphylococcus aureus* is a priority. Combining antibiotics with virulent (lytic) bacteriophages may be considered a viable alternative to conventional antibiotic therapy. The study was aimed to assess the combined effects of the lytic bacteriophage vB_SauM-515A1 of *Herelleviridae* family and antibiotics of various classes on the *Staphylococcus aureus* clinical strains. Strains (n = 4) belong to the clinically significant sequence types ST1, ST8, ST121 and are characterized by multidrug resistance. Efficiency of the combination use of two antibacterial agents was assessed by comparison of optical densities of the test samples and controls after 24 hrs. of incubation. Mutually enhancing activities of bacteriophage used in combination with oxacillin, tetracycline and linezolid were revealed, in contrast to the separate use of each agent. Efficiency generally increased with the selected optimum multiplicity of infection values. No antagonism was revealed when combining the phage with antibiotics. Thus, virulent bacteriophage vB_SauM-515A1 can be considered as a possible auxiliary therapeutic agent for antimicrobial-resistant strains of *Staphylococcus aureus*.

Keywords: bacteriophage therapy, *Staphylococcus aureus*, *Herelleviridae*, combined effects, gentamicin, tetracycline, vancomycin, oxacillin, linezolid, levofloxacin

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Compliance with ethical standards: the study was carried out in accordance with the sanitary and hygienic guidelines SP 1.3.2322-08 “Safety of Working With Microorganisms of III-IV Groups of Pathogenicity (Danger) and Causative Agents of Parasitic Diseases”; sanitary and hygienic guidelines SP 1.3.2518-09 "Additions and Amendments № 1 to the guidelines SP 1.3.2322-08 "Safety of Working With Microorganisms of III-IV Groups of Pathogenicity (Danger) and Causative Agents of Parasitic Diseases"; sanitary and hygienic guidelines "Sanitary and Epidemiologic Requirements for the Handling of Medical Waste" (SanPiN 2.1.7.2790-10); Federal Clinical Guidelines "Rational Use of Bacteriophages in Clinical and Epidemiological Practice".

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КОМБИНИРОВАННОЕ ВОЗДЕЙСТВИЕ БАКТЕРИОФАГА VB_SAUM-515A1 И АНТИБИОТИКОВ НА КЛИНИЧЕСКИЕ ИЗОЛЯТЫ *STAPHYLOCOCCUS AUREUS*

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Поиск новых вариантов терапии инфекционных заболеваний, вызванных *Staphylococcus aureus* с множественной лекарственной устойчивостью, на сегодняшний день является приоритетной задачей. В качестве одной из перспективных альтернатив классической антибиотикотерапии может быть рассмотрена комбинация антибиотиков с вирулентными (литическими) бактериофагами. Целью работы было оценить результат совместного воздействия литического бактериофага vB_SauM-515A1 семейства *Herelleviridae* и антибиотиков различных классов на клинические штаммы *Staphylococcus aureus*. Штаммы (n = 4) относятся к клинически значимым сиквенс-типам ST1, ST8, ST121 и характеризуются множественной лекарственной устойчивостью. Эффективность комбинированного воздействия двух антибактериальных агентов оценивали при сравнении значений оптической плотности опытных и контрольных образцов после 24 ч инкубации. Наличие взаимодополняющих эффектов было показано при совместном использовании бактериофага с оксациллином, тетрациклином и линезолидом, по сравнению с использованием каждого из агентов по отдельности. Эффективность повышалась в основном в рамках подобранных оптимальных значений множественности инфекции. Антагонистические эффекты комбинации фага и антибиотиков не были выявлены. Таким образом, вирулентный бактериофаг vB_SauM-515A1 можно рассматривать в качестве возможного вспомогательного терапевтического агента против устойчивых к антибактериальным препаратам штаммов *Staphylococcus aureus*.

Ключевые слова: бактериофаговая терапия, *Staphylococcus aureus*, *Herelleviridae*, комбинированное воздействие, гентамицин, тетрациклин, ванкомицин, оксациллин, линезолид, левофлоксацин

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Staphylococcus aureus is a pathogenic microorganism causing severe inflammatory disorders of the skin and soft tissues, as well as invasive infections, such as pneumonia, endocarditis, osteomyelitis, etc. [1]. It is difficult to treat such diseases due to wide spread of the multidrug-resistant (MDR) strains, among which methicillin-resistant *Staphylococcus aureus* (MRSA) is the most clinically significant. About 4.95 million people died due to antibiotic-resistant infections in 2019. Staphylococcal infections were the major cause of deaths, and more than 100,000 deaths were caused by methicillin-resistant strains [2]. In 2020 in Russia, the share of bacteria of genus *Staphylococcus* resistant to such antibiotics as tetracycline, gentamicin, erythromycin and oxacillin was 15–25%. The vast majority of strains showed intermediate resistance to levofloxacin and ciprofloxacin [3]. More recently isolated cases of acquired resistance to vancomycin and linezolid used as drugs of choice in treatment of MRSA infections have been reported [4, 5]. These statistics highlight the need to search for alternative antimicrobial agents. Bacteriophage preparations might be considered as such agents [6, 7].

Bacteriophages (phages) are viruses that naturally infect prokaryotic cells. Only virulent (lytic) phages are used as therapeutic agents due to the need to avoid possible horizontal transmission of antibiotic resistance determinants and genes encoding bacterial toxins [8]. Phage preparations have some advantages over antibiotics. Thus, virulent bacteriophages are capable of lysing bacteria regardless of their sensitivity to antibiotics. This makes phages a powerful tool for combating resistant strains. Another advantage is no side effects on the patient's body. This enables safe use of virulent bacteriophage preparations even in complex clinical cases [9].

Currently, the use of bacteriophages is one of the promising approaches to treatment of staphylococcal infections caused by MDR strains [10]. Successful implementation of these approaches has been confirmed by clinical experiments, both animal [11] and human [12]. We should also mention the effectiveness of bacteriophage preparations against biofilms formed by *Staphylococcus aureus* [10].

The combined use of bacteriophages and antibiotics is considered the most promising strategy for treatment of disorders caused by drug resistant strains [13, 14]. A number of papers about various pathogens report that the combined use of median lethal doses of antibiotics and bacteriophages is more effective compared to separate use [13, 15]. Beneficial effects of such combination were first reported in 2007 [13]. Studies have now shown that the combined use of bacteriophage and antibiotic may also result in neutral and adverse effects [16, 17].

The increased efficiency associated with the combination use of antibacterial agents (mutually enhancing actions) can be explained by one of the following effects: additive or synergistic. More active suppression of bacterial growth associated with additive effects is achieved through summing up antibacterial effects exerted by the agents. Synergism happens when the efficiency of the combination is significantly higher compared to the separate use of individual components or their sum. Neutral effects happen when there are no significant differences between the combination use of drugs and the use of at least one antimicrobial agent. Antagonism happens when the effects of one agent suppress the effects of another one. It should be noted that only isolated cases of antagonistic interactions between bacteriophages and antibiotics have been reported [17].

To date, the described effects were observed when using the combinations of bacteriophages and some antibiotics (vancomycin, daptomycin, oxacillin) against *S. aureus* [12, 17]. However, taking into consideration the genetic and phenotypic heterogeneity of the pathogen, even the laboratory strains, it is important to test suitability of the phage-antibiotic pairs using the larger set of bacterial isolates to reveal the patterns underlying the emergence of this or that resulting effect.

The study was aimed to assess the combined effects of the lytic bacteriophage of *Herelleviridae* family and antibiotics of various classes on the multidrug-resistant (MDR) clinical strains of *Staphylococcus aureus*.

METHODS

Bacterial strains

The study used *S. aureus* strains (SA64, SA413, SA1050, and SA515/1) obtained from the collection of the Laboratory of Molecular Genetics of Microorganisms, Federal Research and Clinical Center of Physical-Chemical Medicine of FMBA of Russia. Bacteria were grown in the LB (lysogeny broth) culture medium (Oxoid; UK) for 18–24 hrs at 37 °C. Typing of the strains was performed by multilocus sequence typing (MLST) using the standard scheme [14]. Minimum inhibitory concentrations (MICs) of antibiotics were defined by the CLSI serial dilution method [18]. MICs of six antibiotics (oxacillin, vancomycin, gentamicin, tetracycline, levofloxacin, linezolid (Sigma-Aldrich; USA)) were defined.

Bacteriophage

Bacteriophage vB_SauM-515A1 (*Herelleviridae* family) was earlier isolated from the commercial complex phage preparation "Staphylococcal bacteriophage" P332 (Microgen; Russia) on the SA515 S. aureus host strains. The detailed bacteriophage characteristics were reported earlier [19, 20].

Determining the studied bacteriophage titer on the tested strains

The titer was determined by the previously reported method of Grazia [21]. For that aliquots (5 μL) of the bacteriophage preparation ten-fold sequential dilutions (stock 2 × 10^9 plaque-forming units (PFU)/mL) were applied onto the surface of plates with semi-solid LB agar (0.6% agar) containing 0.1 mL of the tested strain overnight culture (10^6 colony-forming units

Table 1. Characteristics of the *Staphylococcus aureus* strains

Strain	ST	EOP	Susceptibility to antibiotics, μg/mL					
			Oxacillin	Vancomycin	Gentamicin	Tetracycline	Levofloxacin	Linezolid
SA64	1	267%	< 0.125 (S)	8 (I)	128 (R)	64 (R)	8 (R)	4 (I)
SA413	8	283%	< 0.125 (S)	0.5 (S)	128 (R)	32 (R)	4 (R)	8 (R)
SA1050	121	72%	< 0.125 (S)	8 (I)	< 0.125 (S)	64 (R)	< 0.125 (S)	4 (I)
SA515/1	8	100%	4 (R)	8 (I)	128 (R)	32 (R)	< 0.125 (S)	4 (I)

Note: R — resistant strains, I — strains showing intermediate resistance, S — susceptible strains.

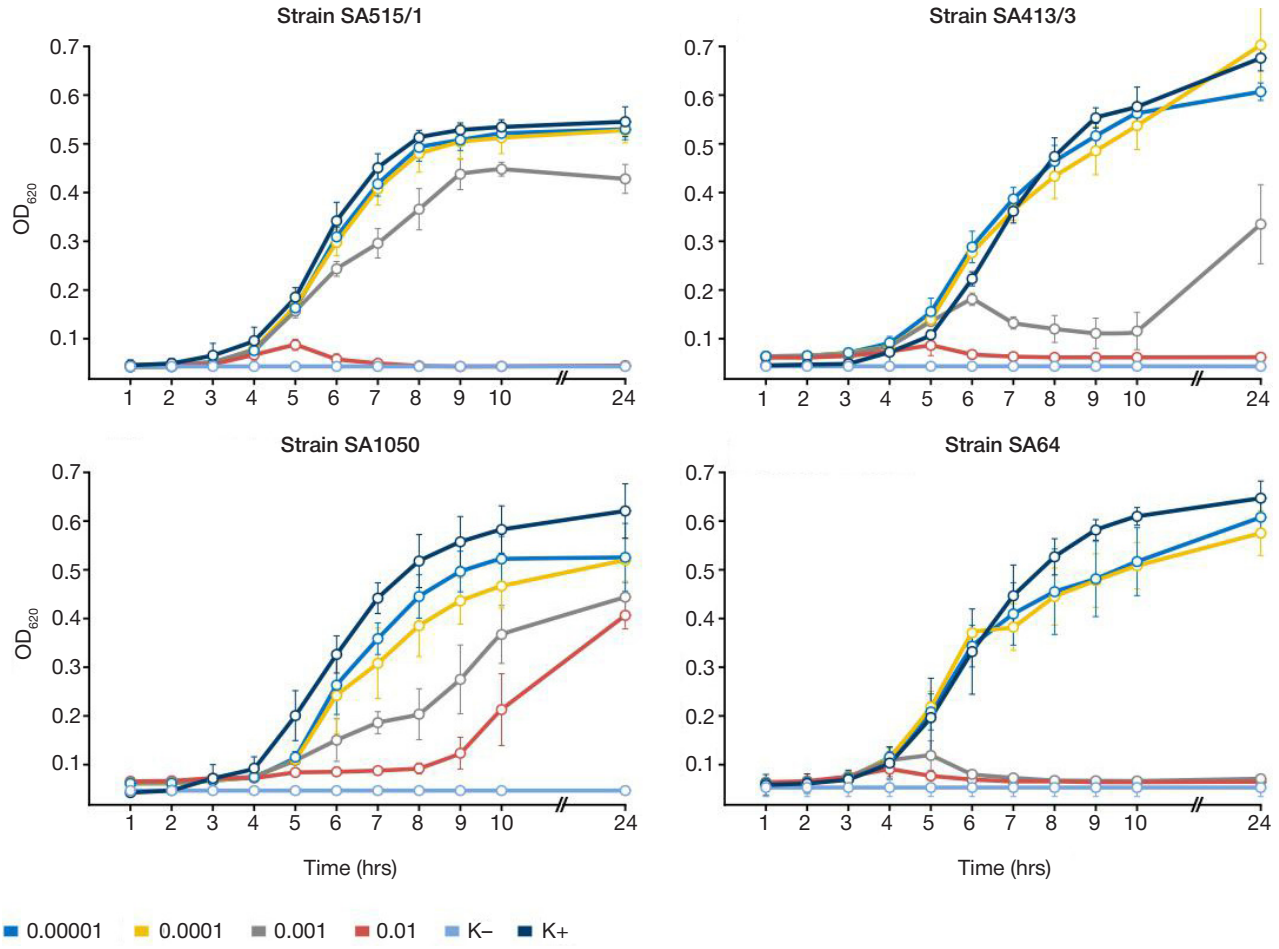

Fig. 1. Growth curves of the *S. aureus* infected with bacteriophage vB_SauM-515A1 with various MOI values

(CFU/mL) and incubated at 37 °C for 24 hrs. The concentrations of phage particles for the tested strains were measured in PFU/mL. The effectiveness of the tested strain lysis by bacteriophage was assessed based on efficiency of plating (EOP) [19]. EOP is defined as a relationship of the phage titer on the tested strain to the phage titer on the host strain (SA515/1), expressed as a percentage. Plating efficiency was tested three times.

Studying the combined effects of antibiotics and bacteriophage

The combined effects of antibiotics and bacteriophages were assessed as previously described [17]. Experiments were carried out in the 96-well flat bottom plates (Thermo Scientific; USA) in 200 µL in the LB medium. Bacterial cells were inoculated during the exponential growth phase (OD_{620} = 0.2; 5 × 10^8 CFU/mL) to the final concentration of 10^4 cells per well. Bacteria were infected separately with the phage at four multiplicity of infection (MOI) values (0.01; 0.001; 0.0001; 0.00001), then exposed to different antibiotics and a combination of two antibacterial agents in various concentrations. Antibiotic concentrations of 1/8 MIC, 1/4 MIC, 1/2 MIC were used. Inoculated culture medium with no added antibacterial agent was used as a positive control, while pure growth media was used as a negative control. The dynamics of the phage and antibiotic effects on bacteria were defined by continuous measurement of optical density (OD) at 620 nm for 10 hrs and after 24 hrs of incubation at 37 °C using the Multiscan Ascent Microplate Reader (Thermo Electron Corporation; Finland). Growth curves for the S. aureus strains infected with bacteriophage at various MOI values were plotted based on the OD values. In certain cases, mutually enhancing activities were confirmed by comparison of the finite OD values in the final point (24 hrs) as previously reported [15].

Statistical analysis

Statistical analysis was performed in the Graph Pad Prism software package, v. 8.0.1 (GraphPad Software Inc.; USA) based on the *t*-test. The analysis involved comparison of OD values obtained after 24 hrs of incubation for samples exposed to only one antimicrobial agent (antibiotic/bacteriophage) with similar values of the samples simultaneously exposed to both agents.

RESULTS

Bacterial strains were characterized based on the sequence types (ST) and tested for susceptibility to bacteriophage and antibiotics (Table 1). MLST showed that *S. aureus* strains fell into sequence types ST1, ST8, and ST121. All samples were MDR, there were strains resistant to oxacillin (SA515/1), gentamicin (SA64, SA413, SA515/1), levofloxacin (SA64, SA413), and linezolid (SA413) among them. All the studied bacteria showed resistance to tetracycline. Intermediate resistance to vancomycin and linezolid of three strains (SA64, SA1050, SA515/1) was revealed. Bacteriophage vB_SauM-515A1 lysed all the studied bacteria. The highest efficiency of lysis exceeding the value obtained for the host strain (SA515/1) more than 2.5 times was shown for strains SA64 (267%) and SA413 (283%). Bacteriophage lysed strain SA1050 less actively (72%).

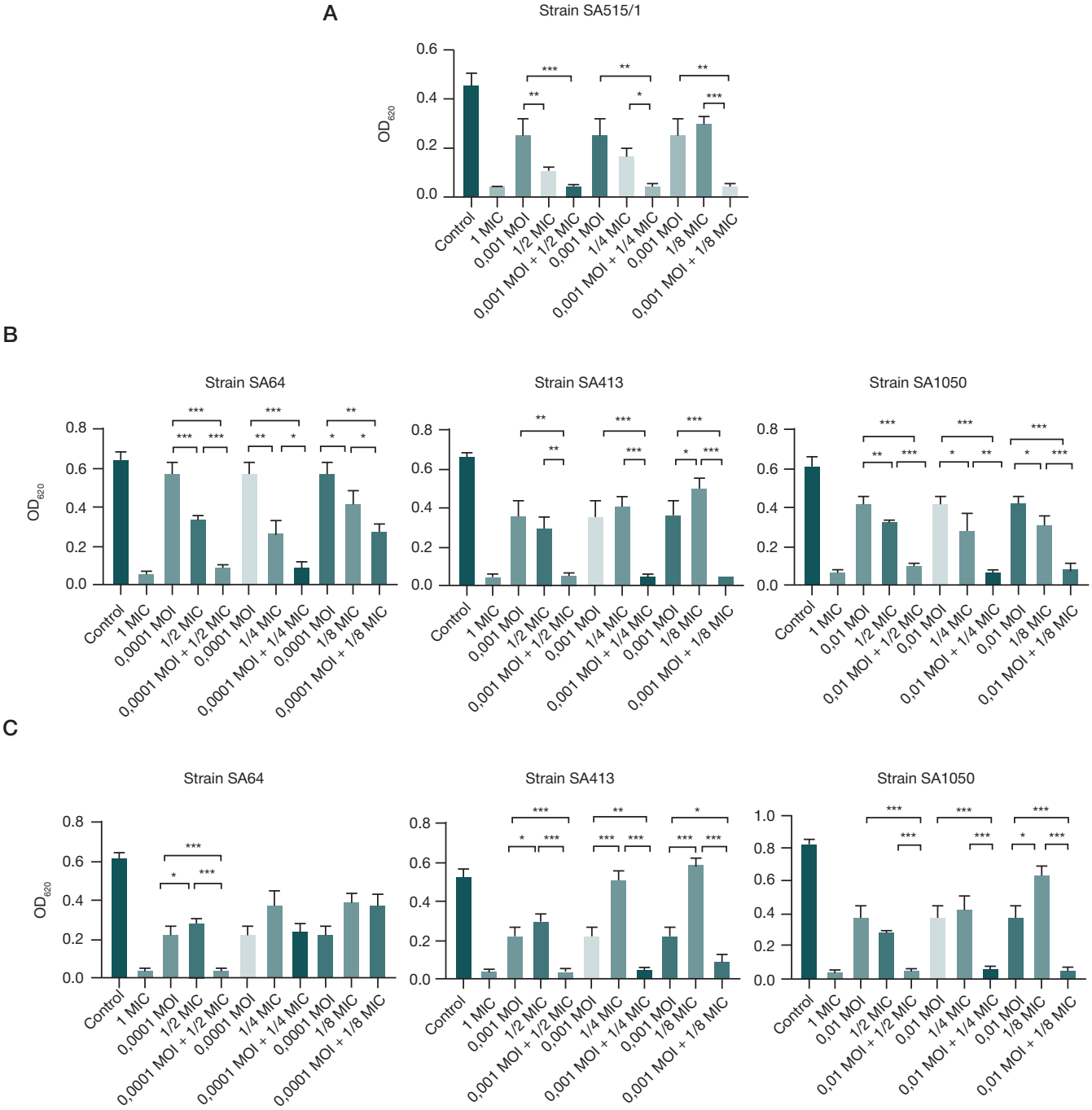

Fig. 2. Combined effects of the lytic bacteriophage vB_SauM-515A1 and antibiotics (oxacillin (**A**), tetracycline (**B**), linezolid (**C**)) on the *S. aureus* strains at optimum MOI values. Statistical significance: * — $p \leq 0.05$; ** — $p \leq 0.01$; *** — $p \leq 0.001$

Optimum MOI values were defined using the growth curves of bacterial cultures infected with bacteriophage in order to assess the combined effects of antimicrobial agents (Fig. 1). Reduced OD compared to non-infected control with MOI values of 0.01 и 0.001 was reported for the host strain SA515/1, moreover, bacteriophage-cell ratio that corresponded to MOI = 0.01, completely suppressed growth by hour 24. Thus, experiments involving the use of MOI = 0.001 were the most interesting in terms of assessing mutually enhancing activities of bacteriophage and antibiotic against the SA515/1 strain cell culture. The efficiency of the SA1050 strain lysis by bacteriophage vB_SauM-515A1 was lower than that reported for the host strain, therefore, only partial suppression of cell growth was achieved with MOI = 0.01 and MOI = 0.001: in contrast to the non-infected control, OD dropped from 0.6 to 0.44 and 0.4, respectively, by hour 24. More effective lysis was reported for strains SA413 and SA64 than for the host strain, the most optimal vB_SauM-515A1–SA413 cell ratio was MOI = 0.001, and the most optimal ratios for strain SA64 were 0.0001 and 0.00001.

The efficiency of the combination use of antibiotic (oxacillin, vancomycin, tetracycline, gentamicin, levofloxacin and linezolid) and bacteriophage vB_SauM-515A1 was assessed for strains resistant to the selected antibiotic or showing intermediate resistance. The mutually enhancing activities of oxacillin and bacteriophage vB_SauM-515A1 were considered using the only oxacillin-resistant strain SA515/1 as an example. Bacteriophage enhanced the effects of antibiotic with the MOI value of 0.001 that was optimal for this strain and the concentrations of antibiotic of 1/4 and 1/8 MIC (Fig. 2, Table 2).

Table 2. Resulting effects of the combination use of various vB_SauM-515A1 bacteriophage and antibiotic concentrations on the *S. aureus* clinical strains

Strain	MOI	Oxacillin, MIC share			Vancomycin, MIC share			Gentamicin, MIC share			Tetracycline, MIC share			Levofloxacin, MIC share			Linezolid, MIC share		
		1/8	1/4	1/2	1/8	1/4	1/2	1/8	1/4	1/2	1/8	1/4	1/2	1/8	1/4	1/2	1/8	1/4	1/2
SA64	0.00001	S																	
	0.0001										+	+	+						+
	0.001				L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
	0.01				L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
SA413	0.00001	S			S														
	0.0001											+	+						+
	0.001										+	+	+				+	+	+
	0.01							L	L	L	L	L	L	L	L	L	L	L	L
SA1050	0.00001	S						S						S					
	0.0001												+						
	0.001											+	+					+	+
	0.01										+	+	+				+	+	+
SA515/1	0.00001													S					
	0.0001																		
	0.001	+	+																
	0.01	L	L	L	L	L	L	L	L	L	L	L	L				L	L	L

Note: + — mutually enhancing activities; empty cell — lack of mutually enhancing activities; L — culture completely lysed by bacteriophage; S — antibiotic-susceptible strain.

Similar effects were observed for oxacillin concentration of 1/2 MIC, however, this finding was non-significant.

The majority of mutually enhancing activities against other strains were reported for the combination use of bacteriophage and tetracycline or linezolid (Fig. 2, Table 2). When combined with bacteriophage, these antibiotics more effectively lysed strains SA64, SA413 и SA1050 that any of antimicrobial agents taken separately: this was true for various combinations of concentrations (Table 2). It should be noted that mutually enhancing activities were most often observed with optimum MOI values for each strain and antibiotic concentration of 1/2 MIC.

When using bacteriophage in combination with vancomycin, gentamicin and levofloxacin, no mutually enhancing activities against the *S. aureus* strains were observed. Furthermore, no antagonism was revealed in any of the strains when using antibiotic (oxacillin, vancomycin, tetracycline, gentamicin, linezolid, and levofloxacin) in combination with bacteriophage vB_SauM-515A1 (Table 2).

DISCUSSION

High prevalence of infections caused by MDR *S. aureus* strains is a major challenge faced by modern health care. The combination use of antibiotics and bacteriophages is a solution. We used an earlier characterized member of the family *Herelleviridae*, the lytic bacteriophage vB_SauM-515A1, to study the combined effects of two agents on the MDR *S. aureus* strains [20, 22]. Staphylophages of the family *Herelleviridae* are one of the most effective for therapy [19]. These obligate virulent phages show a broad spectrum of lytic activity [19]. The latter is in line with our findings: bacteriophage vB_SauM-515A1 effectively suppressed growth of all the studied *S. aureus* strains belonging to highly prevalent clinically significant sequence types (Table 1) [23, 24].

Medications used for treatment of various infectious diseases caused by staphylococci (oxacillin, vancomycin, gentamicin, tetracycline, levofloxacin, linezolid) were selected to assess the combined effects of the lytic bacteriophage and antibiotics [25, 26]. The above-mentioned antibiotics belong to different classes, each of them is characterized by specific mechanism underlying the effect on bacterial cells. It is important to note that the study involved both bacteriostatic (tetracycline, gentamicin, linezolid) and bactericidal (oxacillin, vancomycin, levofloxacin) medications. The studied strains were generally resistant to these antibiotics.

The study revealed cases of mutually enhancing activities shown by medications (oxacillin, tetracycline, linezolid) and bacteriophage vB_SauM-515A1, which is consistent with the reports by other authors. Thus, it was shown that the use of oxacillin and linezolid in combination with the lytic bacteriophage Sb-1 more effectively inhibited growth of the *S. aureus* strains in the majority of cases [17, 25]. In its turn, the combination of tetracycline in certain concentration and bacteriophage of *Herelleviridae* family ensured more effective growth suppression in the *S. aureus* biofilm-forming strains than the phage [27].

The results of the recent study conducted by colleagues were opposite [28]. The authors showed that simultaneous use of antibiotic and lytic bacteriophage never significantly increased the efficiency of bacterial growth inhibition, regardless of the antibiotic type. Such discrepancies may be explained by the outcome dependance on the target bacterial strain [9]. Thus, in our study, strain SA515/1 exposure to the combination of tetracycline, linezolid and the phage never resulted in growth suppression, while the same combination showed mutually enhancing activities against other strains.

Upon detection of beneficial effects associated with the combination use of antibiotics and bacteriophages, it is important to select optimal median lethal doses of both agents. When used in appropriate concentrations, their antibacterial effects are probably summed up, as observed during the study (Fig. 2, Table 2). The effectiveness of combining bacteriophages and antibiotics may be also due to bypassing the mechanisms underlying antibiotic resistance during interaction between cells and virus particles. It has previously been shown that the lytic phage of the resistant *Pseudomonas aeruginosa* strain uses the

membrane protein, porine, essential for efflux of antibiotics, as a receptor. To acquire resistance to phage, the bacterium gets rid of the efflux system and becomes antibiotic-susceptible again [29]. Therefore, the effects of bacteriophage on the cell may provide clone selection, thus increasing the bacterial culture susceptibility to antibiotics.

It is important to note, that no antagonism, i.e., reduced efficiency of some antibacterial agent (antibiotic/bacteriophage) in presence of another one, was observed in any of the studied combinations. Low rate of such negative cases has been also reported in other papers [17, 27].

For now, it remains unclear, what is the basis of mutual activities of phages and antibiotics against bacterial cells. Higher efficacy may be explained by both simple summation of effects exerted by individual antibacterial agents and more complex mutual effects resulting in more active suppression of cell growth. In-depth study of the causes of emerging mutually enhancing activities is essential for further practical use of phages as therapeutic agents in combination with antibiotics.

CONCLUSIONS

The findings show that the combination use of the lytic bacteriophage vB_SauM-515A1 and antibiotics of various classes can be more effective than the separate use of antibacterial agents. Thus, the studied phage can be considered as a promising therapeutic agent for the *S. aureus* MDR strains. The data obtained may be used for further study of effects resulting from the combination use of two antibacterial agents of different types.

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GEOGRAPHIC DISTRIBUTION OF THE *LZTFL1* SNP MARKERS ASSOCIATED WITH SEVERE COVID-19 IN RUSSIA AND WORLDWIDE

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The correlation between the risk of death from COVID-19 and the patient's ethnogeographic origin has been previously detected. *LZTFL1* gene was identified as a potential marker of a two times higher risk of severe COVID-19. The study was aimed to assess spatial variation in the *LZTFL1* SNP markers in indigenous populations of Russia and the world. Spatial variation in the *LZTFL1* polymorphic markers was analyzed in 28 metapopulations (97 ethnic groups) of North Eurasia (n = 1980) and 34 world's metapopulations (n = 3637) by bioinformatics, statistical and cartographic methods. In North Eurasia, the major geographic variation vectors, North–South and West–East, are generally in line with the Caucasoid–Mongoloid anthropological vector. Global variation also corresponds to anthropological features: each cluster of indigenous populations includes only those from the place where it originates: Africa, Asia, or America. Indo-European cluster integrates Caucasoid populations of Europe and Asia. All four clusters of the world's indigenous population are separated from each other. The huge genetic diversity of Russia peoples and neighboring countries forms a bridge between three continents: Europe, Asia and America. Cartographic atlas for spatial variation in 11 *LZTFL1* markers in the populations has been created. The following major patterns have been revealed: a) the world's extrema fall on the indigenous populations of Africa and America; 2) Eurasia constitutes a transition zone between these two extrema, but has its own patterns and shows enormous scale of variation shows enormous variation on a global scale; 3) the genetic landscape of Russia tends to be seamlessly integrated into the Eurasian landscape.

Keywords: COVID-19, LZTFL1, SNP, gene geography, populations, indigenous people, Russia, world

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Compliance with ethical standards: the study was approved by the Ethics Commitee of the Research Centre for Medical Genetics (protocol № 1 of 29 June 2020); all subjects submitted the informed consent to study participation.

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ГЕНОГЕОГРАФИЯ В РОССИИ И МИРЕ SNP-МАРКЕРОВ ГЕНА *LZTFL1*, АССОЦИИРОВАННЫХ С ТЯЖЕЛЫМ ТЕЧЕНИЕМ COVID-19

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Ранее была обнаружена корреляция между риском смерти от COVID-19 и этногеографическим происхождением пациента. Ген *LZTFL1* отмечается как потенциальный маркер, ассоциированный с двухкратным увеличением риска тяжелого течения COVID-19. Целью исследования было изучить пространственную изменчивость SNP-маркеров гена *LZTFL1* в коренном населении России и мира. Биоинформатическими, статистическими и картографическими методами был проведен анализ пространственной изменчивости полиморфных маркеров гена *LZTFL1* в 28 метапопуляциях (97 этносов) Северной Евразии (n = 1980) и 34 метапопуляциях мира (n = 3637). В Северной Евразии основные географические векторы изменчивости «север–юг» и «запад–восток» в целом согласуются с антропологическим вектором «европеоидность–монголоидность». Глобальная изменчивость тоже соответствует антропологии: каждый кластер коренного населения включает популяции только «своей» части света — Африки, Азии или Америки. «Индоевропейский» кластер объединяет европеоидные популяции Европы и Азии. Все четыре кластера коренного населения мира отдалены друг от друга, и только огромное генетическое разнообразие народов России и сопредельных стран является мостом, связующим три части света: Европу, Азию и Америку. Создан картографический атлас пространственной изменчивости 11 SNP-маркеров *LZTFL1* в популяциях. Выявлены основные закономерности: а) мировые экстремумы приходятся на коренное население Африки и Америки; 2) Северная Евразия является переходной зоной между мировыми экстремумами, но обладает собственными закономерностями и огромным размахом изменчивости в мировом масштабе; 3) генетический ландшафт России, как правило, органично вписан в Евразийский ландшафт.

Ключевые слова: COVID-19, LZTFL1, SNP, геногеография, популяции, коренное население, Россия, мир

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The COVID-19 pandemic, with its high mortality and severe complications, forced the world's scientific community to engage in the search for DNA markers associated with the SARS-COV-2 infection. COVID-19 severity varies among representatives of various world populations, that is why the COVID-19 Host Genetics Initiative international project has started gathering information about the frequency of genome variants associated with severe COVID-19 [1]. Among these gene *LZTFL1* [2] is specified as a potential marker of a two times higher risk of severe COVID-19 [3].

LZTFL1 is expressed in human lungs and encodes a protein involved in transport of other proteins to the primary cilia of the ciliated epithelial cells [4]. The *LZTFL1* gene clinical significance has been discvered earlier: the gene is associated with Bardet-Biedl syndrome-17 (OMIM 615994) [5], the autosomal recessive ciliopathy [6, 7]. Seven BBS and BBIP10 proteins form a stable complex referred to as BBSome. This complex ensures protein transport to the ciliary membrane [8, 9], while the reduced *LZTLF1* function can compensate for a lack of BBS proteins and restore ciliary motility [10].

Alterations related to severe COVID-19 and associated with *LZTFL1* were found in the patients' lung epithelial cells [3, 11]: alterations in the chromosome 3p21.31 region carrying gene *LZTFL1* resulted in the twofold increased risk of respiratory failure [12, 13] and more than twofold increased risk of mortality in people under the age of 60 [11]. The study of polymorphism of one of the *LZTFL1* gene variants in the UK population revealed association between the risk of death from COVID-19 and the patient's origin: ones from South Asia had a four times higher risk than patients of European descent. These differences partly explain higher mortality rates among the representatives of South Asian peoples living in the UK [3].

A significant association between *LZTFL1* and severe COVID-19, as well as therapeutic potential and ethnogeographic differences, calls for examining interpopulation variations among the world's population. The research team has an information base that includes both own and literature data on the world's peoples' genomes. The information base, that has already enabled the analysis of variation in SNP markers (*rs11385942*, *rs657152*) associated with severe COVID-19 among the world's population (more specifically in Russia) [14], makes it possible to perform similar study of 11 *LZTFL1* SNP markers.

The study was aimed to assess spatial variation in SNP markers of the LZTFL1 gene associated with severe COVID-19 [15] in the human population: 1) to perform the search for polymorphic *LZTFL1* SNP markers provided information about the SNP marker abundance in indigenous peoples of the world and North Eurasia (in more detail); 2) to provide two representative pools of population data on these SNP markers; 3) to perform multivariate statistical analysis of these data; 4) to create a cartographic atlas of the *LZTFL1* polymorphic SNP marker distribution among indigenous populations of North Eurasia and the world.

METHODS

Two original pools of DNA markers

The pool of data on the indigenous population of North Eurasia is represented by the populations of 97 ethnic groups, mostly of Russian origin, but also from the majority of post-Soviet states and Mongolia. DNA samples were provided by Biobank of North Eurasia. The sampling method was described earlier [16]: the samples comprised specimens obtained exclusively from unrelated individuals, whose grandparents belonged to the studied ethnic group. Specimens obtained from geographically and historically proximate populations, but from small samples, were merged into metapopulations [17]. The resulting pool of data on the indigenous population of North Eurasia included 28 metapopulations (n = 1980) with the average sample size of 140 chromosomes.

The pool of data on the indigenous population of other world's regions (n = 1657) comprises data on the genome-wide panels by Illumina reported in scientific literature and accumulated in the GG-Base [18]. The geographically and historically proximate groups were merged into metapopulations in order to provide a representative sample. The resulting pool of data on the world's indigenous population (n = 3637) included 34 metapopulations (64 populations of the maps).

Selection of polymorphic *LZTFL1* markers

Bioinformatics analysis of both data pools revealed 10 *LZTFL1* SNPs characteristically represented in the indigenous population of North Eurasia and other regions of the world. Of these two SNP markers are specified as strongly associated with COVID-19: *rs1058961* (3′-untranslated region) and *rs12493471* (intron 2) [1]. The other eight SNP markers were studied for the first time: *rs11130077* (intron 3), *rs17078408*, *rs1860264*, *rs2191031*, *rs2236938*, *rs6441929*, *rs7614952* and *rs9842595* (intron 2). There was no information about the *rs17713054* marker associated with severe COVID-19 [3] (*LZTFL1* enchancer) in the GG-Base, so that analysis was provided only for North Eurasia.

Evaluation of linkage disequilibrium (LD R2) for the studied 11 *LZTFL1* SNP markers is based on the North Eurasian data pool (appendix, Table 1). Tight linkage to *rs12493471* associated with severe COVID-19 [1], as well as to *rs2191031* and *rs11130077* was revealed for the *rs17713054* marker [3].

Statistical and cartographic analysis

Multivariate statistical analysis was performed using the frequencies of 10 *LZTFL1* SNP markers, for which information about the populations in North Eurasia and the world was available (appendix, Tables 2, 3): multidimensional scaling (MDS) was used for North Eurasia to perform analysis based on the frequencies of all 10 SNPs and 5 SNPs showing the least tight linkage with each other (appendix, Table 1); principal component analysis (PCA) and MDS were used for the world to perform analysis based on the frequencies of all 10 markers. The MDS algorithm involved calculations based on pairwise Nei's genetic distances (d).

The *LZTFL1* SNP marker frequencies were used to create maps of marker distribution in indigenous populations of North Eurasia (11 SNP markers) and the world (10 SNP markers) in the original GeneGeo software package. In maps and tables (Appendix, Tables 2, 3; Fig. C) each population was assigned a number making it easy to identify. The maps were created based on the digital grid model representing the matrix of interpolated marker frequency values at the regular grid nodes calculated by the weighted average interpolation with the weights decreasing with the cube of the distance based on all values at all the reference points which fell into the circle of radius R (R = 3000 km for North Eurasia, R = 4200 km for the world). Uniform color and numerical scales used all maps ensured the atlas unity.

RESULTS

Heterogeneity of North Eurasian populations based on the *LZTFL1* SNP markers

The data on certain SNP marker frequencies in the North Eurasian populations are provided in Table 2 of Appendix.

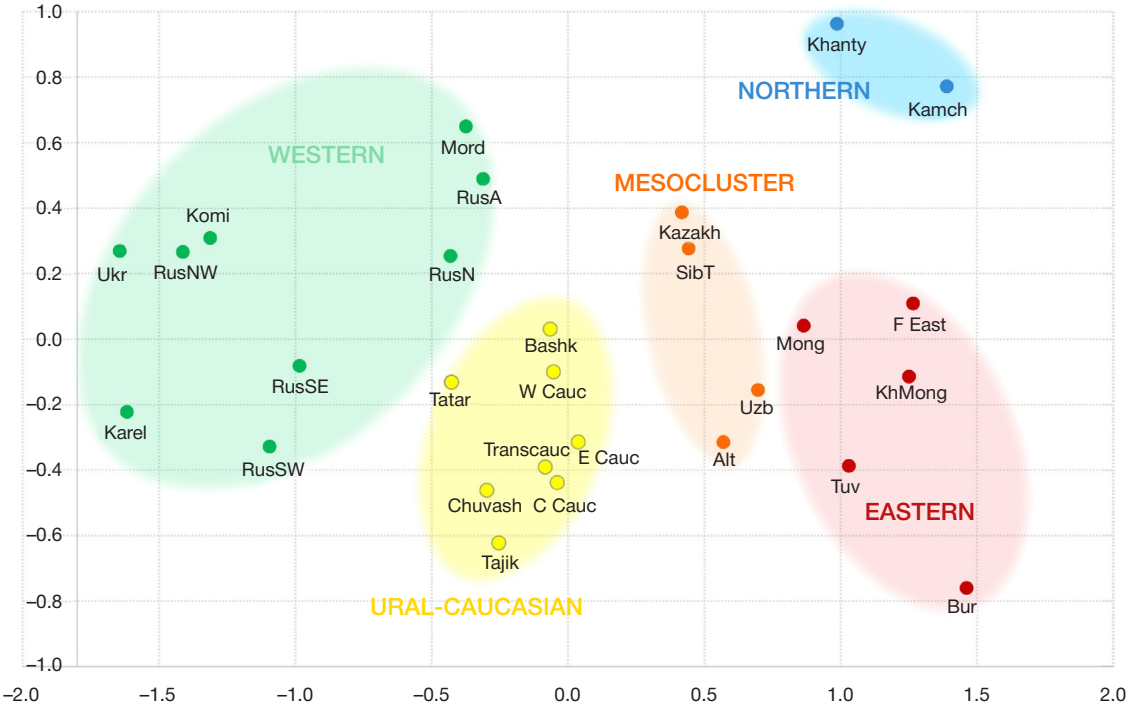

Fig. 1. Position of the populations of Russia and neighboring countries in the genetic space mapped by multidimensional scaling based on the data on 10 *LZTFL1* SNP markers. Note: multidimensional scaling plot, coefficient of alienation 0.1, stress SO = 0.09. Northern cluster is highlighted in blue, Western cluster in *green*, Ural-Caucasian cluster in *yellow*, Mesocluster in *orange*, Eastern cluster in *red*

However, it is necessary to recognize the patterns of variation in the entire marker set prior to analysis of the genetic landscape for each of the *LZTFL1* SNP markers (Fig. 1).

Their compliance with geographic variation along both axes, North–South and West–East, has turned out to be the most important feature of the relative position of populations from Russia and neighboring countries on the the MDS plot (Fig. 1).

The Northern cluster, which brings together the northernmost population of Western Siberia (Nenets, Mansi, Khanty) and the Far East (Itelmes, Koryaks, Chukchi), is opposed to all other clusters located strictly along the West–East axis. However, in each of the "southern" clusters the populations do not comply to their geographical position.

The Western cluster includes all Eastern Slavs (Belarusians, Russians, Ukranians) and Finnic-speaking peoples of Russia (Besermyans, Karelians, Komi, Mordvins, Udmurts).

The Ural-Caucasian cluster includes all peoples of the Caucasus, Transcaucasia and Tajikistan, Turks of the Urals (Bashkirs, Volga Tatars, Chuvash), and the Finnish-speaking Mari people included in the same metapopulation with the Chuvash.

Mesocluster visualizes gene pool transition from the Western to the Eastern cluster, bringing together those peoples of South Siberia and Eurasian Steppe, whose gene pools comprise both ancient Caucasoid stratum and potent later strata of Mongoloid populations (Altaians, Kazakhs, Karakalpaks, Kyrgyz, Nogais, Siberian Tatars, Turkmens, Uzbeks, Uyghurs, Khakas).

The Eastern cluster includes all the Mongolic-speaking peoples of North Eurasia (Buryats, Kalmyks, peoples of Mongolia), Tuvans (who were a part of Mongolia until the mid XX century) and small ethnic groups of the Far East (Nanais, Nivkhs, Ulchis, Evens).

Similar analysis performed based on the frequencies of 5 SNP markers showing the least tight linkage (LD $R^2 < 0.2$) reveals the same structure (Appendix, Fig. A), except the Volga Tatars' transition to the Western cluster.

In general, the overall trend of the *LZTFL1* SNP marker variation over North Eurasia is also in line with the West–East geographical vector and the Caucasoid–Mongoloid anthropological vector (Fig. 1).

Heterogeneity of the world's population based on the *LZTFL1* SNP markers

Global variation of distinct SNP markers is presented in Table 3 of Appendix, while the patterns for the set of SNP markers are provided in the PCA (Fig. 2) and MDS (Appendix, Fig. B) plots. Since the results and cluster structures obtained by both methods are similar, let us consider the PCA plot (Fig. 2).

What is striking is how high the variation of the populations of Russia and neighboring countries is on a global scale. These populations did not fit in any of five clusters within the space of principal components 1 and 2 of the world's gene pool (Fig. 2). The Western cluster of Russia is located in the upper part of the world's Indo-European cluster between the populations of North and Central Europe. The Ural-Caucasian cluster of Russia is in the opposite part of the Indo-European cluster, it is surrounded by the populations of Western Asia and South Europe. Three "Asian" clusters of North Eurasia, arranged in accordance with their origin, form their own North Asian cluster in the global genetic space: Russian Mesocluster gravitates to the world's Indo-European cluster, Eastern cluster of Russia to the world's South Asian Cluster, and Northern cluster of Russia (includes peoples of Chukotka and Kamchatka) is close to American cluster.

In general, the world's indigenous population is distributed over four clusters based on the parts of the world, however, it is adjusted to the anthropological features of the population (Fig. 2). Three clusters of indigenous populations includes only those from the place where it originates: Africa, Asia, or America.

However, Indo-European cluster juxtaposes geography and history of the populations. It includes Caucasoid populations of both Europe and Asia (India, Pakistan, Afghanistan, Middle East).

In other words, the main trend in variation of the whole *LZTFL1* SNP marker set across the world fits well with the

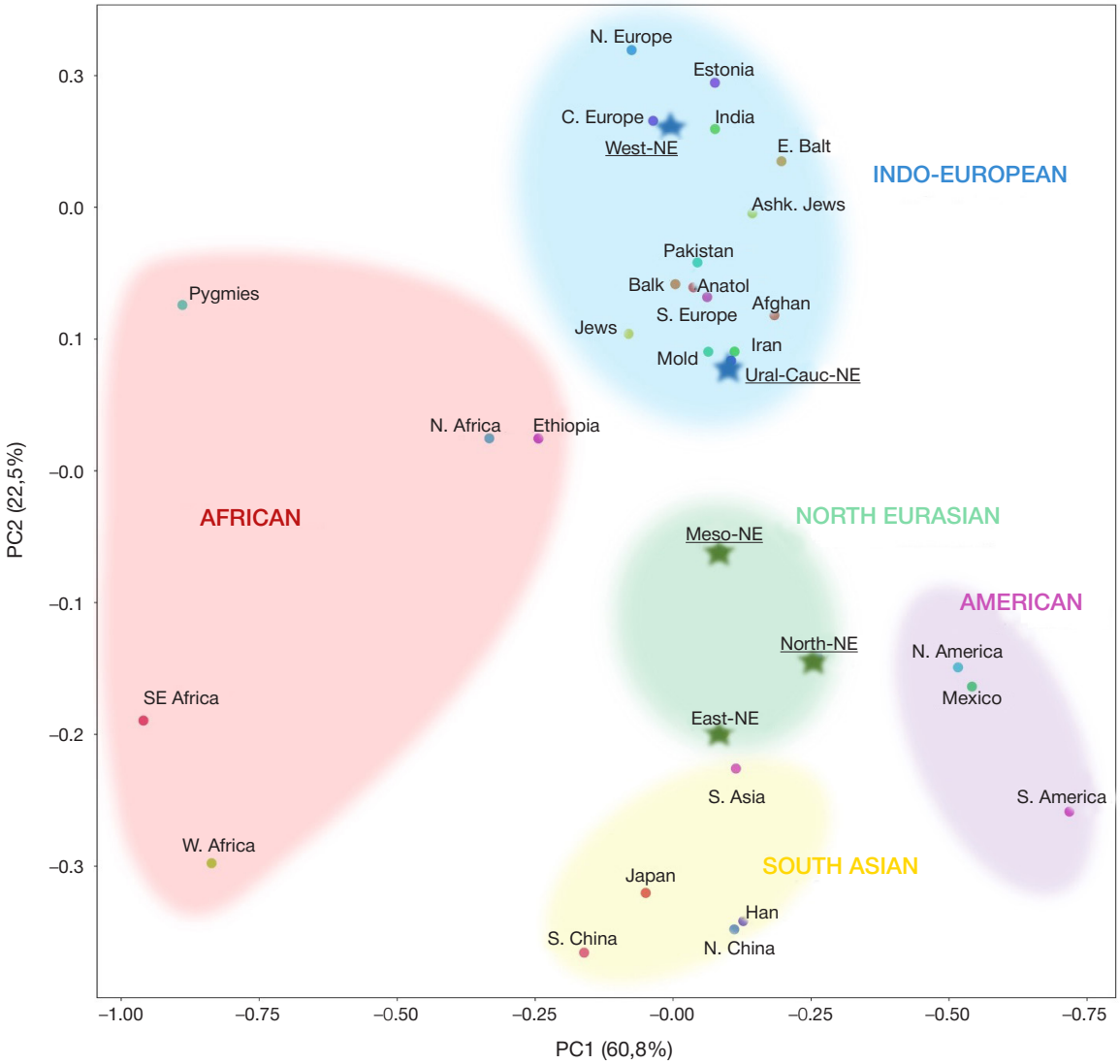

Fig. 2. Position of the world's indigenous populations in the space formed by principal components (PC) 1 and 2 based on variation in 10 *LZTFL1* SNP markers. Five clusters identified by multidimensional scaling of the populations of Russia and neighboring countries are marked with *asterisks*. Note: share of the described variance: PC 1 (61%), PC 2 (22%). Indo-European cluster is highlighted in *blue*, African cluster in *red*, North Asian cluster in *green*, South Asian cluster in *yellow*, American cluster in *violet*

world population anthropological division. Moreover, all four world's clusters are separated from each other. It's just huge genetic diversity of the peoples of Russia and neighboring countries that forms a bridge connecting three parts of the world: Europe, Asia and America.

Gene geography of 11 *LZTFL1* SNP markers in the populations of Russia and the world

The maps are not an illustration. They add two more dimensions of the geographic space to the tables to become an effective and powerful analysis tool. The ability to quickly capture a huge amount of information due to non-verbal representation is a specific advantage of this tool. We have constructed two variation maps per *LZTFL1* SNP marker (except *rs17713054*, since no information about the marker is available from global databases): for indigenous populations of North Eurasia and the world. Map comparison makes it possible both to reveal the global patterns and not to lose sight of the Russian genetic landscape. Each of 28 North Eurasian populations is marked with the number, allowing one to update both metapopulation name and the frequency of SNP marker in the population, in all maps (Appendix, Table 2). Information helps to navigate the world's metapopulations (Appendix, Table 3, Fig. C). The maps are arranged in the same order as in Table 1 of Appendix.

rs17713054. Spatial variation in the *rs17713054*(*A*) frequency across North Eurasia (Fig. 3A) is low, however, it fits the West–East trend: with its minimum in the Far East and its maximum in the European part, where high frequency values are found in the west, (Ukraine, 16%), northwest (Karelia, 14%), Urals region (16%), and Caucasus (14%). That is why the European part of North Eurasia can be considered the region showing the highest frequency of this SNP marker. The other region of increased frequency is emerging in Tajikistan ($p = 0.15$), which could indirectly confirm the earlier conclusions [3] about the high *rs17713054* frequency in the southern regions of Asia.

rs1058961. The *rs1058961*(*A*) genetic landscape in North Eurasia (Fig. 3B) showing higher average frequency (30%) reflects similar, but more smoothed clinal variation in the form of frequency decline from the west (43% in Karelians and Veps) to the northeast (20% in the Far East). The local minima are found in the north of Western Siberia (8%), while the local maxima are observed in Central Asia (37%).

Comparison with global variability (Fig. 3C) shows that the North Eurasian genetic landscape is almost fully integrated into the overall pattern of world population. The frequency decline

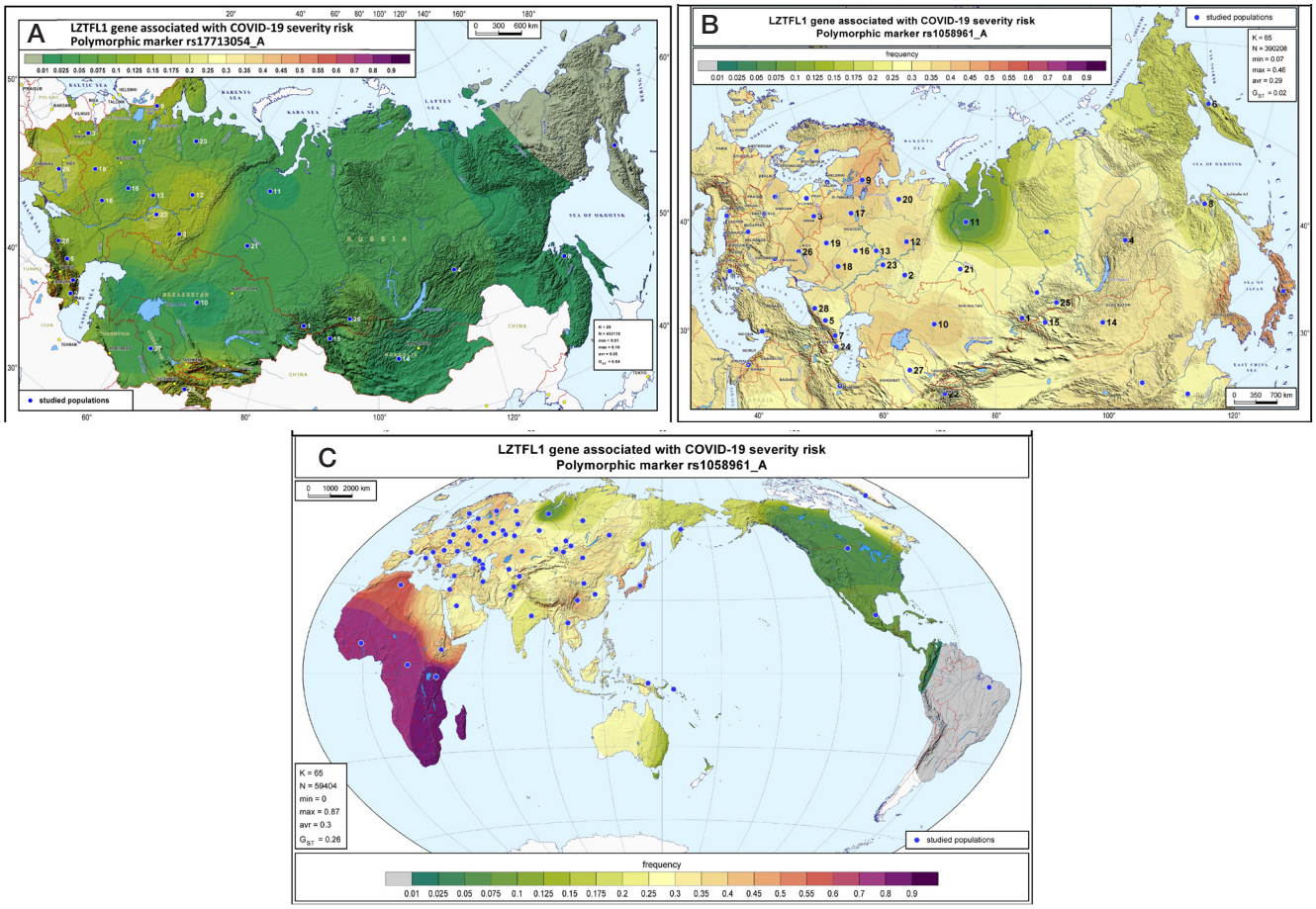

Fig. 3. Geographic variation in the frequencies of *LZTFL1* SNP markers in indigenous population: 3A — *rs17713054*(*A*) variation in the population of North Eurasia; 3B — *rs1058961*(*A*) variation in the population of North Eurasia; 3C — *rs1058961*(*A*) variation in the world's population. Note: The numbers of the populations on the map of North Eurasia correspond to that presented in Table 1 of Appendix, the numbers on the map of the world's population correspond to that provided in Table 2 and Fig. 1 of Appendix. No literature data on the indigenous population of Australia have been found, that is why the data on Oceania are interpolated on this region

found in the Far East smoothly turns into frequency decline in Alaska, decline in the indigenous population of North America, and decline to zero in South America. High frequencies found in Europe gradually turn into maximum frequencies (87%) found in Africa. Even the local maximum found in Japan (46%) (Fig. 3B) is reflected in the increasing frequency found in South-East Asia (42%).

rs12493471. The same West–East clinal variation across North Eurasia has been found in *rs12493471*(*A*) (Fig. 4A) linked with *rs17713054*(*A*) (Fig. 3A; Appendix, Table 1). However, the frequency drop gradient between west and east is much clearer: the maxima (≈ 50%) covering the European part of Russia barely move beyond the Urals. The frequency decline found in the east covers all regions and drops to zero in the Far East, Japan and China. The peak frequency found in Eastern Europe and Fennoscandia decreases in Western and Southern Europe.

The global variation map (Fig. 4B) shows that the peak frequency found in Eastern and Northern Europe is a world's maximum, from which the frequency decrease goes in all directions showing local maxima (40%) in Hindustan (the impact of which reaches Pamir) and Oceania.

rs11130077. In North Eurasia, the West–East clinal variation is also typical for *rs11130077*(*G*) (Fig. 4C). Minor differences are associated with the maximum shift to Fennoscandia (54%), however, the maxima do not move beyond the Urals and do not enter the Caucasus. Yet, the pattern observed in the Asian part is less clear than the patterns found in the previous maps. When the frequency is reduced to 16–17% in Siberia, there is a slight increase to 20% in the Far East and to 27% in South Siberia.

The *rs11130077*(*G*) variation in the world's population (Fig. 4D) is generally in line with the previous map. However, there is one exception: the world's maximum fall not on Europe (53%), but on the African population showing significant differences based on this marker (40% in North Africa to 82% in Pygmies of Africa).

rs7614952. Unlike the previous maps, the *rs7614952*(*A*) genetic landscape (Fig. 4E) in North Eurasia shows no clear pattern. Despite the maximum values are still found in the western part of the region and decrease toward the Far East, the frequency minimum falls on the northern part of West Siberia, and the local maxima are found in both Europe and Transbaikal.

In the context of global variation (Fig. 4F) we can see that the Baikal maximum is a high frequency part of the entire East Asian region. This is the most marked difference with the previous map: minimum *rs11130077*(*G*) frequencies are found in East Asia (Fig. 4G), but the frequencies of *rs7614952*(*A*) are high (Fig. 4F). In contrast, were see a rapid *rs7614952*(*A*) frequency drop instead of high frequencies in Hindustan and West Asia. However, the world's maximum is still in Africa, while the world's minimum is in America.

rs2191031. The *rs2191031*(*A*) variation in North Eurasia is unimpressive (Fig. 5A) due to alternation of local maxima and minima. Minimum is once again found in the Far East (10%), as well as in the western part of the region (18%) and in West Siberia (22%). High frequencies that stretch from Transbaikal

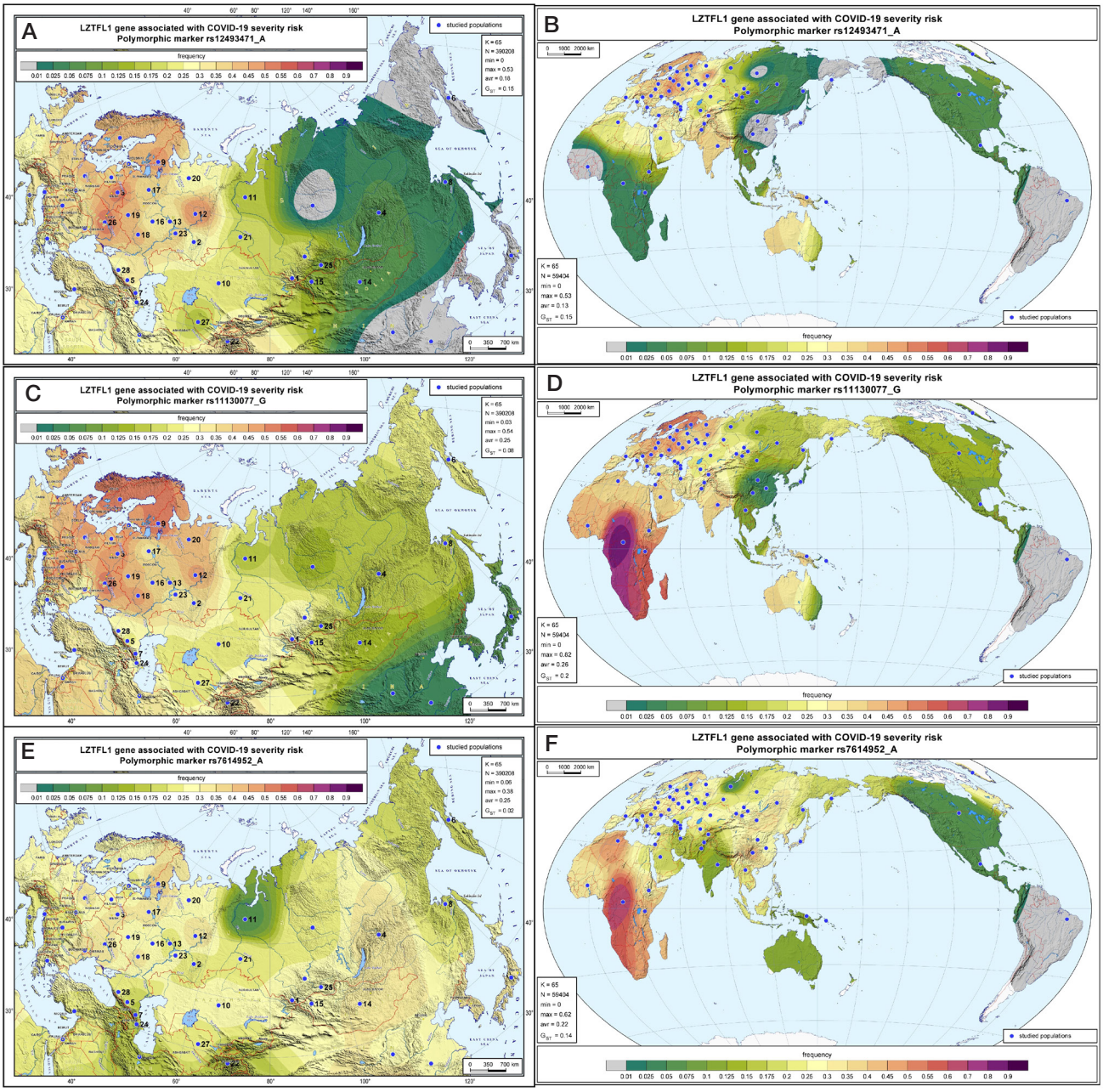

Fig. 4. Geographic variation in the frequencies of *LZTFL1* SNP markers in indigenous population: 4A — *s12493471*(*A*) variation in the population of North Eurasia; 4B — *rs12493471*(*A*) variation in the world's population; 4C — *rs11130077*(*G*) variation in the population of North Eurasia; 4D — *rs11130077*(*G*) variation in the world's population; 4E — *rs7614952*(*A*) variation in the population of North Eurasia; 4F — *rs7614952*(*A*) variation in the world's population. Note: The numbers of the populations on the map of North Eurasia correspond to that presented in Table 1 of Appendix, the numbers on the map of the world's population correspond to that provided in Table 2 and Fig. 1 of Appendix. No literature data on the indigenous population of Australia have been found, that is why the data on Oceania are interpolated on this region

(36%) through South Siberia (34%) to Central Asia (36%) are also typical for Povolzhye (37%) and the Caucasus (34%).

However, this pattern is fully integrated into the global variation landscape (Fig. 5B): the frequency decline observed in Western Europe (16%) turns into minimum in the West and East Africa (3%), while the frequency decline found in the Far East transforms into minima observed in the indigenous population of America (0–4%). The maxima found in the southern Siberia and Central Asia are a part of the high frequency region of Southeast and South Asia (35–40%), and the world's maximum is reached in Oceania (53%).

rs9842595. The *rs9842595*(*A*) genetic landscape is even less impressive (Fig. 5C): high frequencies are distributed through North Eurasia: Far East (19%), northern (17%) and southern (15%) parts of Europe, Ural region (12%) and the Caucasus (11%).

Similar is the global genetic landscape (Fig. 5D), covered almost entirely by the low frequency region, except sub-Saharan Africa, where the marker frequency rises to 33%.

rs1860264. In contrast, the *rs1860264*(*C*) marker frequency does not drop below 22% in North Eurasia (Fig. 5E) and generally fits the common West–East trend, although the local minima and maxima are scattered over various regions. Thus, the minimum frequency band stretches across the entire West Siberia towards Kazakhstan, but also shows up in the Ural region and Baltic States. High frequencies have been revealed not only in the western part of the region (50%), but also in Central Asia, South Siberia and the Baikal region (40–42%).

The global genetic landscape map (Fig. 5F) shows that Eurasia represents a gradual transition from the African maximum (97%) to American minimum (0%).

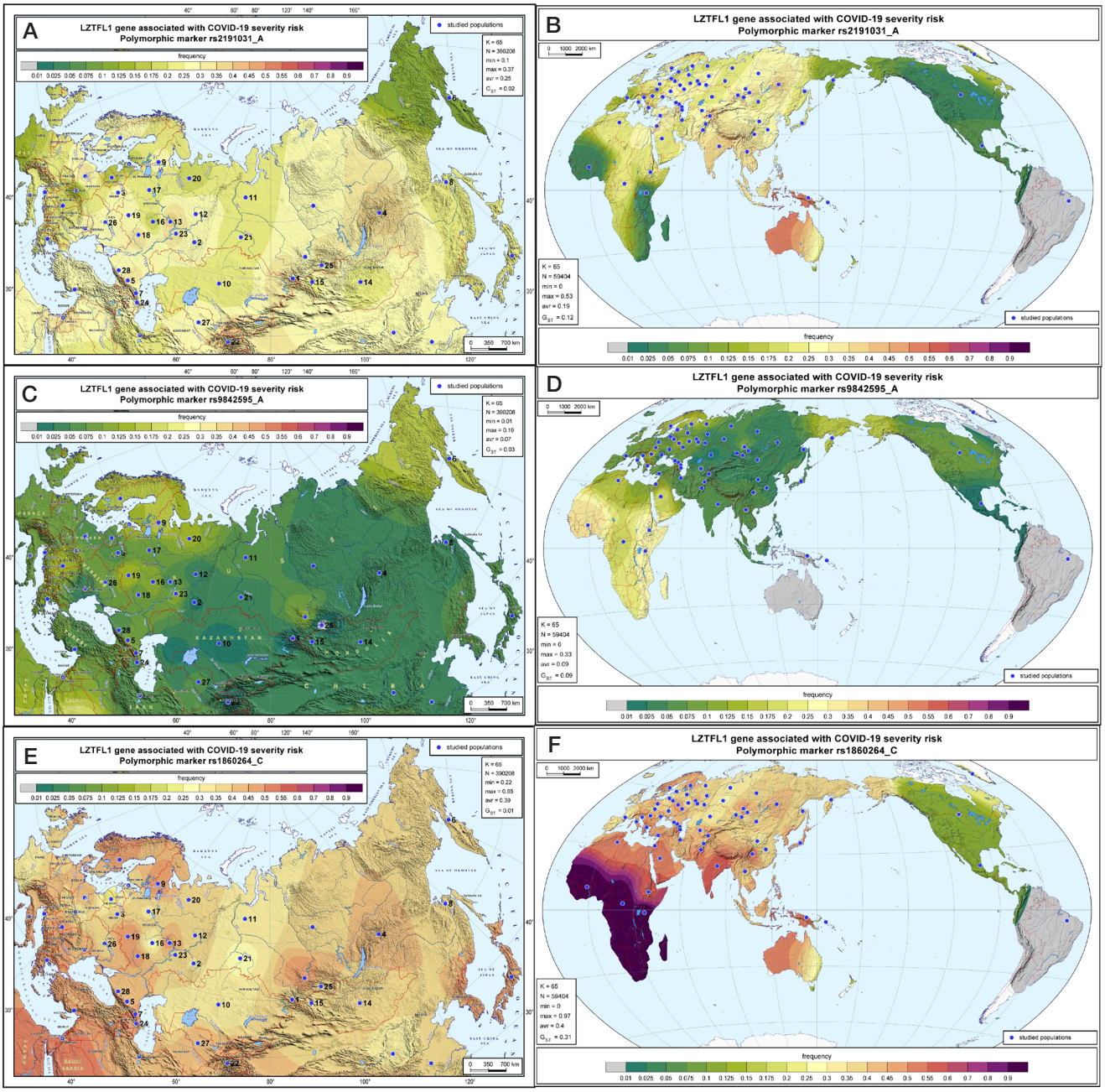

Fig. 5. Geographic variation in the frequencies of *LZTFL1* SNP markers in indigenous population: 5A — *rs2191031*(*A*) variation in the population of North Eurasia; 5B — *rs2191031*(*A*) variation in the world's population; 5C — *rs9842595*(*A*) variation in the population of North Eurasia; 5D — *rs9842595*(*A*) variation in the world's population; 5E — *rs1860264*(*C*) variation in the population of North Eurasia; 5F — *rs1860264*(*C*) variation in the world's population. Note: The numbers of the populations on the map of North Eurasia correspond to that presented in Table 1 of Appendix, the numbers on the map of the world's population correspond to that provided in Table 2 and Fig. 1 of Appendix. No literature data on the indigenous population of Australia have been found, that is why the data on Oceania are interpolated on this region

rs6441929. The *rs6441929*(*G*) variation pattern also fits the West–East trend (Fig. 6A), however, the highest values are found in the east with their maximum in Transbaikal and minimum frequencies in the Urals, Caucasus and Baltic States.

This landscape is fully integrated into the global one (Fig. 6B): the division of Eurasia into western, showing low values, and eastern, showing higher values, continues to the south down to the border between Hindustan and Southeast Asia. However, Eurasia globally contains no extrema. These are once again found in Africa (maxima) and America (minima).

rs2236938. The *rs2236938*(*A*) genetic landscape is similar to the previous one (Fig. 6C). The western zone of minima is the only clear one, while the zone showing a rapid frequency drop and gravitating towards the American world's minimum has emerged in the northeastern part of the eastern high frequency zone.

The *rs2236938*(*A*) global landscape, which is also similar to the previous one, appears to be more contrasting (Fig. 6D). In Africa the frequency rises to 53%, while Arabia, northern and northeastern Africa accede to the western Eurasian low frequency zone.

rs17078408. Finally, the marker is considered that is virtually abscent all over the world (Fig. 6E, F), except Africa, where the *rs17078408*(*C*) frequency reaches 48%.

DISCUSSION

Summarizing genetic landscapes of all the discussed *LZTFL1* markers associated with severe COVID-19, we shall refer to the main patterns: 1) the world's extrema are most typical for indigenous populations of Africa and America and are

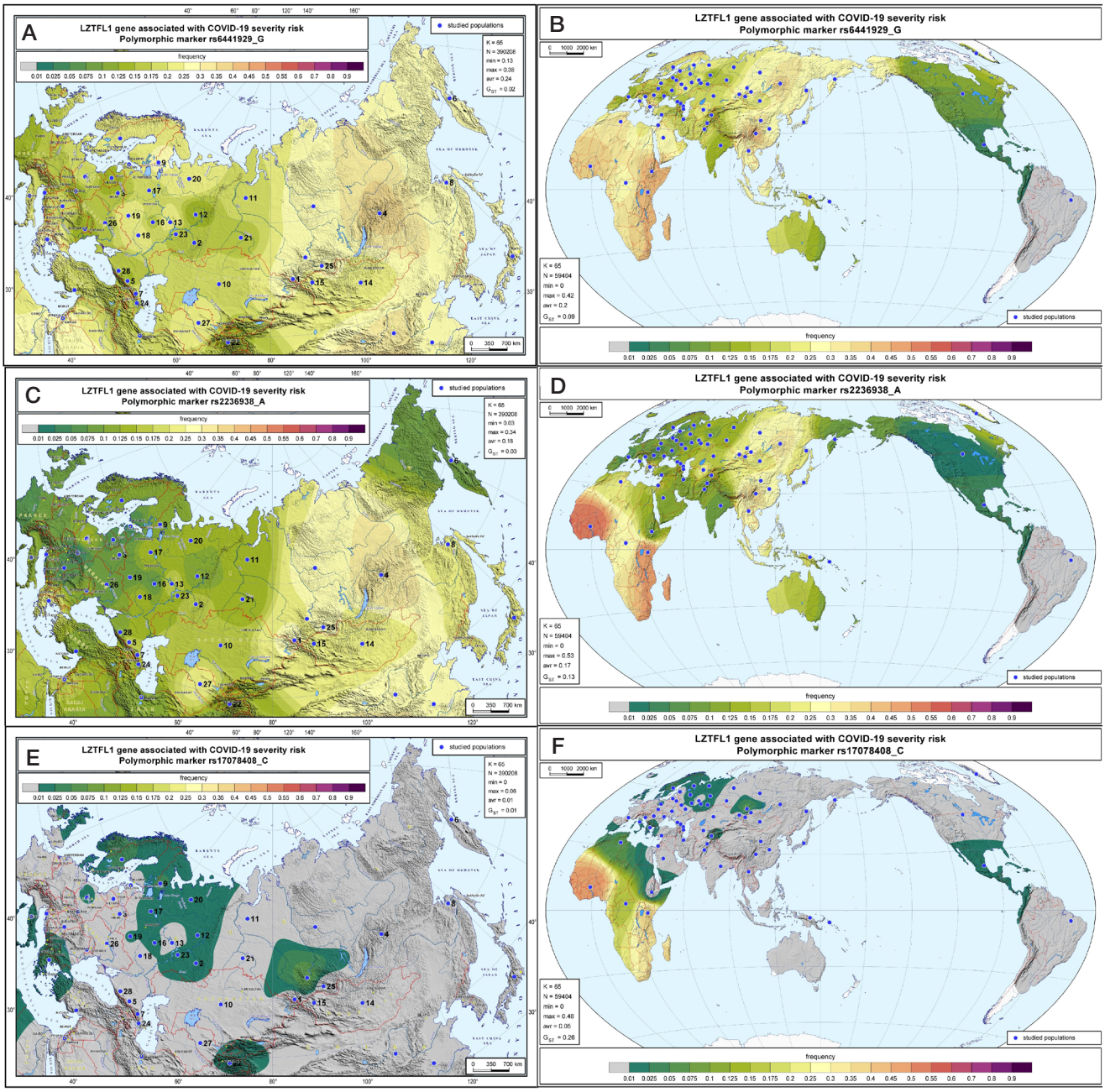

Fig. 6. Geographic variation in the frequencies of *LZTFL1* SNP markers in indigenous population: 6A — *rs6441929*(*G*) variation in the population of North Eurasia; 6B — *rs6441929*(*G*) variation in the world's population; 6C — *rs2236938*(*A*) variation in the population of North Eurasia; 6D — *rs2236938*(*A*) variation in the world's population; 6E — *rs17078408*(*C*) variation in the population of North Eurasia; 6F — *rs17078408*(*C*) variation in the world's population. Note: The numbers of the populations on the map of North Eurasia correspond to that presented in Table 1 of Appendix, the numbers on the map of the world's population correspond to that provided in Table 2 and Fig. 1 of Appendix. No literature data on the indigenous population of Australia have been found, that is why the data on Oceania are interpolated on this region

usually alternative; 2) Eurasia usually constitutes a transition zone between these two extrema, but shows its own patterns and enormous variation on a global scale; 3) the genetic landscape of Russia is seamlessly integrated into the Eurasian landscape.

These main patterns cannot always be relied on.

There are two exceptions to the first pattern. The minimum *rs12493471*(*A*) frequencies are clustered in Africa, America and East Asia, while the maximum values are clustered in Europe and Hindustan. Likewise, the *rs2191031*(*A*) minima are found in Africa and America; high frequencies are found across the entire Eurasia, however, the maximum is centered in Oceania. It should be noted that indigenous population of America always shows the common pattern: low frequencies of all the discussed markers drop to zero in South America.

There are also exceptions to the second pattern. For example, the world's maximum of *rs11130077*(*G*) is typical not only for Africa, but also for North Europe. Eurasia, like North America, represents a confinement of low *rs9842595*(*A*) frequencies. The *rs6441929*(*G*) Eurasian landscape is in sharp contrast to the term "transition zone", although it is an intermediate between two extrema: the maximum frequencies of the African continent share borders with the maximum frequencies found in Europe and Hindustan, while the world's lowest frequency is found in America which shares borders with high frequencies found in East Asia. The same Eurasian "patchwork" is observed for *rs2236938*(*A*). The division into west and east along the 80th meridian, separating Hindustan from Southeast Asia in the south and gradually blurring on its way to the Arctic Ocean, is most typical for Eurasia. The

following markers do not fit to this pattern: *rs1058961*(*A*) (high frequencies are observed in almost all Eurasia); *rs9842595*(*A*) and *rs17078408*(*C*) (the entire Eurasia is homogenous based on low frequencies of this marker); *rs2191031*(*A*) (frequency increases towards the south and turns into the Oceanic world's maximum); the pattern of *rs9842595*(*A*) is unclear. However, this pattern is clear in a half of the *LZTFL1* markers.

There are also exceptions to the third pattern. These are usually related to the local extrema found in the northern part of West Siberia, southern part of Middle Siberia and in the Ural region. In general, these patterns do not negate, but cast light on the overall integration of the Russian genetic landscape into the Eurasian landscape.

CONCUSIONS

1. The patterns typical for indigenous populations of Russia and the world were revealed in the spatial variation of the studied *LZTFL1* SNP markers associated with severe COVID-19. 2. The main pattern revealed in the North Eurasian genetic space is the compliance with geographic variation along two axes, North–South and West–East. This trend fits well with the Caucasoid–Mongoloid anthropological vector. 3. The main vector of global variation is fully in line with the world's population anthropological division. The clusters of indigenous populations of Africa, Asia and America include only the populations of their own parts of the world. The Indo-European cluster juxtaposes the population's geography and history, it includes Caucasoid populations of both Europe and Asia. 4. All four clusters of the world's indigenous population are separated from each other. It's just huge genetic diversity of the peoples of Russia and neighboring countries that forms a bridge connecting gene pools in three parts of the world: Europe, Asia and America. 5. A cartographic atlas for spatial variation of 11 *LZTFL1* markers in the populations of North Eurasia and the world showing the main patterns of the genetic landscapes has been created: a) the world's extrema fall on the indigenous populations of Africa and America; b) Eurasia constitutes a transition zone between these two extrema, but has its own patterns; c) the genetic landscape of Russia is seamlessly integrated into the Eurasian landscape.

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ANTIPHOSPHOLIPID ANTIBODIES AND OUTCOMES OF ASSISTED REPRODUCTIVE TECHNOLOGY PROGRAMS IN PATIENTS WITH A HISTORY OF COVID-19

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Investigation of the effect COVID-19 mediated with autoantibodies has on reproductive outcomes is important. This study aimed to evaluate the profile of antiphospholipid antibodies (aPL) and their association with the outcomes of assisted reproductive technology (ART) programs in patients with a history of COVID-19. The study included 240 patients: 105 of them did not have a history of COVID-19 (group 1) and 135 of them had a history of COVID-19 (group 2) with a mild course (subgroup 2a, *n* = 85) or moderate course (subgroup 2b, *n* = 50). With the help of ELISA, serum antibodies (M, G) to cardiolipin, β_2-glycoprotein-I, annexin V (AnV), phosphatidylethanolamine (PE), phosphatidylserine, and phosphatidylserine/prothrombin complex were determined. The evaluated parameters were the indices of oogenesis, embryogenesis, ART intervention outcomes. In group 2, growing levels of anti-AnV and anti-PE IgG were observed more often (in 28 (20.7%) and 8 (5.9%) patients) than in group 1 (in 10 (9.5%) and 1 (0.95%); $p = 0.02$ and $p = 0.045$, respectively). In subgroup 2b we registered a higher level of anti-PE IgG and a higher incidence of early miscarriages (in 6 (12%) patients) than in group 1 (in 3 (2.9%)) ($p = 0.024$). Weak inverse correlations were found between the level of anti-PE IgG and the number of oocytes and zygotes. The results of this study suggest a negative impact of aPL-mediated COVID-19 on the outcomes of ART programs and the course of early pregnancy.

Keywords: COVID-19, SARS-CoV-2, assisted reproductive technologies, ART intervention outcomes, antiphospholipid antibodies

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АНТИФОСФОЛИПИДНЫЕ АНТИТЕЛА И ИСХОДЫ ПРОГРАММ ВСПОМОГАТЕЛЬНЫХ РЕПРОДУКТИВНЫХ ТЕХНОЛОГИЙ У ПАЦИЕНТОК С COVID-19 В АНАМНЕЗЕ

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Исследование влияния COVID-19, опосредованного аутоантителами, на репродуктивные исходы имеет важное значение. Целью исследования было оценить профиль антифосфолипидных антител (аФЛ) и их связь с исходами программ вспомогательных репродуктивных технологий (ВРТ) у пациенток с COVID-19 в анамнезе. В исследование включили 240 пациенток: 105 из них не болели COVID-19 (группа 1), 135 перенесли COVID-19 (группа 2) в легкой (подгруппа 2а; *n* = 85) или среднетяжелой форме (подгруппа 2б; *n* = 50). С использованием ИФА определяли сывороточные антитела (M, G) к кардиолипину, β_2-гликопротеину-I, аннексину V (АнV), фосфатидилэтаноламину (ФЭ), фосфатидилсерину, комплексу фосфатидилсерин/протромбин. Оценивали показатели оогенеза, эмбриогенеза, исходы ВРТ. В группе 2 повышение уровня анти-АнV и анти-ФЭ IgG наблюдалось чаще (у 28 (20,7%) и 8 (5,9%) пациенток), чем в группе 1 (у 10 (9,5%) и 1 (0,95%); $p = 0,02$ и $p = 0,045$ соответственно). В подгруппе 2б был отмечен более высокий уровень анти-ФЭ IgG и более высокая частота ранних выкидышей (у 6 (12%) пациенток), чем в группе 1 (у 3 (2,9%)) ($p = 0,024$). Выявлены слабые обратные корреляционные связи между уровнем анти-ФЭ IgG и числом полученных ооцитов и зигот. Результаты исследования предполагают негативное влияние COVID-19, опосредованное аФЛ, на исходы программ ВРТ и течение беременности на ранних сроках.

Ключевые слова: COVID-19, SARS-CoV-2, вспомогательные репродуктивные технологии, исходы ВРТ, антифосфолипидные антитела

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The new coronavirus infection (COVID-19) pandemic, caused by the SARS-CoV-2 virus, affected over half a billion people throughout the world. In this connection, the task of studying the effect COVID-19 has on the female reproductive system is a very urgent one. Individual studies have reported discrepant data that suggest that COVID-19 increases the risk of pregnancy complications such as spontaneous miscarriages and preterm birth [1, 2]. The incidence of preterm birth in pregnant women with COVID-19 reaches 11.5–17% [3, 4], and the frequency of early spontaneous

miscarriages is 1.7 times higher compared to women not infected with SARS-CoV-2 [5].

The number of studies evaluating impact of COVID-19 and post-COVID syndrome on fertility and outcomes of assisted reproductive technology (ART) programs is insufficient. There is a paper [6] describing separate cases of development of premature ovarian failure (POF) in women after COVID-19, but the origin of the disorder is unclear. A 2022 meta-analysis did not register the impact of COVID-19 on the outcomes of ART programs [7]. However, the results of another study suggest the disease does have a negative effect and the number of oocytes received through an ART intervention depends on the time elapsed after COVID-19 [8].

It is known that infection caused by SARS-CoV-2 increases production of cytokines, such as IL6, TNFα, which can translate into a cytokine storm and adversely affect reproductive function through cytokine-driven suppression of the hypothalamic-pituitary-gonadal axis, development of systemic vasculitis and autoimmune lesions of the gonads [9, 10].

Autoimmune response is one of the possible mechanisms that can result in damage to the woman's reproductive system under the influence of the infection. It has been shown that people with a certain HLA haplotype are most susceptible to the development of autoimmune processes after COVID-19 [11]. Activation and maturation of autoreactive B lymphocytes from naïve B cells may follow an extrafollicular pathway lacking some tolerance checkpoints [12], which is proven by higher levels of extrafollicular B cells and plasma cells in patients with severe COVID-19.

A recent discovery of 28 human proteins containing domains homologous to SARS-CoV-2 peptides supports the hypothesis about the role of autoimmunity in a COVID-19 case. These peptides can act as autoantigens and trigger production of autoantibodies that is based on molecular mimicry [13]. COVID-19 patients have shown to have significant amounts of autoantibodies of various specificities, including antinuclear antibodies, antineutrophil cytoplasmic antibodies, antibodies to cardiolipin (CL), and β_2-glycoprotein-I (β_2-GP-I) [14]. Thyroid peroxidase antibodies have also been found in patients with post-COVID syndrome [15].

Vascular complications associated with COVID-19, such as deep vein thrombosis, stroke, disseminated intravascular coagulation, were initially linked to antiphospholipid antibodies (aPL) [16], primarily antibodies to CL and β_2-GP-I, classified as laboratory criteria for antiphospholipid syndrome (APS) [17]. However, it has been shown that aPL in COVID-19 patients are not detected frequently (8.9%), they may be transient and are not always associated with thrombosis [18]. At the same time, genetically predisposed patients may have a long-term persistence of pathogenic aPL, as well as an autoimmune disease developed [19].

In infertile women, autoimmune processes can affect fertilization, implantation, and placental development [20]. The pathogenetic mechanisms that link autoimmunity and infertility remain unclear. The association of aPL with infertility is an actively discussed matter currently. A 2016 scientific literature analysis has showed that, in most studies, infertility was associated with antibodies to β2-GP-I and "non-criteria" aPL, including phosphatidylethanolamine (PE) [21]. Five out of 18 studies reported detrimental effects aPL ("criteria" aPL, mainly) may have on the outcome of ART programs. Thus, the study of various autoantibodies in infertile patients that had COVID-19 and investigation of their impact on reproductive outcomes is of great scientific and practical importance.

This study aimed to evaluate the profile of antiphospholipid antibodies and their association with the outcomes of ART programs in patients with a history of COVID-19.

METHODS

The study was conducted at the V.I. Kulakov National Medical Research Center for Obstetrics, Gynecology and Perinatology of the Ministry of Health of Russia. The design of the study was prospective observational; it included a total of 240 infertile patients, who were stratified into two groups depending on the history of COVID-19: group 1 included patients who did not have COVID-19 (n = 105), group 2 included patients who had COVID-19 (n = 135) for 12 and less months prior to an ART intervention. Group 2 was further divided into two subgroups: subgroup 2a comprised of patients who had a mild form of COVID-19 (n = 85) and subgroup 2b where the form of the disease was moderate (n = 50).

The inclusion criteria were: age 18–40 years; normal ovarian reserve (anti-Müllerian hormone (AMH) ≥ 1.2 ng/mL, follicle-stimulating hormone (FSH) < 12 mIU/mL, antral follicle count (AF) ≥ 5 in both ovaries). The exclusion criteria were: vaccination against COVID-19; contraindications to ART, morbid obesity (BMI ≥ 40.0 kg/m^2); participation in donor programs, surrogacy programs; HIV infection. All patients were examined as prescribed in the 2021 Female Infertility clinical guidelines.

The patients' testimony of their COVID-19 cases were cross-checked with the Uniform State Health Information System and proven by determining antibodies to SARS-CoV-2 in the blood serum with the help of "Kit of reagents for detection of class G antibodies to SARS- CoV-2 spike protein by ELISA" (DS-ELISA-ANTI-SARS-CoV-2-G(S)) (Diagnostic Systems; Russia), which enables qualitative determination of antibodies to SARS-CoV-2 using enzyme-linked immunosorbent assay (ELISA). To evaluate the results of the analysis, we calculated positivity index (PI) using the formula: PI = sample OD / Cut-off, where sample OD is the optical density of the sample. With PI > 1.2 the result was considered positive, with PI < 0.8 — negative, with PI in the range from 0.8 to 1.2 — doubtful (uncertain).

The aPL study relied on ELISA and kits for quantitative determination of antibodies of classes M and G to CL, β_2-GP-I, annexin V (An V), phosphatidylserine (PS) (ORGENTEC Diagnostika GmbH; Germany). The reference values (RV) of blood antibody content were as follows: anti-CL IgM — < 7 MPL-U/ml, anti-CL IgG — < 10 GPL-U/ml; anti-β_2-GP-1 IgM and IgG — < 5 U/ml; anti-AnV IgM and IgG — < 5 U/ml; anti-PS IgM and IgG — < 10 U/ml. The quantity of antibodies (M, G) to PE and the phosphatidylserine/prothrombin complex (PS/PT) in blood serum was determined with the help of enzyme immunoassay kits (AESKU Diagnostics; Germany). The reference range for anti-PE antibodies and anti-PS/PT antibodies was < 12 U/mL.

Ovarian stimulation followed the protocol with gonadotropin-releasing hormone (ant-GnRH) antagonists, recombinant FSH (rFSH) preparations and/or preparations containing a luteinizing hormone (LH) component: human menopausal gonadotropin (hMG) or a combined preparation containing rFSH/rLH. On average, all patients who recovered from COVID-19 underwent ovarian stimulation six months (two to nine months) after the disease. The dose of gonadotropins was selected individually, taking into account age, medical history and ovarian reserve parameters. Ovulation was triggered with a single dose of chorionic gonadotropin (CG), 8,000–10,000 IU, or a combination of CG and a gonadotropin-releasing hormone (a-GnRH) agonist. Transvaginal follicle puncture (TFP) was

Table 1. Serum aPL level growth incidence in the study groups

Parameter	Group 1, *n* = 105	Group 2, *n* = 135		*p* value
		Subgroup 2a, *n* = 85	Subgroup 2б, *n* = 50	
anti-CL IgM	2 (1,9%)	4 (2,9%)		0,60*
		3 (5,2%)	1 (2,0%)	0,75**
anti-CL IgG	0 (0,0%)	0 (0,0%)		–
		0 (0,0%)	0 (0,0%)	–
anti-β_2-GP-1 IgM	3 (2,9%)	3 (2,2%)		0,75
		2 (2,4%)	1 (2,0%)	0,95
anti-β_2-GP-1 IgG	4 (3,8%)	7 (5,2%)		0,61
		3 (3,5%)	4 (8%)	0,43
anti-AnV IgM	9 (8,6%)	10 (7,4%)		0,74
		3 (3,5%)	7 (14%)	0,09
anti-AnV IgG	10 (9,5%)	28 (20,7%)		0,02
		20 (23,5%)	8 (16%)	0,03
anti-PS IgM	1 (0,9%)	0 (0,0%)		0,26
		0 (0,0%)	0 (0,0%)	0,52
anti-PS IgG	0 (0,0%)	0 (0,0%)		–
		0 (0,0%)	0 (0,0%)	–
anti-PE IgM	23 (21,9%)	23 (17,0%)		0,34
		11 (12,9%)	12 (24,0%)	0,28
anti-PE IgG	1 (0,95%)	8 (5,9%)		0,045
		5 (5,9%)	3 (6%)	0,13
anti-PS/PT IgM	3 (2,9%)	2 (1,5%)		0,46
		2 (2,3%)	0 (0,0%)	0,49
anti-PS/PT IgG	4 (3,8%)	4 (2,9%)		0,72
		4 (4,7%)	0 (0,0%)	0,32

Note: abs (%), χ^2 test; * — comparison of groups 1 and 2; ** — comparison of group 1 and subgroups 2a and 2b.

performed under ultrasound guidance 36 hours after triggering the ovulation.

In the aspirated follicular fluid we determined the resulting number of oocyte-cumulus complexes (OCC), and then, after oocyte denudation, assessed the degree of cell maturity. All mature oocytes were fertilized by in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI); after 16–18 h, if there were two symmetrical pronuclei in the cytoplasm, normal fertilization was recorded. The zygotes were then transferred to the COOK culture medium (COOK Medical; USA) for further cultivation. Morphological evaluation of the embryos was done after 120–122 hours (on the fifth day) of cultivation, relying on the Gardner blastocyst grading system (degree of blastocyst maturity, quality of trophectoderm and intracellular mass).

On the fifth day of cultivation one or two embryos were transferred (embryo transfer, ET) into the uterine cavity. Post-transfer support measures involved administration of micronized progesterone (600 mg daily) or dydrogesterone (30 mg daily). Pregnancy was identified by the level of β-CG in the blood serum 14 days after ET. A pregnancy test was considered to have returned positive when the level of β-CG exceeded 20 IU/L. Twenty-one days after ET, if ultrasound examination revealed a fetal egg in the uterine cavity, a clinical pregnancy was recorded.

Statistica 10 (StatSoft Inc.; USA) was used for statistical analysis. To evaluate qualitative data, we calculated proportions (%). The χ^2 test enabled comparison of categorical data and assessment of the differences between them. The analysis of quantitative data in comparison groups relied on the Kolmogorov–Smirnov test and graphical data evaluation, which allowed learning data distribution. For abnormal data distribution we applied nonparametric statistical methods: determination of median with an interquartile range ($Me(Q_{25}–Q_{75})$), Mann–Whitney test or Kruskal–Wallis test to compare data in unrelated populations. Spearman's rank correlation coefficient enabled evaluation of correlation dependence between variables. The differences between statistical values were considered significant at $p < 0.05$.

RESULTS

The mean age of the patients was 34 years (34 (30–36) years in group 1 and 34 (31–37) years in group 2 ($p = 0.39$). In group 1, 24 (22.8%) women were in the late reproductive age (35 years and older), and in group 2 there were 36 (26.7%) such patients ($p = 0.49$). Group 2 patients had a significantly higher serum level of specific antiviral antibodies compared to patients in group 1: the mean PI values in groups 1 and 2 were 0.16 ± 0.13 and 6.8 ± 4.1 ($p < 0.001$), respectively. Patients who had COVID-19 had greater body mass index than those who did not have the disease (22.9 (20.4–25.5) kg/m^2 and 21.9 (20.0–24.5) kg/m^2; $p = 0.009$); the former also exhibited higher incidence of ENT diseases (24 (17.8%) and 9 (8.6%); $p = 0.04$) and allergic conditions (23 (17%) and 9 (8.6%); $p = 0.055$).

As for the gynecological diseases, the most common diagnosis for women of groups 1 and 2 was endometriosis (in 25 (23.8%) and 38 (28.1%) patients, respectively; $p = 0.45$) and uterine myoma (in 21 (20%) and 33 (24.4%) patients; $p = 0.41$), followed by chronic salpingo-oophoritis (in 13 (12.4%) and 15 (11.1%) patients; $p = 0.76$), chronic endometritis (in 11

Table 2. The average level of blood serum aPL, both study groups

Parameter	Group 1, $n = 105$	Group 2, $n = 135$		p value
		Subgroup 2а, $n = 85$	Subgroup 2б, $n = 50$	
anti-CL IgM, MPL-U/ml	3,03 (1,94–4,05)	2,52 (1,59–3,91)		0,14*
		2,43 (1,59–1,04)	3,04 (1,50–3,83)	0,30**
anti-CL IgG, GPL-U/ml	1,87 (1,41–2,56)	2,10 (1,59–3,01)		0,06*
		2,01 (1,50–2,86)	2,14 (1,68–3,31)	0,08**
anti-β_2-GP-1 IgM, U/ml	1,51 (0,81– 2,43)	1,41 (0,98–2,17)		0,87
		1,41 (0,95–2,38)	1,42 (1,06–2,07)	0,96
anti-β_2-GP-1 IgG, U/ml	2,98 (2,12– 3,59)	2,37 (1,21–3,26)		0,001
		2,09 (0,94–2,30)	2,52 (1,94–3,54)	0,001
anti-AnV IgM, U/ml	2,52 (1,76–3,52)	2,22 (1,23–3,22)		0,03
		2,22 (1,26–3,18)	2,25 (1,45–3,35)	0,07
anti-AnV IgG, U/ml	2,88 (2,26–3,94)	3,37 (2,13–4,65)		0,19
		3,37 (2,20–4,95)	3,23 (2,00–4,58)	0,25
anti-PS IgM, U/ml	2,53 (1,56–3,76)	2,54 (1,25–4,01)		0,64
		2,31 (1,11–3,78)	2,95 (1,50–4,14)	0,33
anti-PS IgG, U/ml	1,76 (1,41–2,15)	1,69 (1,32–2,16)		0,76
		1,67 (1,27–2,10)	1,73 (1,51–2,23)	0,32
anti-PE IgM, U/ml	12,23 (8,70–16,98)	11,85 (8,67–15,58)		0,54
		11,93 (7,78–15,2)	11,61 (9,09–7,89)	0,51
anti-PE IgG, U/ml	3,63 (2,96–4,80)	4,78 (3,27–6,82)		0,001
		4,39 (3,20–5,89)	5,20 (3,74–7,93)	0,001
anti-PS/PT IgM, U/ml	1,72 (1,10–3,28)	2,39 (1,47–3,58)		0,01
		2,39 (1,53–3,73)	2,33 (1,28–3,55)	0,03
anti-PS/PT IgG, U/ml	4,24 (3,00–5,36)	3,38 (2,28–5,31)		0,03
		3,43 (2,32–5,48)	3,02 (2,24–5,11)	0,06

Note: Me (Q_{25}–Q_{75}), тест Манна–Уитни или Краскела–Уоллиса; * — при сравнении групп 1 и 2, ** — при сравнении группы 1 и подгрупп 2а и 2б.

(10, 5%) and 8 (5.6%) patients; $p = 0.19$) and PCOS (in 9 (8.6%) and 6 (4.4%) patients; $p = 0.19$). The share of patients with primary infertility was similar in both groups: 61 (58.1%) and 79 (58.5%) women; $p = 0.95$. There were no differences in terms of the average number of pregnancies, deliveries and miscarriages between the groups.

According to the results of the serum aPL content study, patients of both groups frequently had the level of antibodies growing above the RV. Cumulatively, 66 (62.9%) women in group 1 had at least one aPL of class M or G increased, and for group 2 this value was 81 (60%) ($p = 0.65$). A simultaneous increase in the level of several aPL was observed in 18 (17.1%) and 33 (24.4%) patients of groups 1 and 2, respectively ($p = 0.17$). Fifty-five (52.4%) patients of group 1 exhibited an increased level of antibodies to PE most frequently, whereas in group 2 there were 66 (48.9%) such patients; a less frequent observation — increased level of antibodies to An V, which was registered in 20 (19%) and 37 (27.4%) women, respectively ($p < 0.001$). The level of other antibodies (different specificity) has been registered growing up not as often as that to An V: antibodies to β2-GP-I in 6 (5.7%) and 8 (5.9%) cases, respectively, to the PS/PT complex — in 6 (5, 7%) and 6 (4.4%) cases, to CL — in 2 (1.9%) and 4 (3.0%) women, to PS — only a single case (0.95%) in group 1 ($p < 0.001$). A comparative analysis of incidence of increased level of either aPL class M or aPL class G revealed significant differences only in IgG antibodies to PE and AnV (Table 1); the incidence was significantly higher in group 2 than in group 1.

Assessment of serum aPL in patients of groups 1 and 2 has shown that, for all the studied antibodies, the mean levels with interquartile ranges were within the RV. Herewith, the average level of anti-β_2-GP-I IgG, anti-AnV IgM and anti-PS/PT IgG was higher in group 1, while the average level of anti-PE IgG and anti-PS/PT IgM, on the contrary, was higher in group 2 (table 2). A slightly higher level of IgG antibodies to AnV was noted in group 2 compared to group 1.

The indicators of spermatogenesis, oogenesis and early embryogenesis in groups 1 and 2 did not differ significantly. A factor that should be noted here is the high incidence of pathospermia in partners of the participants: it was registered in 72 (68.6%) partners of group 1 patients and 86 (63.7%) partners of group 2 patients ($p = 0.43$). The average number of

mature oocytes recorded in groups 1 and 2 was, respectively, 8 (5–11) and 7 (4–11) (p = 0.26), the level of fertilization was 0.90 (0.75– 1.0) and 0.92 (0.80–1.0) (p = 0.39), the number of zygotes — 6 (4–9) and 6 (4–10) (p = 0.37), the number blastocysts — 3 (1–5; similar in both groups), the number of excellent quality blastocysts — 1 (0–3) and 1 (0–2) (p = 0.19), the number of poor quality blastocysts — 1 (0–2) in both groups.

Investigation of the relationship between the level of aPL and the parameters of oogenesis and embryogenesis revealed a significant weak negative correlation between the level of anti-PE IgG antibodies and the number of mature oocytes (r = –0.129, p = 0.045) and zygotes (r = –0.132, p = 0.041). In other cases, correlations between other aPL and parameters of oogenesis and embryogenesis were not significant. It should be noted here that the mean level of anti-PE IgG antibodies was significantly higher in group 2 patients and especially in subgroup 2b (patients who had moderate COVID-19). In addition, the share of patients with elevated levels of anti-PE IgG antibodies in group 2 was significantly higher than in group 1.

Evaluation of the outcomes of ART programs has shown that the frequency of pregnancy occurrence (FPO) and childbirth did not differ significantly between the groups: biochemical pregnancy was observed in 32 (30.5%) and 39 (28.9%) women (p = 0, 79), clinical pregnancy in 30 (28.6%) and 39 (28.9%) (p = 0.96), childbirth in 27 (25.7%) and 30 (22.2%) (p = 0.53). It is important to note that in subgroup 2b (with a history of moderate COVID-19) the incidence of spontaneous abortions up to 12 weeks of pregnancy was higher than in group 1: 6 (12%) and 3 (2.9%) women, respectively (p = 0.024). Compared to group 1, the OR for spontaneous miscarriage in subgroup 2b was 2.1 (95% CI = 1.1; 19.4) (p = 0.036). A fact to be underscored here is that emphasized that 3 out of 6 miscarrying patients had elevated serum levels of IgM antibodies to PE and AnV.

DISCUSSION

Some scientific studies demonstrate the absence of a negative impact of COVID-19 on the outcomes of ART programs [2, 7, 22]. Similar statements can be found in papers comparing the results of ART programs in pre-covid and covid periods [23, 24]. However, there are publications that discuss the negative effect the infection may have on the reproductive function of women [6, 25, 26]. Post-COVID syndrome may manifest in reproductive dysfunction, including, inter alia, impaired fertility and miscarriage [6]. The scientific literature describes individual cases when young fertile patients became infertile or suffered from POF after recovery from COVID-19 [25, 26]. The severity of COVID-19 may have an impact on the incidence of complications of pregnancy [27].

It is assumed that COVID-19 can trigger autoimmune processes in genetically predisposed people [28]. Researchers describe development of such autoimmune pathologies as immune thrombocytopenic purpura, Guillain–Barré syndrome, and Miller–Fischer syndrome in patients that recovered from COVID-19 [16]. Those who contracted SARS-CoV-2 exhibit a significant prevalence of autoantibodies of different specificity: antinuclear autoantibodies — in 57.5%, antineutrophil cytoplasmic antibodies — in 25%, anti-CL antibodies — in 12.5%, anti-β_2-GP-I antibodies — in 5% [14].

This study shows a high overall rate of detection of aPL classes M and G in infertile patients that did or did not have COVID-19. The most common observation is an elevated level of "non-criteria" antibodies to PE and AnV, significantly less frequently registered — elevated level of classic aPL, namely, antibobies to CL and β_2-GP-I, which are currently considered to be laboratory criteria for APS. These results are consistent with our earlier findings, which demonstrate that in patients with COVID-19, antibodies to CL and β_2-GP-I are detected less frequently compared to antibodies to AnV [29].

An individual analysis of the frequency of identification of aPL classes M and G has shown that, compared to women who did not have a history of COVID-19, patients who recovered from the disease less than 12 months before ART intervention have higher levels of IgG antibodies to PE and AnV significantly more often. It is known that COVID-19 patients are predisposed to pro-inflammatory and hypercoagulable conditions and run a higher risk of thrombotic events and impaired coagulation. Activation of vascular endothelial cells, externalization of phospholipids and higher content of natural anticoagulants that bind phospholipids on the surface of the damaged endothelium, in particular AnV, can induce increased formation of autoantibodies to AnV [29].

In addition, patients with a history of COVID-19 had a higher average level of IgG antibodies to PE than those who did not have COVID-19 in anamnesis; moreover, the former had that level increasing more often than the latter. Antibodies to PE are known to form in infectious and inflammatory processes of a viral or bacterial nature and can persist for a long time in the human body. This is due to the fact that PE is the main lipid component of microbial membranes and is also abundant in human cell membranes with asymmetric distribution. Production of pro-inflammatory mediators and damage to cells and tissues in the context of infectious and inflammatory processes contribute to the exposure of PE in cell membranes and formation of autoantibodies to PE.

The revealed negative correlation between the level of IgG antibodies to PE and the number of mature oocytes and zygotes may indirectly indicate the possible negative effect some aPL persisting after COVID-19 have on the outcomes of ART programs.

The present study showed that the parameters of oogenesis, embryogenesis, FPO and frequency of childbirth did not differ significantly in the groups of patients who did and did not have a history of COVID-19, which is consistent with the data reported by other researchers and mentioned above. However, patients who had COVID-19 in its moderate form were more prone to spontaneous abortion up to 12th week of gestation; for them, the risk of miscarriage was 2.1 times higher compared with patients who did not have COVID-19. Since half of the patients that miscarried early were found to have antibodies to PE and AnV, it can be assumed that autoimmune mechanisms are involved in the development of these pregnancy complications. As is known, antibodies to PE and AnV are associated with recurrent miscarriage; they are significant risk factors for this complication of pregnancy.

CONCLUSIONS

Infertile patients often have antiphospholipid antibodies detected in them. Patients who had a moderate form of COVID-19 before entering the ART program have elevated serum levels of IgG antibodies to PE and AnV more often than patients who did not have COVID-19. It is assumed that COVID-19 may have a negative impact on reproductive outcomes, decrease the number of mature oocytes, zygotes and, as a result, embryos in the context of ART interventions, as well as increase the risk of spontaneous abortion in the early stages of pregnancy, which may be associated with the involvement of autoimmune mechanisms mediated by the formation of aPL, in particular antibodies to PE and AnV.

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CHANGES IN SEXUAL FUNCTIONING IN WOMEN OF REPRODUCTIVE AGE WITH INFERTILITY AND DIMINISHED OVARIAN RESERVE

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Androgens play a key role in the physiology of the female body and the reproductive system. Androgen receptor expression in the various tissues points to the importance of androgens in the regulation of the female sexual and social functioning. The study aimed to evaluate sexual functioning in women with infertility and diminished ovarian reserve (DOR) using the Female Sexual Functioning Index questionnaire (FSFI). A cross-sectional study of 496 patients with infertility and DOR assessed the degree of sexual dysfunction in conjunction with the changes in the androgenic profiles as indicated by the androstenedione levels in the blood serum. Women with infertility and DOR were significantly more likely to report changes in sexual functioning, including a decrease in libido and in the quality and frequency of sexual relations. Furthermore, patients with normal androstenedione levels generally significantly outscored patients with decreased androstenedione levels (average questionnaire scores 21.2 ± 7.2 and 15.17 ± 3.0 respectively), indicating a lesser degree of sexual dysfunction in the former group; on the other hand, the latter group reported increased pain and decreased attraction, arousal, lubrication, orgasm, and satisfaction. Hormonal profile changes in patients with DOR, including decreased androstenedione levels, significantly impact sexual functioning, and their detection in clinical practice will allow to objectify complaints at an earlier state in order to assess the severity of sexual dysfunction and determine further personalized management tactics.

Keywords: androgens, androgen deficiency, reproductive age, infertility, diminished ovarian reserve, ART, questionnaire, sexual dysfunction, FSFI

Author contribution: Gavisova AA — analysis, manuscript writing, final approval; Gavisova AA, Dolgushina NV — study design; Stenyaeva NN, Gardanova ZR, Nazarenko TA, Dolgushina NV — review.

Compliance with ethical standards: the study was approved by the ethical review board at the Kulakov National Medical Research Center for Obstetrics, Gynecology and Perinatology (protocol № 2 of 07 February 2019).

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ВЛИЯНИЕ ГОРМОНАЛЬНОГО СТАТУСА НА СЕКСУАЛЬНУЮ АКТИВНОСТЬ ЖЕНЩИН РЕПРОДУКТИВНОГО ВОЗРАСТА С БЕСПЛОДИЕМ

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Андрогены играют одну из ключевых ролей в физиологии женского организма и репродуктивной системы. Экспрессия андрогенных рецепторов в различных тканях свидетельствует о важной роли андрогенов в регуляции сексуального и социального функционирования женщин. Целью исследования было оценить сексуальное функционирование у женщин с бесплодием и сниженным овариальным резервом (СОР) по результатам опросника «Индекс женской сексуальной функции» (Female Sexual Function Index, FSFI). В одномоментном исследовании у 496 пациенток с бесплодием и СОР провели оценку нарушений сексуального функционирования и их взаимосвязи с изменениями андрогенного профиля, основанного на концентрации андростендиона в сыворотке крови. Женщины с бесплодием при СОР статистически значимо чаще отмечали изменение сексуального функционирования, в том числе снижение способности и частоты сексуальных отношений, либидо. Для женщин с бесплодием и измененным овариальным резервом с нормальным уровнем андростендиона характерен суммарно больший общий балл (21,2 ± 7,2), что говорит о меньшей степени выраженности нарушений сексуального функционирования по сравнению с группой со сниженным уровнем андрогенов, средний балл в которой статистически значимо ниже (15,17 ± 3,0). Кроме того, наблюдаются снижение влечения, возбуждения, удовлетворения, оргазма, любрикации и увеличение болевых ощущений. Изменение гормонального профиля у пациенток с СОР и снижением уровня андрогенов вносит значимый вклад в сексуальное функционирование, и его выявление в клинической практике позволит на более раннем этапе провести объективизацию жалоб и оценить выраженность сексуальных нарушений у молодых женщин с бесплодием с целью определения дальнейшей персонифицированной тактики ведения.

Ключевые слова: андрогены, андрогенный дефицит, репродуктивный возраст, бесплодие, сниженный овариальный резерв, ВРТ, опросник, сексуальное функционирование, FSFI

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Many modern women prioritize career development and then partner choice in their lifestyle; the decision to have a child is made later in life, by the age of 35–38 years, as dictated by changes in levels of reproductively significant hormones before the occurrence of disturbances in the menstrual rhythm. This hormonal profile change clinically manifests by the age of 40 years. It is associated with changes in folliculogenesis and contributes to the structure of infertility [1], which is defined by the inability to achieve pregnancy after at least a year of regular sexual intercourse. In women with diminished ovarian reserve (DOR), changes in sexual functioning are associated with the anti-Müllerian hormone (AMH) levels.

Androgens, which form certain behavioral features including sexual functioning, most prominently so in males, play a major role in folliculogenesis. The production of androgens under the influence of the luteinizing hormone (LH) stimulates the appearance of follicle-stimulating hormone (FSH) receptors on granulosa cells. The process of differentiation and maturation

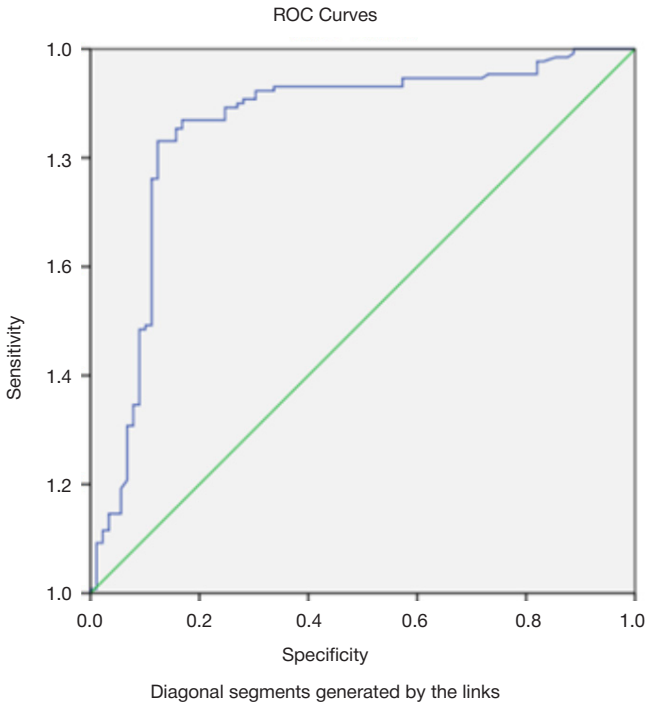

Fig. ROC curve for androstenedione

of follicles, especially in FSH-dependent early antral stages, likewise involves androgens [2].

Expression of androgen receptors (AR) in the various tissues, such as the central nervous system and reproductive organs, indicates the importance of androgens in the regulation of the female sexual and social functioning. Gonadectomy leads to changes in hippocampal neuroplasticity, depressive behavior, and suppression of sexual motivation.

The brain is among the main targets for sex hormones, both for estradiol as a central modulator of sexual desire, and for testosterone, the so-called "king" of sexuality. Androgens are instrumental in maintaining all phases of sexual functioning and significantly impact many neural and behavioral functions through both genomic and non-genomic effects [3].

ARs in the medial preoptic region of the hypothalamus, the region of the brain that regulates sexual behavior, have a high affinity for dihydrotestosterone (DHT). The neuroendocrine mechanisms underlying the effect of testosterone on female sexual functioning are studied through direct stimulation of ARs or through conversion of androgens to estrogens and subsequent binding to estrogen receptors [4].

Androstenedione (A) is a precursor of androgens (including testosterone) and estrogens in the body. Due to the difficulties in determining the level of androgens and the absence of lower reference values in women of reproductive age, focus on androstenedione levels is an acceptable approach to androgenic profile analysis in patients with DOR.

The Female Sexual Function Index questionnaire (FSFI) is commonly used to assess sexual functioning in women, and allows assessment of the severity of sexual dysfunction caused by low androgen levels [5, 6].

All of the above led to the current study, which aimed to assess the severity of sexual dysfunction in patients with infertility and DOR with psychodiagnostic testing.

METHODS

The cross-sectional study with a parallel group design enrolled 496 female patients aged 18–42 years with confirmed infertility and DOR. Exclusion criteria: surgical menopause (bilateral oophorectomy or hysterectomy); hormone-producing tumors; body mass index (BMI) ≤ 18 kg/m^2 or ≥ 30 kg/m^2; HIV and other immunodeficiency conditions; rheumatic diseases; immunomodulatory therapy; glucocorticoids, combined oral contraceptives, other hormonal drugs; intrauterine contraception; oncological diseases; pregnancy and lactation. Medical history collection accounted for current age, age of menopause onset in the mother, and BMI.

The hormonal profile was determined by immunochemiluminescent analysis (ICLA). ROC analysis was performed for all hormones, with AUC > 0.6 as the primary criterion for prognostic significance. The sensitivity and specificity of the models were calculated using logistic regression for use as prognostic factors for low androgen levels. The concentration of androstenedione turned out to be the most prognostically significant parameter (cut off point = 7.034) (see Fig.).

Patients were assigned into groups depending on A levels based on the results of the hormone analysis: group 1 — 256 women with reduced A levels (≤ 7.0 nmol/l), group 2 — 240 women with normal A levels (> 7.0 nmol/l).

Female sexual functioning was assessed in six domains: attraction, arousal, lubrication, orgasm, satisfaction, and pain. Domain-specific scores were calculated for each domain by multiplying the initial score (0(1)–5) by a factor, with the sum of scores in all six domains as the total score. Each patient answered the questionnaire twice with an interval of one month in order to confirm the result. The threshold value for healthy women with no sexual dysfunction is 29 points; a low total score corresponds to more pronounced sexual dysfunction [5, 6].

Statistical data processing was performed using the statistical software package Statistica V10 (StatSoft Inc.; USA). The type of quantitative data distribution was determined using the Kolmogorov-Smirnov test and graphical analysis of the data before conducting comparative analysis. For normal data distribution, the mean value (M) with standard deviation (SD) was calculated; the differences between the two groups were assessed with t-test. For data distribution patterns differing from normal, the median and interquartile range were determined; the differences between the two groups were assessed with the Mann–Whitney U test. The differences were considered statistically significant at $p < 0.05$.

RESULTS

The study participants were women of reproductive age with infertility and DOR who applied to achieve pregnancy in the IVF/ICSI program at the Kulakov National Medical Research Center for Obstetrics, Gynecology and Perinatology, Moscow, Russia. The average age of the women was 37.3 ± 2.4 years. All patients had a regular menstrual cycle with the average duration of 27.4 ± 2.1 days. All patients demonstrated a high level of intelligence and social responsibility.

30% of the patients had a history of surgical interventions (appendectomy or diagnostic laparoscopy, including linked with tubal-peritoneal factor). One in three patients noted a history of ureaplasma parvum while providing information on past inflammatory and infectious diseases of the genital organs. On average, more than three programs of assisted reproductive technologies (ART) (IVF and cryoprotocol) terminating with a negative result at the stage of hormonal verification of pregnancy were noted in medical history. The duration of infertility was 6.8 ± 5.9 years; 349/496 (70.6%) patients had primary infertility. 2.7 ± 2.2 ART cycles were performed. A history of pregnancies ending in childbirth was noted in 49/147 (33.3%) patients;

Table 1. Hormonal characteristics of patients in the study group

n = 496	A ≤ 7.0 nmol/l (*n* = 256)	A > 7 nmol/l (*n* = 240)	*p*
LH, mIU/ml	5.3 (2.8–8.3)	5.1 (3.2–9.8)	0.112
FSH, mIU/ml	7.9 (6.3–9.5)	6.7 (4.6–8.8)	0.1009
T_{tot}, nmol/l	0.7 (0.5; 1.2)	1.1 (0.6; 1.7)	0.0586
T_{fr}, pg/ml	1.7 (0.6; 2.1)	2.1 (0.7; 3.3)	0.4696
DHT, pg/ml	294 (152; 554)	269 (201.5; 422.0)	0.0768
DHEAS, μmol/l	4.5 (2.4; 6.8)	4.6 (2.6; 7.7)	0.2019
17-OP, nmol/l*	2.2 (1.0; 4.3)	3.15 (2.3; 3.8)	0.0174
A, nmol/l*	4.5 (2.5; 7.0)	8.9 (7.1; 11.6)	< 0.001
AMH, ng/ml*	1.0 (0.4; 4.2)	2.7 (1.3; 5.6)	< 0.001

Note: data presented as median (lower and upper quartile); * — Mann–Whitney test, $p < 0.05$; LH — luteinizing hormone; FSH — follicle-stimulating hormone; T_{tot} — total testosterone; T_{fr} — free testosterone; DHT — dihydrotestosterone; DHEAS - dehydroepiandrosterone sulfate; 17-OP — 17-hydroxyprogesterone; A — androstenedione; AMH — anti-Müllerian hormone.

medical abortion for various indications in 28/147 (19%) patients; pregnancy terminations in the early stages (6–7 weeks gestation) in 52/147 (35.4%) patients. BMI in patients was 24.6 ± 5.4 kg/m^2.

The hormonal profile analysis revealed statistically significant differences in 17-OP, A, and AMH levels between the study groups (Table 1).

All patients were tested to assess sexual functioning. Women with infertility and DOR at A > 7.0 nmol/l generally scored higher (21.2 ± 7.2), indicating a lesser degree of sexual dysfunction. In the group with A ≤ 7.0 nmol/l, the average score was significantly lower (15.17 ± 3.0). Domain-specific scores for attraction, arousal, lubrication, orgasm, and satisfaction were higher in the former study group; the domain-specific score for pain was higher in the latter study group.

The questionnaire data links laboratory-confirmed A < 7,0 nmol/l to sexual dysfunction in women with infertility and DOR (Table 2).

DISCUSSION

The study aimed to determine the severity and prevalence of sexual dysfunction in patients of reproductive age with infertility and DOR.

The tissues with androgen receptors, including those in the nervous system, are the first to respond to changes in androgen levels, which corresponds to the areas of sexual dysfunction identified in our study, such as decrease in libido, in amount and quality of orgasms, and in satisfaction with intercourse [7].

Female sexual dysfunction is multifaceted; its occurence depends on the age and ethnicity of the woman. In a study of 1749 patients aged 18-59 years, sexual dysfunction was found more often in women (43%) than in men (31%) [8]. With the rhythm of the menstrual cycle remaining the same, the number of antral follicles and AMH decreases, and estrogen levels depend on the presence of a leading follicle. Hypoestrogenism occurs close to the menopausal period, while a reduced level of androgens, in particular dehydroepiandrosterone (DHEA), manifests long before the onset of menopause. Numerous studies show a reduced level of DHEA (which has weak androgenic activity and is involved in sex hormone synthesis) observed in women as young as 30 years [9].

Reduced synthesis of sex steroids in the blood serum, as an additional component to the sexual dysfunction, further aggravates the psychological discomfort of a woman [10]. We show that sexual dysfunction is more common in patients with low androgen levels. In particular, problems in all domains covered by the FSFI questionnaire, including decrease in arousal and libido, are significantly more frequent. These results are consistent with those of another study where low androgen levels were found in post-menopausal women and linked with a decrease in libido with a relative preservation of physiological mechanisms of sexual function [11].

In our study, decrease in sexual functioning correlated with low levels of AMH, 17-OP, and androsteneodine. Similarly, a study of women with hypoactive sexual dysfunction revealed significantly lower levels of two testosterone precursors, A and DHEA-C. Other authors likewise associate the existing relationship between low androgen levels and sexual desire with DHEAS levels [12].

Use of estrogens as part of menopausal hormone therapy (MHT) in postmenopausal women is associated with slight improvement in sexual functioning without effect on libido [13], while in a randomized placebo-controlled study during combined MHT with testosterone medications in postmenopausal women, resulting normalization of levels

Table 2. Comparative assessment of the women's sexual functioning according to the FSFI questionnaire data (M ± SD)

	A ≤ 7.0 nmol/l (*n* = 256)	A > 7 nmol/l (*n* = 240)	*p*
Attraction*	2.58 ± 0.95	3.45 ± 1.55	0.003
Arousal*	2.15 ± 0.8	2.98 ± 1.39	0.003
Lubrication*	2.88 ± 0.49	3.96 ± 0.87	< 0.001
Orgasm*	2.4 ± 0.8	3.48 ± 1.36	< 0.001
Satisfaction*	2.68 ± 0.98	3.54 ± 1.43	0.002
Pain*	2.45 ± 1.0	3.79 ± 1.26	< 0.001
Total*	15.17 ± 3.0	21.2 ± 7.2	< 0.001

Note: * — Student's *t*-test, $p < 0,05$.

of total and free testosterone was linked to improved sexual functioning, including sexual satisfaction, general well-being, and mood [14], which also confirms the role of androgens in maintaining sexual functioning.

Decreased levels of androgens contribute significantly (possibly decisively) to impaired sexual functioning. It is not always possible to determine the early changes in the androgenic profile using only laboratory data, since clinical diagnosis is generally retrospective, when the changes are already pronounced [15]. The probable decrease in the concentration of sexual receptors may also narrow the window of therapeutic possibilities.

CONCLUSIONS

Psychodiagnostic testing conducted in women of reproductive age with infertility and DOR using the FSFI questionnaire allows to identify changes in sexual functioning and can be considered a method for early assessment of androgen levels. The impaired sexual functioning in women with infertility and DOR is associated with a physiological decrease in the levels of androstenedione as a testosterone and estradiol precursor and is pathogenetically explained by a decrease in the functional activity of the ovaries and low androgen levels. Further research into timely personalized treatment strategies is required.

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COMPARATIVE ASSESSMENT OF RMI-IV AND RMI-V IN PREOPERATIVE PREDICTION OF OVARIAN TUMOR TYPE IN PREGNANT WOMEN

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Prediction of ovarian tumor type in pregnant women is of great clinical significance, however, it is vastly difficult. In the last 5–10 years gynecologists were suggested to use RMI (Risk of Malignancy Index) in non-pregnant women, however the value of the test for obstetric practice has yet to be established. The study was aimed to determine RMI-IV and RMI-V during preoperative non-invasive prediction of ovarian tumor type in pregnant women. Retrospective and prospective clinical and laboratory data of 114 pregnant women aged 20–38 were collected. Among them 15 patients had malignant ovarian tumors (MOTs), 28 had borderline ovarian tumors (BOTs), and 71 had benign ovarian tumors. Color Doppler and pulsed wave Doppler ultrasound was performed. The levels of CA-125 were defined by enzyme immunoassay. Models IV, V were used to assess the risk of ovarian cancer. A moderate non-significant increase in blood levels of CA-125 compared to patients with benign ovarian tumors and BOTs was found in pregnant women with MOTs. Patients with BOTs and MOTs showed higher RMI-IV and RMI-V values compared to the group of pregnant women with benign ovarian tumors. Extreme values are required to guarantee the differences in the diagnosis of tumors (RMI-IV > 3500 indicate the presence of MOTs, the values below 100 indicate no malignancy). Similar RMI-V values are 1500 and 60. However, in most cases, availability of RMI-IV and RMI-V is insufficient for decision making, and a comprehensive approach has to be used. Thus, it is difficult to define ovarian mass type in pregnant women using RMI only. Comprehensive clinical assessment with the use of imaging methods is required for preoperative prediction of ovarian mass type in pregnant women, along with the use of prognostic models taking into account the majority of descriptive “morphological” tumor characteristics.

Keywords: benign and malignant ovarian tumors, ultrasound, RMI

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Compliance with ethical standards: the study was approved by the Ethics Committee of Pirogov Russian National Research Medical University (protocol № 176 of 25 June 2018). All patients submitted the informed consent to study participation.

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СРАВНИТЕЛЬНАЯ ОЦЕНКА RMI-IV И RMI-V ПРИ ДООПЕРАЦИОННОМ ПРОГНОЗИРОВАНИИ ХАРАКТЕРА ОПУХОЛЕЙ ЯИЧНИКОВ У БЕРЕМЕННЫХ

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Прогнозирование характера опухолей яичников у беременных имеет важное клиническое значение, но значительно затруднено. В последние 5–10 лет у небеременных в гинекологии предложено использовать RMI (Risk of malignancy index), однако в акушерской практике ценность этого исследования еще не установлена. Целью исследования было определить RMI-IV и RMI-V при дооперационном неинвазивном прогнозировании характера опухолей яичников у беременных. Ретро и проспективно отобраны данные клинико-лабораторного обследования 114 беременных 20–38 лет, из которых 15 пациенток имели злокачественные опухоли яичников (ЗОЯ), 28 пациенток — пограничные опухоли яичников (ПОЯ) и 71 пациентка — доброкачественные опухоли яичников (ДОЯ). Проводили ультразвуковое исследование (УЗИ) с использованием цветовой допплерографии и импульсноволновой допплерометрии. Определяли концентрацию СА-125 с помощью иммуноферментного анализа. Для оценки риска рака яичников использовали модификации IV, V. В крови беременных с ЗОЯ было выявлено умеренное статистически не значимое повышение СА-125 по сравнению с таковыми значениями у пациенток с ДОЯ и ПОЯ. По сравнению с группой беременных с ДОЯ, пациентки с ПОЯ и ЗОЯ демонстрировали повышенный уровень RMI-IV и RMI-V. Для гарантированного различия в диагностике опухолей необходимы краевые значения (RMI-IV — выше 3500 указывают на ЗОЯ, ниже 100 — на отсутствие злокачественного процесса). Для RMI-V аналогичными значениями являются 1500 и 60. Однако для принятия решения в большинстве наблюдений наличия только показателей RMI-IV и RMI-V было недостаточно и требовалось использовать комплексный подход. Таким образом, определить характер новообразований яичников у беременных трудно, если использовать только индексы RMI. Для дооперационного прогнозирования характера опухолей яичников у беременных требуется комплексное клиническое обследование с использованием визуализационных методов, применение моделей прогнозирования, учитывающих большое количество описательных «морфологических» характеристик опухолей.

Ключевые слова: доброкачественные и злокачественные опухоли яичников, ультразвуковое исследование, RMI

Вклад авторов: авторы внесли равнозначный вклад в проведение исследования и подготовку статьи, прочли и одобрили ее финальную версию перед публикацией

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Differential diagnosis of ovarian neoplasms in pregnancy, which are often treated using pelvic surgery, is of great scientific and practical interest. The increase in the standardized incidence rate of this disorder in Russia over the last 5 years is 4% [1]. There is a trend toward the increase in the number of cases of ovarian tumors (OT) associated with pregnancy [2]. However, 87.0% of young patients have benign tumors. The properly selected strategy (conservative treatment or surgery) is especially important in pregnant women. Strategy is selected based on the ovarian mass type defined by specific assessment methods [3–5]. As is known, there might be false negative and false positive results when forecasting. In obstetric practice, both type I (false positive, diagnosed disorder) and type II (false negative, the disorder is undiagnosed when there is a disorder) errors are unacceptable in patients with ovarian tumors. Type I errors may result in unreasonable surgical treatment of the tumor during early embryogenesis and placentation, as well as in possible loss of a desired pregnancy; without treatment type II errors may result in rapid disease progression.

The improvement of non-invasive ovarian tumor diagnosis methods based on the combination of clinical data, tumor marker levels and imaging techniques resulted in the proposed use of the Risk of Malignancy Index (RMI) for differential diagnosis of ovarian tumors [6]. It is believed that RMI are more informative in differential diagnosis performed in patients with ovarian masses compared to other criteria for malignant ovarian tumors [7]. To date, five RMI models are available. However, the use of those in gynecological practice is not always reliable due to lack of universality and ambiguous results, and there is too little obstetric research.

That is why the study was aimed to determine RMI-IV and RMI-V during preoperative non-invasive prediction of ovarian tumor type in pregnant women.

METHODS

In 2000–2021, retrospective and prospective clinical and laboratory data of 114 pregnant women aged 20–38 (median age 31.3 years) were collected. Among them 15 patients had malignant ovarian tumors (MOTs), 28 patients had borderline ovarian tumors (BOTs), and 71 patients had benign ovarian masses (Table 1). Inclusion criteria: early pregnancy (1^{st}–2^{nd} trimester) and ovarian tumor. Exclusion criteria: no pregnancy.

The study was performed in the Center of Family Planning and Reproduction of the Moscow Healthcare Department.

Transabdominal and transvaginal color Doppler and pulsed wave Doppler ultrasound was performed with the Voluson E8 scanner (General Electric; USA).

CA-125 levels were assessed by enzyme immunoassay using the test system by the manufacturer (Siemens; Germany).

The combined ovarian cancer (OC) risk assessment indicators (Risk of Malignancy Index, RMI) IV, V, were used to assess the risk of OC [8–11].

Statistical processing was performed in the SPSS 15.0 software package (INC; USA). Descriptive statistics and Spearman's rank correlation were used. The search for significant differences between samples was carried out using the Wilcoxon–Mann–Whitney test. The differences were considered significant at $p < 0.01$. Extreme values (series limits) limiting the variational series were presented as ($V_{max} \div V_{min}$). Descriptive statistics for quantitative variables was presented as M (SD) (mean and standard deviation). The method involving estimation of the area under the sensitivity vs. specificity curve (AUC) were used for ROC curve analysis. This method makes it possible not only to assess the diagnostic accuracy, but also to decide on a balance between type I and type II errors.

RESULTS

Epithelial tumors, mostly of benign histologic type, were diagnosed in the majority of pregnant women (n = 71). The share of malignant epithelial neoplasms (6 out of 15) was almost the same as that of germ cell tumors (7 out of 15). Serous tumors prevailed among BOTs (25 out of 28). Stage 1A BOTs and MOTs were identified in the majority of cases, however stage IIIC tumors were found in 7 patients. Distribution of OT histologic types in pregnant women is provided in Table 1.

The data obtained showed that all the surveyed pregnant women were through their fertile life period at almost the same age. Furthermore, a slight non-significant increase in blood levels of CA-125 compared to patients with benign ovarian masses and BOTs was found in pregnant women with MOTs (Table 2).

Patients with BOTs and MOTs showed higher RMI-IV and RMI-V scores compared to the group of pregnant women with benign ovarian tumors.

The case-by-case analysis showed that the highest RMI-IV (540–2888) and RMI-V (200–1444) values in the group of pregnant women with benign ovarian tumors were registered in patients with bilateral deep ovarian endometriosis. In patients with teratomas, RMI-IV varied between 8.3–256, while RMI-V was between 8.3–128. In patients with serous papillary cystadenomas, RMI-IV was within the range of 18.7–397, while RMI-V was between 18.7–198.6. The lowest RMI-IV (13–144) and RMI-V (13–72) values were found in patients with mucinous cystadenomas. In patients with other benign ovarian tumors, RMI-IV and RMI-V varied between the specified values. Both low and high RMI-IV (8.9–1776) and RMI-V (15–888) values were observed in patients with serous borderline tumors. Therefore, it was impossible to differentiate benign from malignant ovarian tumors based on the studied malignancy indices. In patients with MODs, RMI-IV varied between 123.2 and 5631, while RMI-V was within the range of 61.6–2815.6, these values exceeded that of patients with benign ovarian tumors in every third case. Significant differences were revealed in patients with stage IIIC MODs: RMI-IV in patient with yolk sac tumor was 1016, while RMI-V was 508; in patient with serous adenocarcinoma RMI-IV reached 5631.2, and RMI-V was 2815.6. Diagnostic algorithms also revealed high indicators in patients with stage IA dysgerminomas: RMI-IV between 1152–2168, RMI-V within the range of 567–1084.

Distribution of observation rates over the RMI-IV and RMI-V log10 scales shows intervals between the index values, in which the results of three groups overlap (Fig. 1). These values were 100 and 1200 for RMI-IV, while the values for RMI-V were 70 and 1200. It was impossible to define which group this or that tumor found in pregnant woman belonged to using RMI in this interval, that is why it was necessary to use additional examination methods in patients with RMI values between 70–1200. It was impossible to differentiate benign from borderline ovarian tumors based on the diagnostic models (RMI-IV, V).

DISCUSSION

The results of studies focused on using multimodality diagnostic systems indicate the dubious value of RMI for preoperative prediction of the ovarian mass type. This is primarily due to diverse morphological types and phenotypic heterogeneity of ovarian neoplasms. Therefore, one should not use RMI-IV and RMI-V only to predict the ovarian mass type, since prediction is essential for selection of management strategy in patients with OTs found during early pregnancy.

Table 1. Distribution of ovarian tumor histological types in pregnant women

1. Malignant ovarian tumors	
Epithelial	
Serous adenocarcinoma	3
Mucinous adenocarcinoma	2
Clear cell adenocarcinoma	1
Germ cell tumors	
Immature teratoma	2
Dysgerminoma	3
Yolc sac tumor	1
Mixed germ cell tumor	1
Metastatic tumors	2
Total:	15
2. Borderline ovarian tumors	
Serous adenocarcinoma	24
Mucinous adenocarcinoma	1
Endometrioid tumors	1
Serous and mucinous tumors	2
Total:	28
3. Benign ovarian tumors	
Struma ovarii	1
Serous cystadenoma	19
Mucinous cystadenoma	9
Thecoma/fibroma	6
Mature teratoma	16
Endometrioma	20
Total:	71

When trying to predict tumor types in pregnant women, we had to consider two adverse outcomes, thus bringing together type I and type II errors. In other words, the error was critically important for the final decision. This led to the fact that we could not sacrifice specificity (share of false-negatives) in favor of sensitivity and had to use diagnostic methods with maximum accuracy of 90% or more.

When trying to define the cutoff RMI-IV and RMI-V values, we managed to reach the accuracy of determining MOTs of 81%, however, such results were associated with low sensitivity (the number of diagnosed MOTs was lower that the true number of MOTs). With the cutoff values shift towards sensitivity we managed to achieve accuracy of 93% at the expense of the diagnosis of non-malignant tumors: in these

Table 2. Descriptive statistics of CA-125 and Risk of Malignancy indices (RMI-IV and RMI-V) in pregnant women with benign, borderline and malignant ovarian tumors

		Benign tumors	Borderline tumors	Malignant tumors
Number		71	28	15
CA-125	Value	69.7 ± 49.9	63.3 ± 52	95 ± 53.1
	Minimum	5.29	4.8	15.4
	Maximum	361	361	703
	Standard deviation	73.6	75.9	170.9
	Median	40	34.6	73.5
RMI-IV	Value	275.6 ± 56.1	283.3 ± 74.4	1031 ± 357.2
	Minimum	8.3	8.9	123
	Maximum	2888	1776	5631
	Standard deviation	505	393.7	1383.3
	Median	88	104	518.4
RMI-V	Value	125.0 ± 25.2	183.7 ± 42.0	515 ± 178.6
	Minimum	5.29	9.7	61
	Maximum	1444	888	2815
	Standard deviation	227	222.4	691.6
	Median	54	70	259.2

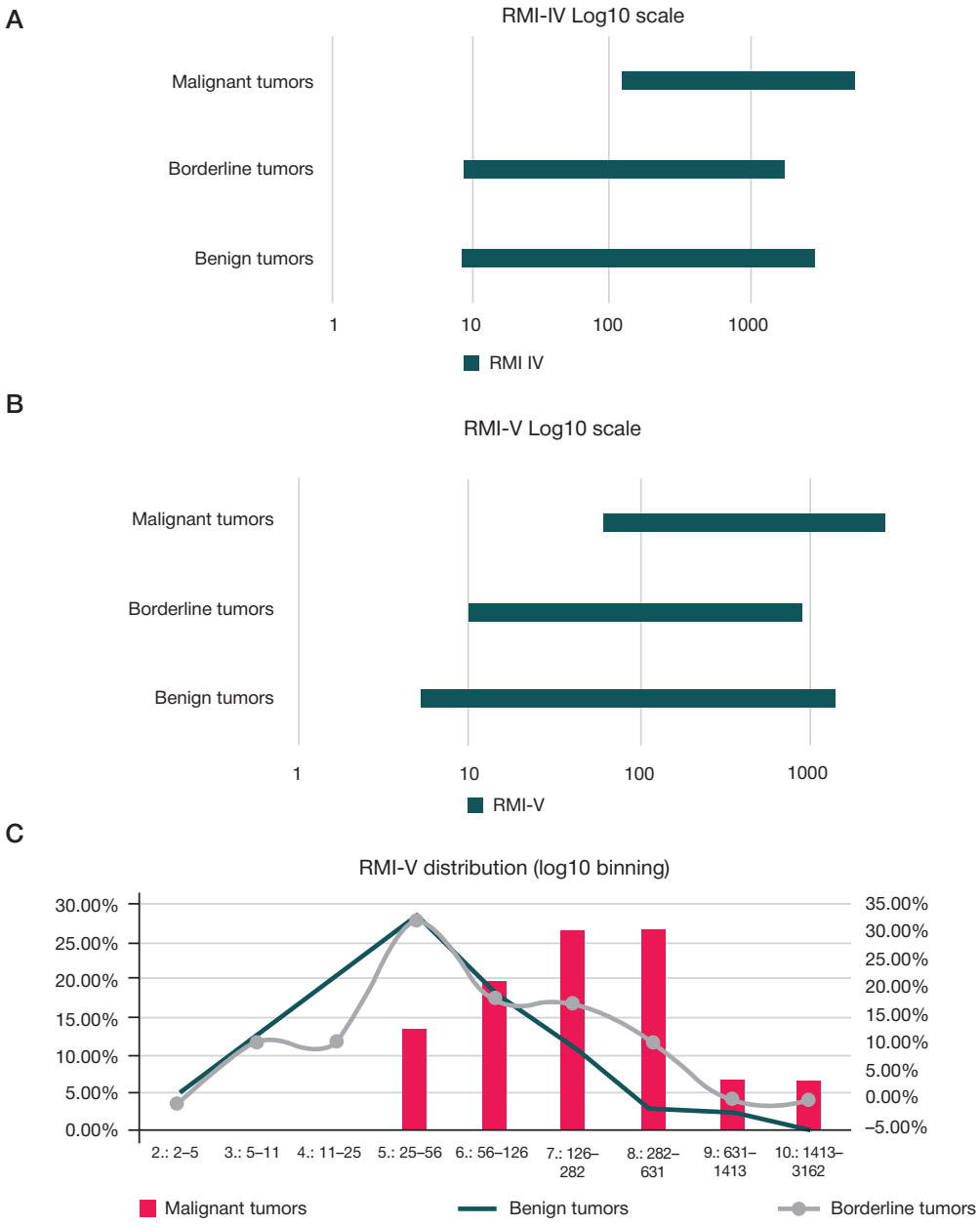

Fig. 1. Frequency analysis of RMI-IV and RMI-V in the surveyed pregnant women

cases accuracy dropped to 70%. Furthermore, 37% of false-positives (suspected MOTs in patients with no MOTs) were observed, which was considered unacceptable (Fig. 1 and 2).

With all the advantages of using RMI-IV and RMI-V, extreme values (series limits) of indices (RMI-IV > 3500 indicate the presence of MODs, RMI-IV below 100 indicate no malignancy) were needed to guarantee the differences in prognosis. Similar RMI-V values were 1500 and 60. Furthermore, lower values had greater significance based on the incidence rate. However, in the majority of cases RMI-IV and RMI-V availability was insufficient, and a comprehensive approach had to be used: ultrasound and MRI data had to be considered along with RMI values, as well as logistic regression models based on the analysis of multiple OT markers, such as earlier reported methods [12–18].

It was shown that RMI (I–III) values in patients with benign ovarian tumors should not be higher than 200, while RMI-IV in patients with MOTs should exceed 450 [11, 19]. Sensitivity was 73%, 81%, and specificity was 93.7%, 89.6%, 93.7%, 92.3%. According to these findings, proper diagnosis is possible in 95% of cases.

However, there are reports that sensitivity exceeds 90% and false negative rate is about 10% in all RMI models when predicting the ovarian mass type [20]. It has been found, though, that RMI-IV is ineffective for prediction of tumor type, even the threshold value of 450. Furthermore, the need to use Doppler techniques, that was later factored in the algorithm together with RMI-V, was substantiated in predicting. Parameters of tumor blood flow, Doppler ultrasound blood flow parameters, and the presence of solid component were malignancy predictors, unlike tumor size and isolated levels of CA-125.

When using RMI-IV, other researchers observed false positive results in non-pregnant patients with benign ovarian tumors: endometrioid cystadenomas, fibromas, serous cystadenofibromas [21]. False negative results were registered in patients with MOTs (clear cell and mucinous carcinomas).

According to other sources, RMI < 25 indicates low risk of malignancy, 25–200 indicates moderate risk, while the levels exceeding 200 may confirm high risk of malignant ovarian

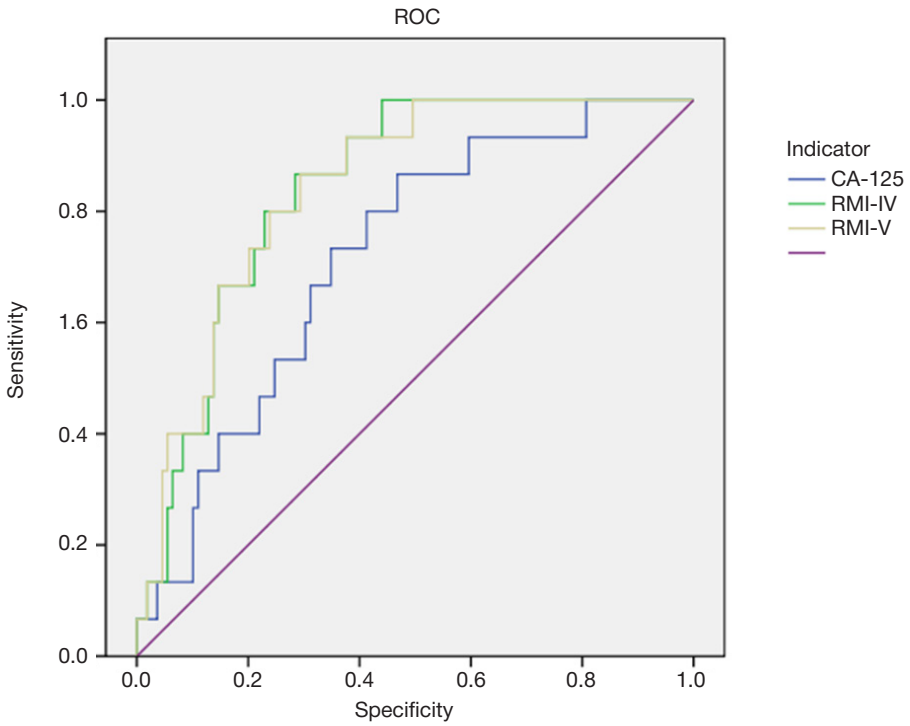

Fig. 2. ROC-curves characterizing sensitivity and specificity of CA-125, RMI-IV and RMI-V as predictors of the successful diagnosis of malignant ovarian tumors in pregnant women

lesion [22]. However, high RMI values have been diagnosed in patients with deep ovarian endometriosis, and low values (< 200) have been found in patients with clear cell carcinoma.

The results reported in the paper showing that RMI should not be used solo during pregnancy due to low sensitivity (50–55.6%) are most similar to our results. Conclusions about benign ovarian tumors, BOTs and MOTs should be drawn based on RMI in combination with clinical features and the results of imaging tests [23].

CONCLUSIONS

It is difficult to define ovarian mass type in pregnant women using RMI only. Comprehensive clinical assessment with the use of imaging methods is required for preoperative prediction of ovarian mass type in pregnant women, along with the use of prognostic models taking into account the majority of descriptive “morphological” tumor characteristics.

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METHODOLOGY OF DETERMINING THE METABOLOMIC PROFILE OF TUMOR-ASSOCIATED MACROPHAGES AND MONOCYTES IN ONCOLOGICAL DISEASES

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Breast cancer is the leading cause of cancer-related death among women worldwide. Tumor-associated macrophages (TAMs) constitute the primary component of innate immunity in breast cancer tissue. During the development of new approaches for breast cancer treatment aimed at editing the epigenome of TAM, precise methods for the analysis of macrophage metabolome are required to examine the effect on new approaches on macrophage metabolism. Our study aimed to develop an HPLC-MS/MS-based analytical approach to characterize the metabolome of human innate immune cells (TAMs and their precursors, monocytes). Analysis of lipid extracts was conducted on a Dionex UltiMate 3000 liquid chromatograph connected to a Maxis Impact qTOF mass analyzer with an ESI ion source. Quantitative analysis of 38 amino acids in the cells was conducted using the Jasem Amino Acids LC-MS/MS Analysis Kit and an HPLC-MS/MS chromatographic system consisting out of an Agilent 6460 triple quadrupole mass spectrometric detector (Agilent), and an Agilent 1260 II liquid chromatograph (Agilent) with Amino acids-HPLC Column (Jasem). The modified Folch method with double extraction was found to be the optimal approached for the sample preparation, since it enables to simultaneously isolate the lipid extract and water-soluble substances, in particular, amino acids. The method of reversed-phase chromatography yielded more useful data on the cell lipid composition than the method of hydrophilic interaction liquid chromatography (HILIC). The minimum number of cells required to determine the metabolome of immune system cells (TAM and monocytes) was identified as 2×10^6. Thus, we have developed the approach to determine the lipid and amino acid composition of modelled human TAMs and primary monocytes isolated out of breast cancer patients using minimal amount of clinical material.

Keywords: mass spectrometry, metabolomics, lipidomics, tumor-associated macrophages

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Author contribution: Frankevich VE, Kzhyshkowska JG — study planning and coordination, manuscript writing; Bragina OD — cancer patient selection and clinical characteristic preparation; Novoselova AV — sample preparation, HPLC-MS/MS; Larionova IV, Patysheva MR — monocyte and macrophage sample preparation and characterization, model TAM system set-up; Rakina MA — obtaining conditioned media of breast cancer cell lines; Starodubtseva NL — HPLC-MS/MS data analysis, manuscript writing; Frankevich VE, Larionova IV, Patysheva MR — discussion of results; Frankevich VE, Kzhyshkowska JG, Starodubtseva NL — manuscript editing.

Compliance with ethical standards: the study was approved by ethical review board of the Federal State Budgetary Educational Institution of Higher Education of the Siberian State Medical University of the Ministry of Health of Russia (protocol № 7 of 14 January 2017), federal laws of the Russian Federation (№ 152, 323, and others), and the 1964 Declaration of Helsinki.

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МЕТОДИКА ОПРЕДЕЛЕНИЯ МЕТАБОЛОМНОГО ПРОФИЛЯ ОПУХОЛЕАССОЦИИРОВАННЫХ МАКРОФАГОВ И МОНОЦИТОВ ПРИ ОНКОЛОГИЧЕСКИХ ЗАБОЛЕВАНИЯХ

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Рак молочной железы — ведущая причина смерти от рака среди женщин во всем мире. Опухолеассоциированные макрофаги (ОАМ) представляют собой основной компонент иммунных клеток в ткани при раке молочной железы. Новые подходы к лечению направлены на редактирование эпигенома ОАМ. Для изучения изменений в клетках иммунной системы при репрограммировании необходимы точные методы анализа метаболома. Целью исследования было создать на базе ВЭЖХ-МС/МС аналитический подход для определения особенностей метаболома иммунных клеток человека (ОАМ и моноцитов). Липидные экстракты анализировали на жидкостном хроматографе Dionex UltiMate 3000, соединенном с масс-анализатором Maxis Impact qTOF с ЭРИ источником ионов. Для количественного анализа 38 аминокислот в клетках использовали набор Jasem Amino Acids LC-MS/MS Analysis Kit и хроматографическую систему ВЭЖХ-МС/МС, состоящую из тройного квадрупольного масс-спектрометрического детектора Agilent 6460 (Agilent) и жидкостного хроматографа Agilent 1260 II (Agilent) с колонкой Amino acids-HPLC Column (Jasem). Модифицированный метод Фолча с двойной экстракцией позволяет одновременно выделить экстракт липидов и водорастворимых веществ, в частности аминокислот. Наиболее информативные данные о липидном составе клеток были получены методом обращенно-фазовой хроматографии по сравнению с гидрофильной хроматографией (HILIC). Для определения метаболома клеток иммунной системы (ОАМ и моноциты) минимальное число клеток оказалось равным 2 млн. Таким образом, разработан подход для определения особенностей липидного и аминокислотного состава ОАМ и моноцитов пациентов с раком молочной железы.

Ключевые слова: масс-спектрометрия, метаболомика, липидомика, опухолеассоциированные макрофаги

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Breast cancer is the most commonly diagnosed malignant disease and the leading cause of cancer-related death among women worldwide [1]. Tumor microenvironment plays a crucial role in breast cancer progression, regulating tumor growth, vascularization, metastasis, and response to therapy [2-4]. Tumor-associated macrophages (TAM) are a major component of the innate immune system in breast cancer tissue; their role in breast cancer progression has been shown in animal models and in patients Larionova et al 2020 [5]. Both the overall number of macrophages and, in particular M2-like macrophages were found to indicate poor prognosis in most studies of breast cancer, which however do not account for intratumoral heterogeneity [2, 5, 6]. Our previous studies showed that macrophages can retain antitumor properties in certain intratumoral compartments, as supported by the negative correlation of breast tissue TAMs with local metastases [6]. Macrophages control tumor growth and metastasis by secreting cytokines, extracellular matrix components, and growth factors; the profile of secreted components is defined by programming on the transcriptional level as well as on the epigenetic and metabolic levels [2, 9–11].

The primary tumor is generally surgically removed without any additional exposure, since such exposure will not have any effect on the micrometastases. TAM metabolize chemotherapeutic agents and significantly increase the growth factors and proangiogenic factors secretion to stimulate tumor growth and further metastasis in response to chemotherapy [12]. The macrophage epigenetic program is the link between the genetic code and interaction with stressful factros, endogenous pathological factors and infectious agents. Macrophage pathological programming can create the conditions, in which even single-cell micro-metastases can develop into deadly secondary tumors resistant to chemotherapy and immunotherapy. New approaches for breast cancer treatment aim to edit the TAM epigenome, using advanced CRISPR/dCas technology for targeted delivery of epigenome editors to target gene promoters. The authors of this article developed a model system of human primary macrophages *ex vivo* and demonstrated long-term programming of human macrophages using a new biomaterial-based cytokine release system [13].

Precise methods of metabolome analysis — in particular of lipids, the main energy component and building material of membranes, and of amino acids, the monomeric units of proteins — are required to study changes in immune system cells during reprogramming. A technique for metabolome analysis in model TAM and in monocytes of patients with tumors of varied localization will allow to study the molecular composition of monocytes in patients with tumors of varied localization and will contribute to the development of technology for clinical application of any discovered correllations. Data that can be obtained with such a technique is of interest for solving both fundamental and applied problems in biology and medicine; it may be particularly useful for studies of mechanism of pathogenesis in malignant neoplasm. Liquid chromatography combined with mass spectrometry (HPLC-MS/MS), the method this study employs, is currently the most effective approach to determining the molecular composition of biological samples [14]. The key problem of existing approaches is the lack of consensus on the minimum number of cells for analysis, which is especially relevant for monocytes and TAM of patients with cancer, since the complex procedure of obtaining monocytes and TAM requires a large amount of whole blood, long-term post-treatment and differentiation in in culture conditions ex vivo in the absence of cell proliferation.

Our study aimed to identify the minimum number of human immune cells (TAM and monocytes) necessary for the metabolomic profile analysis of these cells in oncological diseases using HPLC-MS/MS.

METHODS

Samples from healthy volunteers were used to determine the optimal number of cells to obtain results in metabolomic spectrum study. The inclusion criteria for healthy volunteers: no history of cancer; absence of chronic diseases in the acute phase. In the study of a group of patients, samples were obtained from four patients with breast cancer (BC) T1–4N0–3M0 (stages IIA–IIIB) luminal B-subtype undergoing treatment at the Oncology Research Institute clinic of the Tomsk National Research Medical Center in 2021. Selection criteria: age 45–55 years; no history of cancer of another localization; no hereditary predisposition to breast cancer (based on family history and genetic testing for the 5382insC, 4153delA, 185delAG mutations in the BRCA1 gene); perimenopause or menopause; morphological verification of the nonspecific invasive carcinoma diagnosis (according to the WHO 2019 classification); estrogen receptors (ER) expression ≥ 5 points; Ki-67 ≥ 40%; no hematogenous metastases. Exclusion criteria: age < 45 years or > 55 years; history of cancer; hereditary predisposition; premenopause; all histological subtypes except non-specific invasive carcinoma; ER expression < 5 points; Ki-67 < 40%; hematogenous metastases. An immunohistochemical study was performed before treatment to determine the tumor molecular subtype in accordance with the ASCO/CAP recommendations. BC stage was determined according to the AJCC TNM classification (8th edition, 2017).

We studied the TAM model system [13], blood monocytes from breast cancer patients before treatment and blood monocytes from healthy volunteers. Monocytes were isolated from the whole blood (patients) or from the buffy coat (donors). The $CD14^+$ cell fraction was obtained by positive magnetic separation using the Miltenyi Biotec protocol. Model differentiated M2 macrophages were obtained by culturing monocytes from healthy volunteers at a concentration of 10^6 cells per 1 ml of X-VIVO macrophage medium (Lonza; Germany) supplemented with 1 ng/ml M-CSF (Peprotech; Germany) and 10^{-8} M dexamethasone (Sigma-Aldrich; Germany). Differentiation of macrophages into protumor M2 macrophages was induced by 10 ng/ml IL4 (Peprotech; Germany). Model TAM was obtained by adding conditioned media obtained from MCF7 breast carcinoma cells to the obtained macrophages in a volume of 10% of the total medium amount. Macrophages differentiated for 6 days at 7.5% CO_2 and were afterwards removed from the culture plates with a rubber scraper in the cold.

The resulting monocytes and macrophages were centrifuged in a solution of cold 0.3% ammonium acetate and 0.3% ammonium formate for 5 min at 311g thrice. After separation of the supernate, the precipitate was lyophilized at –197 °C using liquid nitrogen. Samples were stored at –80 °C until further analysis.

Sample preparation for HPLC-MS/MS analysis

Metalobome (lipid and amino acid composition) of TAM and monocytes from patients with breast cancer was determined by HPLC-MS/MS analysis of cell extracts. Lipid extraction was carried out by the modified Folch method [15–17] with double extraction: cells were mixed with 480 µl of chloroform-methanol mixture (2 : 1, v/v) with 20 µl of IS (internal standard) at 4 °C and subjected to ultrasonic treatment for 10 min; then 150 µl of water was added and the sample was centrifuged

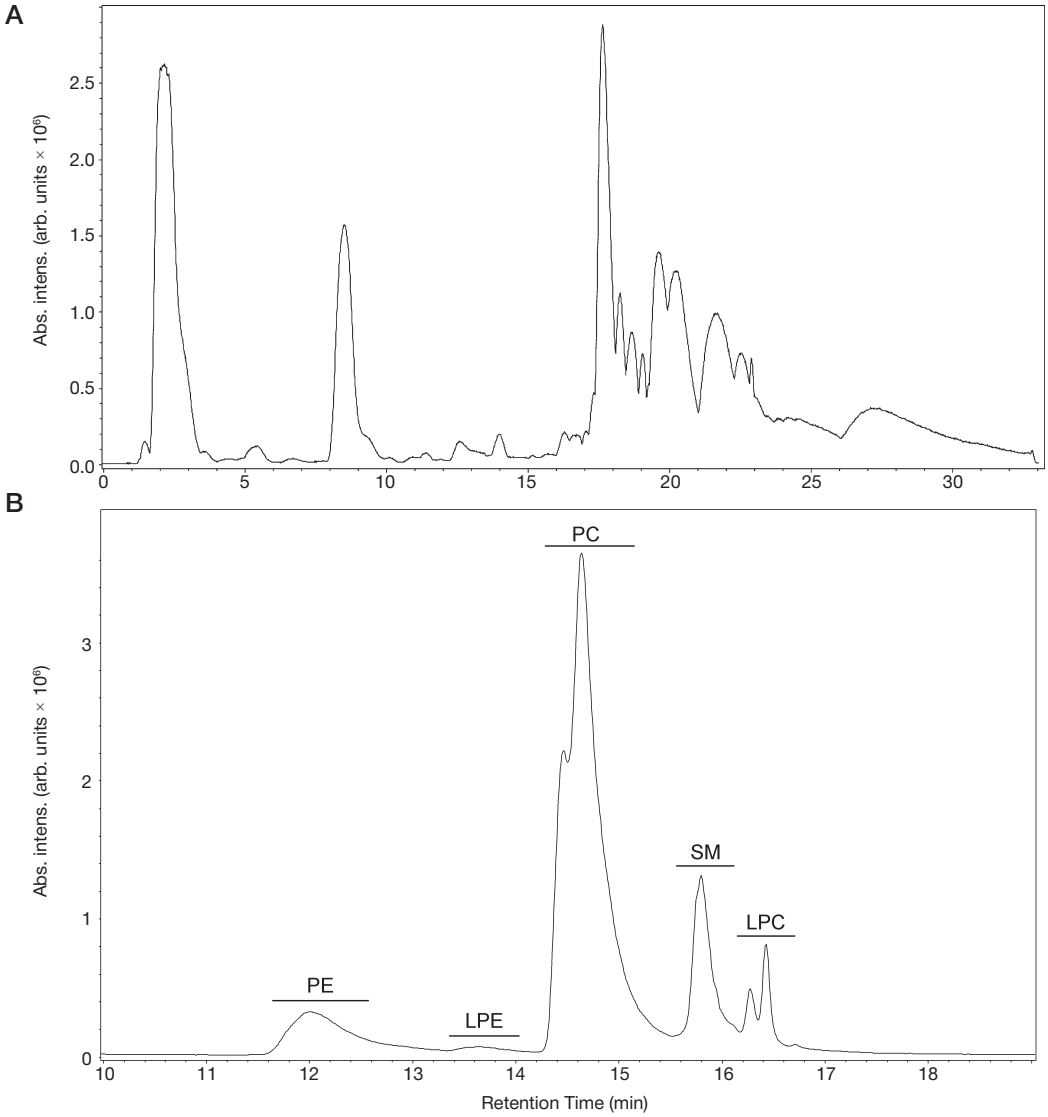

Fig. 1. Characteristic total ion current chromatograms of lipid extracts from monocyte cells recorded in the positive ion mode. (**A**) Reversed-phase chromatography; (**B**) HILIC chromatography. PC — phosphatidylcholines; PE — phosphatidylethanolamines; SM — sphingomyelins; LPC — lysophosphatidylcholines; PE — phosphatidylethanolamines

at 13,000 g for 5 min at room temperature; the lower organic layer containing lipids was taken and the extraction procedure was repeated (adding 250 µl of chloroform-methanol mixture (2 : 1, v/v, 10 min on a vortex; centrifugation at 13,000 g for 5 min); the water-methanol layer was taken into separate tubes; the organic layer was combined with the sample obtained in the first stage and lyophilized in a nitrogen stream. The dried lipid extracts (organic layer) were re-dissolved in 100 µl of isopropanol solution with acetonitrile (1 : 1, v/v). The extracts of water-soluble compounds (water-methanol phase) were used to analyze the amino acid profile: they were redissolved in 100 µl of water with acetonitrile (1 : 1, v/v) and 1% formic acid.

HPLC-MS/MS analysis

Lipid extracts were analyzed on a Dionex UltiMate 3000 liquid chromatograph (Thermo Scientific; Germany) connected to a Maxis Impact qTOF mass analyzer with an ESI ion source (Bruker Daltonics; Germany). Lipid separation by HILIC was performed according to a modified procedure described previously [18, 19]: 3 µL of the sample was injected into the system. Separation was performed on a Spherisorb Si column (150 — 2.1 mm, 5 µm; Waters, USA) at a flow rate of 50 µl/min using acetonitrile as solvent A and aqueous ammonium acetate solution (5 mmol/l) as solvent B (linear gradient from 6 to 23% (v/v) solvent B) for 25 min (column temperature 40 °C). Separation of lipids by reversed-phase chromatography was carried out on a Zorbax C18 column (150 — 2.1 mm, 5 µm; Agilent, USA) with a linear gradient from 30 to 90% of eluent B (solution of acetonitrile/isopropanol/water, 90/8/2 vol., with the addition of 0.1% formic acid and 10 mmol / l ammonium formate) for 20 minutes. A solution of acetonitrile/water (60/40 vol.) with the addition of 0.1% formic acid and 10 mmol/l ammonium formate was used as eluent A. The elution flow rate was 40 µL/min, the injected sample volume was 3 µL. Mass spectra were obtained in the positive and negative ion mode for 100–1700 m/z (capillary voltage — 4.1 kV; spray gas pressure — 0.7 bar; drying gas flow rate — 6 l/min; drying gas temperature — 200 °C). To identify lipids, tandem mass spectrometry was performed in dependent scan mode with a window width of 5 Da.

Amino Acids LC-MS/MS Analysis Kit (Jasem; Turkey) (1-methyl-L-histidine, 3-aminoisobutanoic acid, 3-methyl-L-histidine, β-alanine, DL-5-hydroxylysine, DL-homocystin, ethanolamine, γ-aminobutanoic acid, L-2-aminoadipic acid, L-2-aminobutanoic acid, L-alanine, L-anserine, L-arginine,

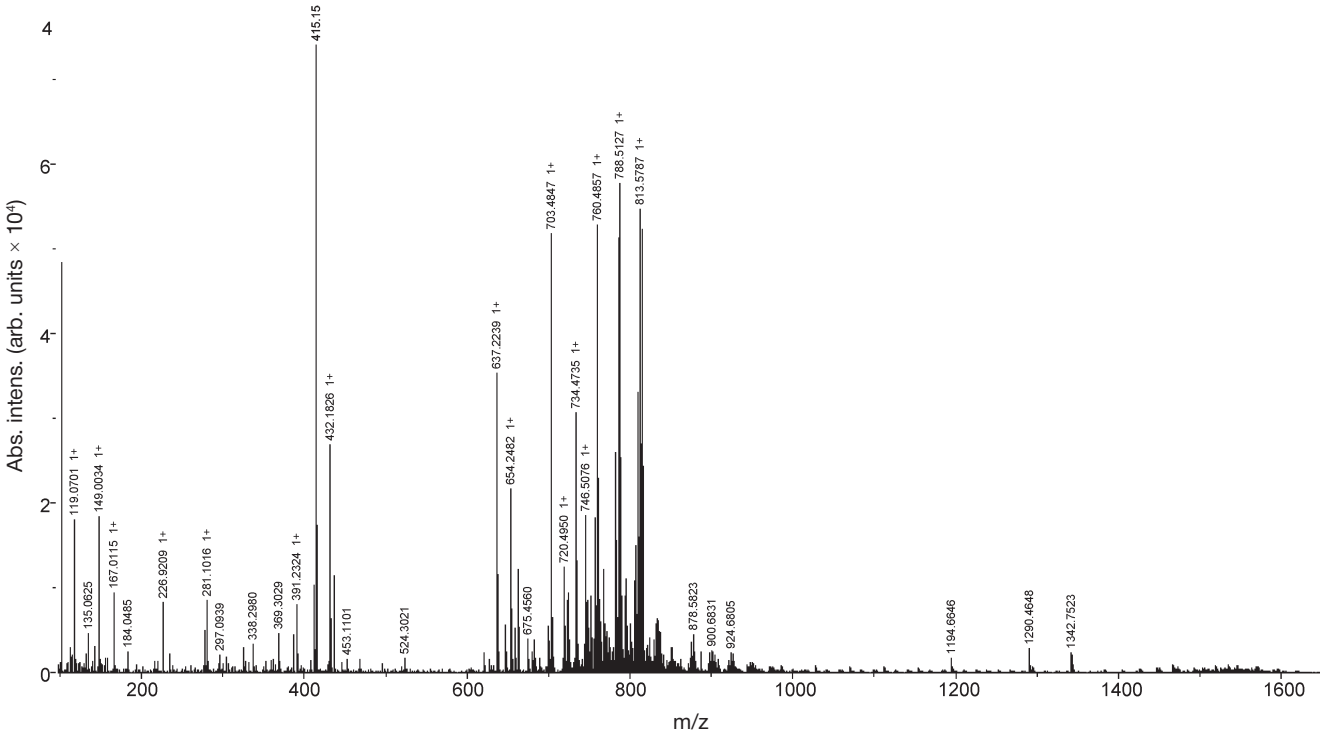

Fig. 2. Time-averaged HPLC-MS mass spectrum of positive ions of a studied sample of macrophage lipid extract, obtained by reversed-phase chromatography

L-asparagine, L-aspartic acid, L-carnosine, L-citrulline, L-cystathionine, L-cystine, L-glutamic acid, L-glutamine, L-glycine, L-histidine, L-homocitrulline, L-lysine, L-norvaline, L-ornithine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, sarcosine, taurine, trans-4-hydroxy-L-proline) was used for quantitative analysis of 38 amino acids in the cells. 40 µl of aqueous cell extract was mixed with 50 µl of internal standard solution and 300 µl of Reagent № 1 (Jasem; Turkey); the resulting mixture was thoroughly shaken for 2 min, then centrifuged at a rotation speed of 13,000 rpm for 10 min; 200 µl of the supernatant was taken into a vial with an insert, 15 µl was injected into an HPLC-MS/MS chromatographic system consisting of an Agilent 6460 triple quadrupole mass spectrometric detector (Agilent; USA) and an Agilent 1260 II liquid chromatograph (Agilent; USA) with a column Amino acids-HPLC Column (Jasem; Turkey).

Conditions for chromatographic separation: temperature — 30 °C, eluent A — 0.1 vol.% formic acid in water; eluent B — acetonitrile 100%, gradient elution from 78% B to 20% B in 4.5 min; return to 78% B and isocratic mode. Amino Acid LC-MS/MS Analysis Kit (Jasem; Turkey) was used for amino acid analysis. Ion source parameters ESI: positive mode; drying gas temperature — 150 °C; capillary voltage — 2 kV. Mass spectrometry was performed in the multiple reaction monitoring (MRM) mode.

Lipid identification and analysis of GC-MS data

For preprocessing of chromato-mass spectrometric data, the msConvert programs from the Proteowizard 3.0.9987 package were used to convert files into the MzXml format, which contains information about the mass spectrum at any moment in time, and the ms2 format, which contains information about tandem mass spectra. In addition, the MzMine program was used to isolate the peaks and create a table containing information on the registered ions: the mass of the ion, the area of its chromatographic peak, and the release time. To identify lipids, the LipidMatch program [20] was used, which took into account the exact mass (0.01 Da) of the corresponding ion, the mass of fragment ions from the tandem mass spectra of the identified ion falling within the specified mass window (3 Da), and the specified vicinity of the retention time (0.5 min). Semi-quantitative analysis of lipids was performed using previously developed R language scripts [11–23].

RESULTS

To develop an optimal methodology for studying human primary macrophage lipidome by LC/MS analysis of lipid extracts, several extraction/chromatography separation methods were compared, including reversed-phase chromatography and hydrophilic interaction liquid chromatography (HILIC), and the minimum required cell number was determined. The efficiencies for three extraction methods were compared in advance. One protocol, adapted from an earlier study [19], used a modified Folch method [2] with double extraction (see Methods). The second protocol was similar to another previously described approach [15], also Folch method-based [16], with single extraction. Shortly, the cells were mixed with 200 µL of ammonium acetate aqueous solution (155 mmol/L) and 990 µL of cold chroloform/methanol (10 : 1, v/v) and sonicated for 10 min; the sample was diluted with 150 µL of water and centrifuged at 13,000 g for 5 min at room temperature; the lipid-containing lower organic phase was dried in a nitrogen flow and re-dissolved in 200 µL of isopropanol/acetonitrile (1 : 1, v/v).

The third protocol employed the Bligh and Dyer extraction principle [24]. Shortly, the cells were mixed with 300 µL of chroloform/methanol (1 : 2); then 100 µL of chloroform were added and the sample was homogenized for 1 min; then 100 µL of water were added, the sample was homogenized for another 1 min and centrifuged at 1,000 g for 10 min at 4 °C. The organic phase was collected and the polar phase was re-extracted with 2 mL of cold chloroform; the organic phase was collected, mixed with the extract obtained at the previous step, dried in a nitrogen flow, re-dissolved in 4 mL of

Table 1. Number of identified lipids in analyzed samples

Cell type	Cells (in millions)	Number of lipids identified by exact mass and characteristic tandem mass spectrum	Number of lipids identified by exact mass
Monocytes	1	80	638
	2	92	701
	5	111	726
	10	183	2727
TAM	1	137	825
	2	176	916
	5	194	900
	10	205	933

cold chloroform/methanol (1 : 1) with added 1.8 mL of NaCl aqueous solution (20 mmol/L) and double-filtered using 0.2 μm PFTE syringe filter.

The second method with a single Folch extraction showed low efficiency for weakly polar lipids (triglycerides, diglycerides and ceramides). The advantages of the third method include the possibility of successful extraction of cardiolipins on a par with other classes of lipids. However, the approach is laborious and involves more stages than the first two protocols, which hampers the reproducibility and extraction yields. Overall, the first protocol turned out to be the most universally applicable and optimal in terms of reproducibility, as well as labor time costs, and accordingly was used as a method of choice in subsequent experiments. Importantly, the water-methanol phase (discarded in the course of lipid extraction) can be preserved for analysis of polar metabolites, notably amino acids, in the same sample.

In this study, we tested two principal techniques of chromatographic separation as applied to lipidome: reversed-phase chromatography and HILIC. Characteristic chromatograms and mass spectra of the studied lipid extracts are given on Figures 1 and 2. In the positive ion mode, about 200 lipids were identified using characteristic tandem mass spectra and about 1000 lipids were identified by exact mass alone. The identified lipids were classified as mono, di- and triglycerides, ceramides and various modifications of phosphatidylcholines and phosphatidylethanolamines including oxidation products. HILIC advantages include default separation of lipids into classes; however, the MS peak intensity obtained by this technique is on average 30% lower compared to reversed-phase chromatography. In addition, HILIC method is of questionable suitability for non-polar lipids (notably triglycerides), poorly bound by the column and often lost (being eluted at a timepoint close to the 'dead time' of the column). Accordingly, the number of lipid identifications by HILIC was 25% lower. Subsequent tests were carried out using reversed-phase chromatography.

Table 1 summarizes the lipidomic data for the analyzed samples. Extracts prepared from 2×10^6 monocytes presented with 15% more lipids than 1×10^6 cell extracts, whereas for 5×10^6 vs 2×10^6 and 10×10^6 vs 5×10^6 the differences constituted 21% and 65%, respectively. For TAM, 2×10^6, 5×10^6 and 10×10^6 cells provided 28%, 10% and 6% more identifications than 1×10^6, 2×10^6 and 5×10^6 cells, respectively. Thus, with limited access to biological material (primary human macrophages), it is necessary to analyze at least 2×10^6 cells, as the 1×10^6 to 2×10^6 transition provides the maximal increment in the number of identifications. For monocytes, no corresponding increment dynamics were encountered.

The results of amino acid profiling of the monocyte and TAM samples are provided in Table 2. The chromatographic peak areas correlate with cell number, with the exception of the smallest monocyte samples containing 1×10^6 cells, which is apparently due to LC/MS sensitivity limits. In this regard, samples suitable for the analysis should contain at least 2×10^6 cells.

CONCLUSIONS

We developed a HPLC-MS/MS-based method for monocyte and cultured TAMs metabolome analysis. The modified Folch

Table 2. Areas of chromatographic peaks of amino acids obtained from the analysis of samples of monocytes and TAM

Sample	Monocytes				TAM			
Cells (in millions)	1	2	5	10	1	2	5	10
3-Methyl-Histidine	7	2	64	187	4	45	76	169
L-Arginine	167.432	85.895	169.003	261.068	192.217	425.313	775.226	798.153
L-Cystine	496	144	158	423	85	386	184	400
L-asparagine	1199	758	1674	3884	70	1443	1265	3290
L-Glutamine	97.176	39.931	105.365	288.808	53.604	145.366	465.023	472.766
L-Histidine	18.103	8496	17.941	34.787	12.253	33.254	114.909	65.534
L-Lysine	97.176	39.931	105.365	288.808	53.604	145.366	465.023	472.766
L-Ornithyne	1354	562	1988	3836	725	2043	2720	1437
L-Phenylalanine	6023	6793	8427	30.896	3393	3052	0	5769
L-Serine	2675	2503	5180	14.425	2658	3134	2617	10.421
L-Threonine	2438	1531	2835	8252	1614	2111	2089	7324
L-Tyrosine	646	1038	884	3756	278	326	0	9464
Sarcosine	6044	5857	12.512	34.552	5858	8329	10.555	28.340
Taurine	92	333	462	1311	204	247	201	944

method with double extraction was chosen as the optimal sample preparation method because it allows to simultaneously isolate the lipid extract (i.e. the organic phase) and the extract of water-soluble substances, in particular amino acids (i.e. the water-methanol phase). The most intense peaks in the mass spectra correspond to lipids and other metabolites, proving the effectiveness of this method of metabolomic profile obtainment. Within the framework of this study, use of both HILIC and reversed-phase chromatography was analyzed: the two methods yield different chromatographic separations for HPLC-MS/MS of lipid extracts of cells, with data yielded by reversed-phase chromatography proving more useful. A comparative analysis of lipid and amino acid composition of monocyte and macrophage samples containing 1×10^6, 2×10^6, 5×10^6, and 10×10^6 cells was carried out; lipids and amino acids were found in all studied samples, but the most effective identification can be performed for samples containing at least 2×10^6 cells. The developed HPLC-MS/MS-based approach for cellular metabolome analysis will be used to determine the metabolomic composition of macrophages and monocytes, and to search for characteristic differences in the metabolomic profile of TAM and monocytes in patients with cancer.

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FREQUENT ASSOCIATION OF VITILIGO WITH AUTOIMMUNE ENDOCRINE DISEASES: PRIMARY DATA OF THE RUSSIAN COHORT OF ADULT PATIENTS

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There is evidence in the literature about more frequent association of vitiligo with autoimmune endocrine diseases (AEDs) compared to general population. No full-fledged studies aimed at assessing the prevalence of AEDs in the Russian cohort of adult vitiligo patients have been conducted. The study was aimed to assess the prevalence of AEDs in the cohort of Russian adult vitiligo patients. Patients with vitiligo monitored in two clinics, the Endocrinology Research Centre (Clinic 1; *n* = 39) and the Moscow Scientific and Practical Center of Dermatovenereology and Cosmetology (Clinic 2; *n* = 26), were enrolled. Along with clinical examination, screening laboratory tests were performed in all patients in order to reveal AEDs. The majority of patients (more than 95% of cases) had nonsegmental vitiligo. Among patients monitored in Clinic 1, AEDs were diagnosed in 85% of cases: isolated AEDs accounted for 39%, while multiple AEDs were found in 46% of cases. Autoimmune thyroid diseases were diagnosed in 69% of cases. Autoimmune adrenal insufficiency was found in 28% of patients, type 1 diabetes mellitus in 21%, hypoparathyroidism in 13%, hypergonadotropic hypogonadism in 10%, endocrine ophthalmopathy in 10% of patients. Among patients monitored in Clinic 2, AEDs were diagnosed in four patients (15% of cases): three patients had primary hypothyroidism in the outcome of autoimmune thyroiditis, one patient had Graves' disease. Thus, the prevalence of AEDs in patients with vitiligo may vary between 15–85%. Vitiligo is most often associated with autoimmune thyroid diseases (15–69%). Vitiligo patients should undergo annual screening aimed at detection of autoimmune endocrine disorders, especially thyroid diseases.

Keywords: vitiligo, autoimmune thyroiditis, type 1 diabetes mellitus, Graves' disease, autoimmune adrenal insufficiency, autoimmune polyglandular syndrome, prevalence

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Compliance with ethical standards: the study was approved by the Ethics Commitee of the Endocrinology Research Centre (protocol № 17 of 27 September 2017); the informed consent was submitted by all patients.

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ЧАСТАЯ АССОЦИАЦИЯ ВИТИЛИГО С ЭНДОКРИННЫМИ АУТОИММУННЫМИ ЗАБОЛЕВАНИЯМИ: ПЕРВИЧНЫЕ ДАННЫЕ В РОССИЙСКОЙ КОГОРТЕ ВЗРОСЛЫХ ПАЦИЕНТОВ

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В литературе имеются данные о более частой, чем в популяции, ассоциации витилиго с эндокринными аутоиммунными заболеваниями (эАИЗ). В российской когорте полноценных исследований, направленных на оценку частоты встречаемости эАИЗ у взрослых пациентов с витилиго, не проводилось. Целью исследования было проанализировать частоту встречаемости эАИЗ в когорте российских взрослых пациентов с витилиго. В него вошли пациенты с витилиго, наблюдавшиеся в двух лечебных учреждениях: «НМИЦ эндокринологии» (центр 1; *n* = 39) и в «МНПЦДК ДЗМ» (центр 2; *n* = 26). Всем пациентам наряду с клиническим обследованием проводили скрининговое лабораторное обследование с целью выявления эАИЗ. У большинства пациентов (более 95% случаев) установлен несегементарный тип витилиго. Среди пациентов, наблюдавшихся в центре 1, эАИЗ диагностированы в 85% случаев: у 39% выявлено одно эАИЗ, у 46% — множественные эАИЗ. Аутоиммунные заболевания щитовидной железы встречались в 69% случаев. У 28% пациентов выявлена аутоиммунная надпочечниковая недостаточность, у 21% — сахарный диабет 1-го типа, у 13% — гипопаратиреоз, у 10% — гипергонадотропный гипогонадизм, у 10% — эндокринная офтальмопатия. Среди пациентов, наблюдавшихся в центре 2, эАИЗ диагностированы у четырех больных (15% случаев): у троих — выявлен первичный гипотиреоз в исходе в аутоиммунного тиреоидита, у одного — болезнь Грейвса. Таким образом, частота встречаемости эАИЗ у пациентов с витилиго может варьировать от 15 до 85%. Наиболее часто (15–69%) витилиго ассоциируется с аутоиммунными тиреопатиями. Пациентам с витилиго показано ежегодное скрининговое обследование с целью выявления аутоиммунной эндокринной патологии, особенно заболеваний щитовидной железы.

Ключевые слова: витилиго, аутоиммунный тиреоидит, сахарный диабет 1-го типа, болезнь Грейвса, аутоиммунная надпочечниковая недостаточность, аутоиммунный полигландулярный синдром, распространенность

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Vitiligo is a common polygenic autoimmune disease characterized by formation of the foci of skin depigmentation, resulting from the death or decreased function of melanocytes, on various parts of the body. Segmental and nonsegmental vitiligo are distinguished. Segmental vitiligo is characterized by unilateral lesions located within one or more body segments. Nonsegmental vitiligo results in a few or multiple foci of depigmentation that are often symmetrically arranged [1]. In foreign literature, there is evidence of the higher incidence of autoimmune endocrine diseases (AEDs) in patients with vitiligo compared to the general population [1–3]. Autoimmune thyropathies are the most common in vitiligo patients (0.3–40% cases) [4–12]: autoimmune thyroiditis (AIT) is diagnosed in 0.3–31% of cases [9, 13, 14], and Graves' disease (GD) is found in 0.3–17.1% of cases [9, 14–16]; thyroid autoantibody positivity is identified in 41.8% of cases [11]. Type 1 diabetes mellitus (T1D) is found in vitiligo patients in 0.1–25% of cases [4, 5, 8–11, 17], autoimmune adrenal insufficiency (AAI) is diagnosed in 0.2–3.2% of cases [4, 5], and anti-adrenal antibodies are detected in 2.5% of cases [18, 19].

Vitiligo can not only be coupled with isolated AEDs, but also be a component of autoimmune polyglandular syndrome (APS), the primary autoimmune disorder that affects two or more peripheral endocrine glands and usually results in the endocrine gland dysfunction. APS type 1 (APS-1) and type 2 (APS-2) are distinguished. Candidiasis involving the skin and mucous membranes, hypoparathyroidism, and AAI are the main components of APS-1. Patients with APS-2 develop such main AEDs, as AAI, T1D, autoimmune thyropathies (GD or AIT), in combination with other autoimmune diseases [2]. Vitiligo often becomes the first component of APS (in 12.6% of cases [2]). APS can occur in 27.4% of vitiligo patients [1].

At the same time, high incidence of vitiligo development in patients with autoimmune endocrine diseases have been reported: it is found in 2.6–2.8% of patients with AIT [20, 21], 1.4–2.6% of patients with GD [20, 22], 23.3% of patients with T1D [23], 37% of patients with APS-1 [24], and 20% of patients with APS-2 [2].

Published research shows that autoimmune endocrine diseases occur mostly in patients with nonsegmental vitiligo [1, 6, 7]. No other factors contributing to the risk of AEDs in vitiligo patients have been identified. According to some reports, [1, 7, 8], the patients' gender and race, as well as vitiligo duration and activity, do not define the rate of AEDs manifestations. Meanwhile, other studies revealed more frequent association of vitiligo with AEDs in females [1, 4, 8, 9] and patients with larger skin lesions [4, 8]. Furthermore, higher prevalence of autoimmune thyropathies associated with the prolonged course of vitiligo and predominant involvement of the skin of the trunk was reported [8]. These data were not confirmed by papers reporting higher incidence of autoimmune thyroid disorders (AITDs) in patients with vitiligo patches located mostly on their limbs and joints [4], as well as predominance of APS in patients with acrofacial vitiligo [1]. The results of some studies suggest that the increased risk of AITD manifestation is associated with the late-onset vitiligo [10, 25]. However, association of vitiligo with GD is most common in young patients [14].

No full-fledged studies aimed at assessing the prevalence of AEDs in adult vitiligo patients and the prevalence of vitiligo in patients with AEDs in the Russian cohort have been conducted. Single studies on the issue were focused on assessing the incidence of vitiligo in patients with diabetes mellitus [26] or the AITD and pancreatic islet autoimmunity marker antibodies carrier state in vitiligo patients [27].

The study was aimed to assess the prevalence of AEDs in the cohort of Russian vitiligo patients.

METHODS

Patients included in the study

The first part of the study involved patients with vitiligo monitored in 2019–2021 in the Endocrinology Research Centre. The second part of the study involved patients with vitiligo monitored in 2019–2021 in the Moscow Scientific and Practical Center of Dermatovenereology and Cosmetology.

The patients were recruited and allocated to certain groups based on their compliance with the inclusion criteria and non-compliance with the exclusion criteria.

Inclusion criteria: age 18 or older; vitiligo; availability of the patient's informed consent.

Exclusion criteria: pregnancy, lactation; acute infections; exacerbation of chronic diseases; severe life-threatening conditions; congenital or acquired immunodeficiency disorders; taking medications affecting the immune system function (glucocorticoids not for vital indications, interleukins, interferons, immunoglobulins, immunosuppressants, cytostatics), and/or vaccination/revaccination within a month prior to enrollment.

Study design: cross-sectional observational descriptive study; the first part involved 39, and the second part involved 26 subjects. Continuous sampling was used during the study.

Clinical assessment

Medical researchers examined all the subjects in order to clarify their compliance with the inclusion criteria or possible non-compliance with the exclusion criteria. Initial examination included patient complaint management and history taking, as well as measuring anthropometric parameters, blood pressure and pulse rate. Family history, acute and chronic diseases, taking medications and dietary supplements, harmful habits, and gynecologic history (in women) were specified.

Dermatovenerologist performed thorough visual examination of the patient that involved assessment of the skin and skin appendages, and photodocumentation of lesions under visible light or Wood's lamp using digital camera.

Laboratory tests

Screening laboratory tests for all the major AEDs were performed in all patients. Biochemical, immunological and hormonal tests were carried out in the clinical diagnostic laboratory at the Endocrinology Research Centre. Blood was collected from the cubital vein in the vacuum tubes containing inert gel and ethylenediaminetetraacetic acid in the morning (between 08:00 am and 10:00 am) in the fasting state (fasting for 8–14 hrs prior to venipuncture). The samples were centrifuged within 15 minutes after blood collection and processed. Complete blood count, biochemical, hormonal, and immunological (thyroid peroxidase (TPO) antibodies, thyroglobulin (TG) antibodies) tests were carried out on the day of blood sampling. Serum samples for further assessment of the levels of 21-hydroxylase antibodies and markers of pancreatic islet autoimmunity had to be temporarily frozen in microtubes at a temperature of –80 °C.

Statistical analysis of the results

Statistical processing of the results was performed by standard methods using the STATISTICA 13 software package (StatSoft; USA).

Table 1. Characteristics of study participants

Group	Patients				
	n	Age (years)	Gender (F/M)		
			n	%	ratio
Patients initially monitored in Clinic 1	39	19–73	26/13	67/33	2.0 : 1
Patients initially monitored in Clinic 2	26	19–71	17/9	65/35	1.9 : 1

Note: M — male, F — female.

Chi-squared test (χ^2) was used to compare qualitative traits. The differences were considered significant at $p < 0.05$.

RESULTS

Characteristics of study participants are provided in Table. 1.

The prevalence of autoimmune endocrine diseases in the cohort of adult patients with vitiligo initially monitored in the Endocrinology Research Centre

In the surveyed cohort, symptomatic AEDs were diagnosed in 85% of cases (*n* = 33). Single AED was diagnosed in 38.5% of cases (*n* = 15), and in 46.1% of cases (*n* = 18) multiple autoimmune endocrine disorders were observed. Another 6 patients (15.4%) with no symptomatic AEDs appeared to be carriers of AITD marker antibodies showing no target organ dysfunction and/or carriers of pancreatic islet autoimmunity marker antibodies showing no carbohydrate metabolism disorders.

AITDs were found in 69% of cases (*n* = 27): 19 patients (70%) were diagnosed with primary hypothyroidism in the outcome of AIT, 8 patients (30%) were diagnosed with GD. AAI was found in 28% of cases (*n* = 11), T1D/LADA (latent autoimmune diabetes in adults) in 21% (*n* = 8), hypoparathyroidism in 13% (*n* = 5), hypergonadotropic hypogonadism (HH) in 10% (*n* = 4), endocrine ophthalmopathy (EOP) in 10% of cases (*n* = 4).

Multiple autoimmune endocrine disorders were represented by APS-2 in 61% of cases (*n* = 11), APS-1 in 22% of cases (*n* = 4); a combination of GD and EOP was found in 17% of cases (*n* = 3). The onset of AEDs was preceded by vitiligo in 30% of patients (*n* = 10), 12% of patients developed vitiligo and AEDs simultaneously (*n* = 4).

The AITDs marker antibodies carrier state with no target organ dysfunction was found in 15% of cases (*n* = 6), while the pancreatic islet autoimmunity marker antibodies positivity with no carbohydrate metabolism disorder was diagnosed in 23% of cases (*n* = 9) (see Fig.). No carriers of 21-hydroxylase antibodies having no disorders of the adrenal cortex were found.

The patients were diagnosed with nonsegmental vitiligo in 100% of cases. However, one patient with APS-2 (AAI, primary hypothyroidism in the outcome of AIT, autoimmune gastritis) was diagnosed with universal vitiligo.

Comparison of the prevalence of AEDs in female and male patients is provided in Table 2.

The prevalence of autoimmune endocrine diseases in the cohort of adult patients with vitiligo initially monitored in the Moscow Scientific and Practical Center of Dermatovenereology and Cosmetology

AEDs were diagnosed in four patients (15%). All the diagnosed AEDs were classified as AITDs. Among them primary hypothyroidism in the outcome of AIT was found in three patients, and Graves' disease was found in one patient.

AITDs marker antibodies positivity with no thyroid dysfunction was diagnosed in 15% of cases (*n* = 4).

Nonsegmental vitiligo was diagnosed in 25 patients (96%). One patient with no symptomatic AEDs or antibody positivity was diagnosed with segmental vitiligo.

Comparison of the prevalence of AEDs in female and male patients is provided in Table 3.

Table 2. The prevalence of AEDs and the AITDs and pancreatic islet autoimmunity marker antibodies positivity in female and male vitiligo patients initially monitored in the Endocrinology Research Centre

AEDs	F *n* = 26	M *n* = 13	*p*
AEDs, *n* (%)	23 (88)	10 (77)	0,347
AITDs, *n* (%)	19 (73)	8 (62)	0,462
Primary hypothyroidism in the outcome of AIT, *n* (%)	14 (54)	5 (39)	0,365
GD, *n* (%)	5 (19)	3 (23)	0,779
AAI, *n* (%)	11 (42)	0	**0,006**
T1D/LADA, *n* (%)	3 (12)	5 (39)	**0,0497**
Hypoparathyroidism, *n* (%)	4 (15)	1 (8)	0,498
HH, *n* (%)	4 (15)	0	0,136
EOP, *n* (%)	2 (8)	2 (15)	0,455
APS-1, *n* (%)	4 (15)	0	0,136
APS-2, *n* (%)	8 (31)	3 (23)	0,615
GD + EOP, *n* (%)	2 (8)	1 (8)	1
AITDs marker antibodies positivity with no target organ dysfunction, *n* (%)	5 (19)	1 (8)	0,347
Pancreatic islet autoimmunity marker antibodies positivity with no carbohydrate metabolism disorder, *n* (%)	5 (19)	4 (31)	0,42

Note: M — male, F — female.

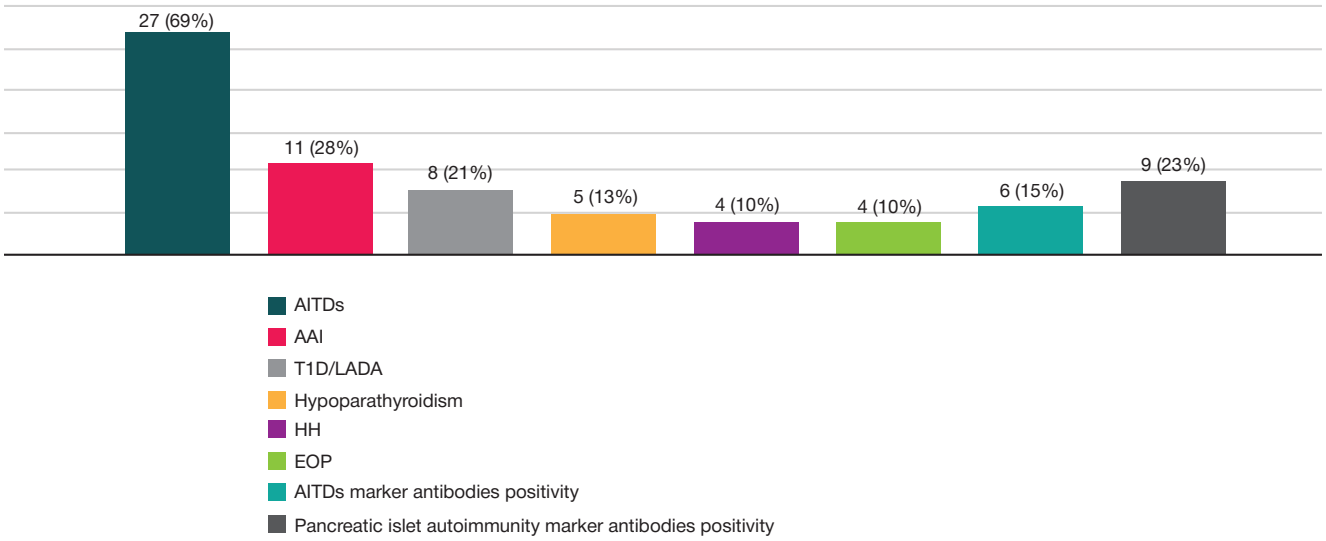

Fig. The prevalence of autoimmune endocrine disorders in the cohort of adult patient with vitiligo initially monitored in the Endocrinology Research Centre

DISCUSSION

Our data on the prevalence of AEDs in patients with vitiligo are consistent with the results of other studies [4, 10, 28]. However, there is a report about one patient with AED (GD) among 204 vitiligo patients [16]. Most probably such low prevalence of AEDs reported by this paper is due to research methods based on the medical history analysis, while we performed active laboratory screening for AEDs.

According to the results of both first and second parts of the study, vitiligo is most often associated with AITDs, which is comparable with the data obtained by other researchers [4, 5, 10, 11]. At the same time, the study that included 50 vitiligo patients [12] revealed no cases of symptomatic AITDs, however, the authors reported high prevalence of TPO antibodies positivity (50% of cases; our study showed that the prevalence of TPO and TG antibodies positivity with no thyroid dysfunction was much lower, 15%).

Unlike other authors [1, 4, 8–10, 26], we found no signficant predominance of women among patients with AEDs associated with vitiligo, including multiple AEDs (except AAI). At the same time, we found that association of vitiligo with T1D was more frequent in the cohort on men compared to women. However, it is necessary to take into account similar gender differences in the general population (higher prevalence of T1D in men [29] and AAI in women [30]).

Furthermore, our findings confirm some data [5] that vitiligo often precedes AEDs manifestation. The results obtained justify the need for regular screening of vitiligo patients for AEDs.

Nonsegmental vitiligo was found in all patients monitored in the Endocrinology Research Centre and 96% of patients monitored in the Moscow Scientific and Practical Center of Dermatovenereology and Cosmetology. However, it must be noted that nonsegmental vitiligo was diagnosed in both patients with symptomatic AEDs or carriers of antibodies against target organs and patients with no symptomatic AEDs or target organ antibodies positivity. Since our findings do not allow an unambiguous conclusion about the risk of AEDs in patients with various types of vitiligo (due to small number of patients with segmental vitiligo), further accumulation of data is required.

CONCLUSIONS

According to our data, the prevalence of AEDs in patients with vitiligo may vary between 15–85%. Vitiligo is most often associated with AITDs. Vitiligo precedes AEDs manifestation in 30% of cases. Among patients with vitiligo and symptomatic endocrine disorders, AAI is most common in women, while T1D is most often found in men. Vitiligo patients should undergo annual screening aimed at detecting autoimmune endocrine disorders, especially of thyroid disease. In is necessary to inform physicians (primarily dermatologists, endocrinologists, and general practitioners) about the possible association of vitiligo with autoimmune endocrine disorders. Patients should be made aware of the need for annual screening and referral to endocrinologist in case of emergence of the AEDs clinical manifestations.

Table 3. The prevalence of AEDs and antibodies positivity in female and male vitiligo patients initially monitored in the Moscow Scientific and Practical Center of Dermatovenereology and Cosmetology

AEDs	F *n* = 17	M *n* = 9	*p*
AEDs, *n* (%)	4 (24)	0	0.114
AITDs, *n* (%)	4 (24)	0	0.114
Primary hypothyroidism in the outcome of AT, *n* (%)	3 (18)	0	0.18
GD, *n* (%)	1 (6)	0	0.458
AITDs marker antibodies positivity with no target organ dysfunction, *n* (%)	1 (6)	3 (33)	0.065

Note: M — male, F — female.

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LOWER EXTREMITY VEIN THROMBOSIS AND ITS CONSEQUENCES IN STROKE RECOVERY PERIOD

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Post-stroke lower extremity vein thrombosis can be the reason behind complications of embolic nature and death. This study aimed to investigate the influence of provoking factors, frequency and localization of acute thrombosis, post-thrombotic changes in the lower extremity veins during stroke recovery period. The study involved 1315 patients, 885 (67.3%) male and 430 (32.7%) female, ages 18–94 years, mean age 59.23 ± 13.7 years. All participants underwent lower extremity venous duplex scanning in the early and late stages of stroke recovery period. We found no evidence of interconnections between presence of signs of thrombosis and/or its consequences and the pathogenetic variant of stroke the patient had. Acute deep vein thrombosis was diagnosed significantly more often ($p < 0.05$) in the early stage of stroke recovery period. The frequency of acute lower extremity vein thrombosis was 7.8%, post-thrombotic changes — 5.6%. Isolated lesion of the lower leg veins was the most common complication associated with deep veins (49.6%). We have discovered a significant relationship between the side of lower extremity paresis (plegia) of and the side of deep vein thrombosis ($p < 0.001$). No relationship was found between lower extremity superficial and deep vein thrombosis and use of anticoagulants and antiplatelet agents ($p > 0.05$). Excess body weight was associated with damage to the lower extremity proximal veins ($p < 0.05$). Women had lower extremity vein thrombosis significantly more often ($p < 0.05$). Repeated lower extremity venous duplex scanning upon admission to the rehabilitation hospital allowed reducing the risk of venous thromboembolic complications that may develop during the stroke recovery period.

Keywords: stroke, rehabilitation, venous thromboembolic complications, vein thrombosis of lower extremities, stroke recovery period, immobilization.

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Compliance with ethical standards: the study was approved by the Ethics Committee of the Federal Center of Brain Research and Neurotechnologies of the Federal Medical Biological Agency of Russia (Minutes of the Meeting #01/19-09-22 of September 19, 2022); All participants of the study signed a voluntary informed consent form.

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ТРОМБОЗ ВЕН НИЖНИХ КОНЕЧНОСТЕЙ И ЕГО ПОСЛЕДСТВИЯ В ВОССТАНОВИТЕЛЬНОМ ПЕРИОДЕ ИНСУЛЬТА

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Тромбоз вен нижних конечностей, развивающийся после перенесенного инсульта, может быть причиной эмболических осложнений и летального исхода. Целью исследования было изучить влияние провоцирующих факторов, частоту и локализацию острого тромбоза и посттромботических изменений вен нижних конечностей в восстановительном периоде инсульта. У 1315 пациентов в раннем и позднем восстановительном периоде инсульта проведено дуплексное сканирование вен нижних конечностей, их которых 885 (67,3%) мужчин и 430 (32,7%) женщин в возрасте 18–94 года, средний возраст 59,23 ± 13,7 года. Показано, что выявление признаков тромбоза и/или его последствий не взаимосвязано с патогенетическим вариантом ишемического инсульта. Достоверно чаще ($p < 0,05$) острый тромбоз глубоких вен отмечен в раннем восстановительном периоде инсульта. Частота острых тромбозов вен нижних конечностей составила 7,8%, посттромботических изменений — 5,6%. Наиболее часто (49,6%) среди поражений глубоких вен наблюдали изолированное поражение вен голени. Обнаружена достоверная взаимосвязь между стороной пареза (плегии) нижней конечности и стороной тромбоза глубоких вен ($p < 0,001$). Взаимосвязи между тромбозом поверхностных и глубоких вен нижних конечностей и приемом антикоагулянтов и дезагрегантов выявлено не было ($p > 0,05$). Избыточная масса тела была ассоциирована с поражением проксимальных отделов вен нижних конечностей ($p < 0,05$). У женщин тромбоз вен нижних конечностей наблюдали достоверно чаще ($p < 0,05$). Результаты повторного дуплексного сканирования вен нижних конечностей при поступлении в реабилитационный стационар позволили снизить риск венозных тромбоэмболических осложнений у пациентов в восстановительном периоде инсульта.

Ключевые слова: инсульт, реабилитация, венозные тромбоэмболические осложнения, тромбоз вен нижних конечностей, восстановительный период, инсульт, иммобилизация

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Embolic complications of venous thrombosis (ECVT) that develop during the acute period of a stroke aggravate the course of the disease and can cause death. Persons with stroke are more likely to develop ECVT among all patients with somatic disease (one of the highest risk groups) [1–4].

The significance of recovery period ECVT is at least comparable to the acute stage ECVT, however, the former were not investigated as thoroughly as the latter, despite the fact that active rehabilitation with undiagnosed thrombosis (primarily deep vein thrombosis, DVT) in the background may be unsafe [5].

Immobilization is one of the main reasons behind slower venous blood flow in stroke survivors. It hinders operation of the lower extremity musculovenous pump system. In such cases, the mobility is restricted due to the severe condition and/or post-stroke paralysis and paresis, causing DVT [2, 6, 7]. It has also been established that the risk of ECVT increases in the first three months after development of the stroke, with immobilization recognized as the main predisposing factor [8, 9]. It should also be taken into account that during the SARS-CoV-2 pandemic, which partially overlapped this study's patient recruitment stage, thrombosis in general becomes a more frequently diagnosed disease (including the lower extremity vein thrombosis [10, 11]), but if the disease was never diagnosed with a PCR test, the fact of infection cannot be established. Currently, there is limited information available regarding the epidemiology, localization and factors influencing the development of thrombosis during the stroke recovery period [12, 13].

Despite the fact that clinical data confirm the relationship between a stroke and development of ECVT, the strength of this relationship and its dependence on time remain to be clarified [8, 14].

Generally, further investigation of the interconnections between historical, constitutional, clinical diagnostic parameters and lower extremity vein thrombosis (and ECVT), as well as its prevalence and peculiarities of localization in stroke survivors, remains an urgent task.

This study aimed to investigate the influence of provoking factors, frequency and localization of acute thrombosis, post-thrombotic changes in the lower extremity veins during the stroke recovery period.

METHODS

The study included data on 1315 patients describing early and late stages of the stroke recovery period. All patients underwent inpatient examination and treatment at the medical rehabilitation departments of the Federal Center of Brain Research and Neurotechnologies of Federal Medical Biological Agency. Eight hundred and eighty five (67.3%) patients were male, four hundred and thirty (32.7%) were female; the ages ranged from 18 to 94 years, the being 59.23 ± 13.7 years. The inclusion criteria were: stroke less than a year ago; 3 point scored on the Rankin scale for neurologic disability; submission of the results of duplex scanning done in other medical establishment, together with a conclusion confirming unimpaired patency of the lower extremity veins, such results and conclusion serving to eliminate the ECVT development risk during active rehabilitation. The patients had been recruited from 2019 to 2021. The exclusion criteria were: no stroke diagnosis in the discharge summary; signs of acute lower extremity vein thrombosis discovered before admission to the rehabilitation departments; over 3 points scored on the Rankin scale for neurologic disability.

During the first days after admission to the medical rehabilitation departments, all patients underwent lower extremity venous duplex scanning. The scanners used for the purpose were Epiq 5 and Epiq 7 (Philips; USA); the broadband multifrequency linear transducer operated at the frequency of 3–12 MHz. The patients were immobilized post-stroke, therefore, all examinations were performed with them in a horizontal position. To detect thrombosis or post-thrombotic changes, the veins (all accessible segments of superficial and deep veins of both lower extremities) were subjected to compression tests every 1–2 cm. When signs of thrombosis and/or post-thrombotic changes were discovered, the factors registered were the side of the lesion and its localization, which could be great/small saphenous veins and their tributaries in case of superficial veins and the following segments in case of deep veins: external iliac vein (EIV) and/or common femoral vein (CFV), popliteal vein (PV) and/or femoral vein (FV), deep veins of the lower leg. Following the detection of signs of acute thrombosis the patients were examined for flotation, and if that was discovered, the length of the floating tip of the thrombus was established.

All participants underwent transthoracic echocardiography (Echo-CG) done with an Epiq 7 scanner (Philips; USA) with a broadband multifrequency sector transducer operating at the frequency of 1–5 MHz.

In addition to the examinations mentioned above, the program included a complex of neuroimaging tests, ultrasound examinations of cerebral vessels and functional diagnostic tests. The results of this complex are not described in this paper.

The data obtained were processed (statistical processing) with the help of SPSS Statistics 26.0 (IBM; USA) and R software 4.0.2 (R Core Team; Austria). The null hypothesis was rejected at the level of significance of $p \leq 0.05$. Frequency and proportion (in percent) were used to describe qualitative and quantitative variables. Pearson's χ^2 test or Fisher's exact test enabled comparison of the frequencies of qualitative dependent variables between categories of independent (grouping) variables. For quantitative dependent variables, the comparison relied on the Mann–Whitney test. A mixed

Table 1. The frequency of occurrence of varieties of ACVA and pathogenetic variants of ischemic stroke (according to the TOAST classification [15]) in the participants of the study

Type of stroke		Frequency, people	Share, %
Hemorrhagic stroke		8	0.6
Ischemic stroke (IS)	atherothrombotic	465	35.4
	cardioembolic	171	13
	lacunar	33	2.5
	cryptogenic	623	47.4
	other established etiology	15	1.1
Total		1315	100

Table 2. Frequency of acute thrombosis and post-thrombotic changes in lower extremity deep and superficial veins

Nature of changes of the lower extremity veins		Frequency, people	Share, %
Acute superficial vein thrombosis	No	1293	98.3
	Yes	22	1.7
Acute deep vein thrombosis	No	1234	93.8
	Yes	81	6.2
Post-thrombotic changes of deep veins	No	1279	97.3
	Yes	36	2.7
Post-thrombotic changes of superficial veins	No	1278	97.2
	Yes	37	2.8
Total number of patients	Total	1315	100

linear model allowed performing a joint analysis of the effect of gender and body mass index (BMI) on the signs of acute deep and superficial vein thrombosis (SVT).

RESULTS

All patients recruited for the study suffered a stroke. In 193 (13.8%) cases it was a second stroke. Depending on the time elapsed from the acute cerebrovascular accident (ACVA), 882 participants (67%) were established to be at the early stage of the stroke recovery period (up to 6 months) and 433 people (33%) were at the late stage thereof (up to 2 years). Table 1 presents characteristics of the types of stroke.

Lower extremity venous duplex scanning revealed echographic signs of deep and superficial vein thrombosis and consequences thereof in 176 (13.4%) patients (Table 2).

We did not discover a significant correlation between pathogenetic variant of IS and the frequency of diagnosed lesion of the lower extremity veins ($p > 0.05$).

The incidence of acute lower extremity deep and superficial vein thrombosis gradually decreased as the number of days passed since the onset of stroke increased (Fig. 1 and 2).

Mann–Whitney test has revealed the stroke onset-dependent differences between groups with and without DVT to be significant ($p < 0.05$) (Fig. 3), while for acute superficial vein thrombosis that was not the case.

Acute and post-thrombotic changes were considered together in the context of analysis of localization of DVT/SVT lesions and investigation of relationships between lesion localization and various factors.

In total, 117 (8.9%) cases of damage to the lower extremity deep veins (acute thrombosis and post-thrombotic changes) were identified.

The most common (49.6%) type of lower extremity deep vein damage were isolated lesions of deep veins of the lower leg (posterior tibial, peroneal, sural veins) (Table 3). There were no cases of damage to the anterior tibial veins registered in our sample.

Seven (6.8%) out of 103 acute thrombosis cases involved a floating thrombus apex, with the length thereof measuring from 7 to 45 mm, the mean being 29 mm.

Chi-squared test allowed identifying a significant relationship between side of the lower extremity paresis (plegia) and side of DVT ($p < 0.001$). No such relationship has been discovered for superficial veins ($p > 0.05$).

Exact T-test allowed identifying significant differences in body weight between groups of patients with distal and proximal deep vein lesions. Individuals with a larger body weight had lesions in the proximal vein segments significantly more often ($p < 0.05$) (Fig. 4).

A total of 59 (4.5%) cases of damage to the main superficial veins (acute thrombosis and post-thrombotic changes) were identified. Table 4 shows the frequency of lesion detection in specific main superficial veins.

Correlation analysis did not reveal significant relationship between SVT/DVT and use of anticoagulants ($rs = 0.045$ at $p = 0.103$; $rs = 0.154$ at $p = 0.113$) and antiplatelet agents ($rs = –0.036$ at $p = 0.195$; $rs = –0.058$ at $p = 0.067$).

Patient groups without echographic signs of damage to the lower extremity veins and with signs of acute thrombosis or post-thrombotic changes in the superficial and deep veins were compared in search for gender-specific dependencies. The chi-squared test revealed that female patients suffer

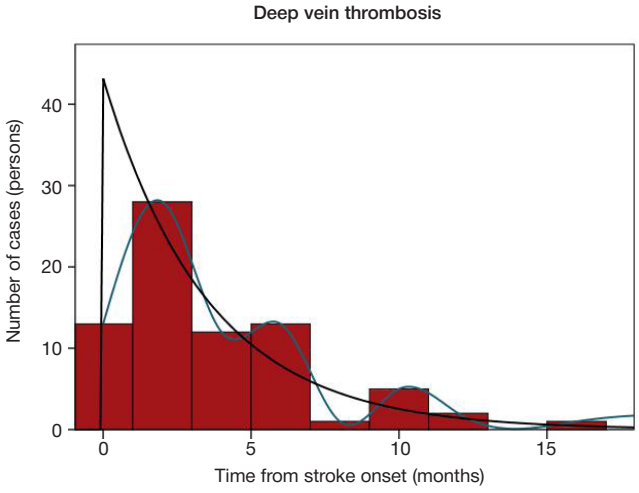

Fig. 1. Time from ACVA to registration of signs of acute deep vein thrombosis

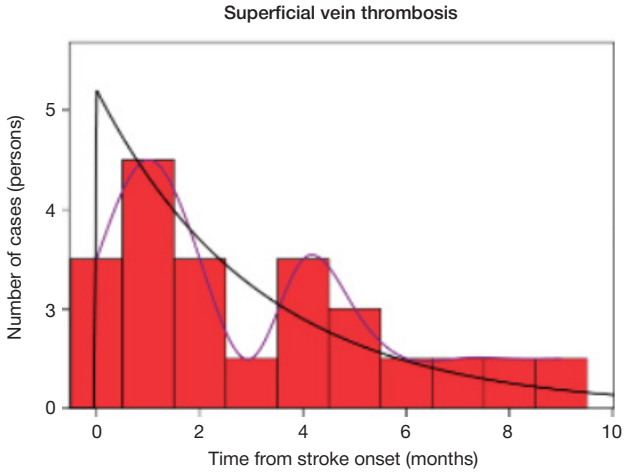

Fig. 2. Time from ACVA to registration of signs of acute superficial vein thrombosis

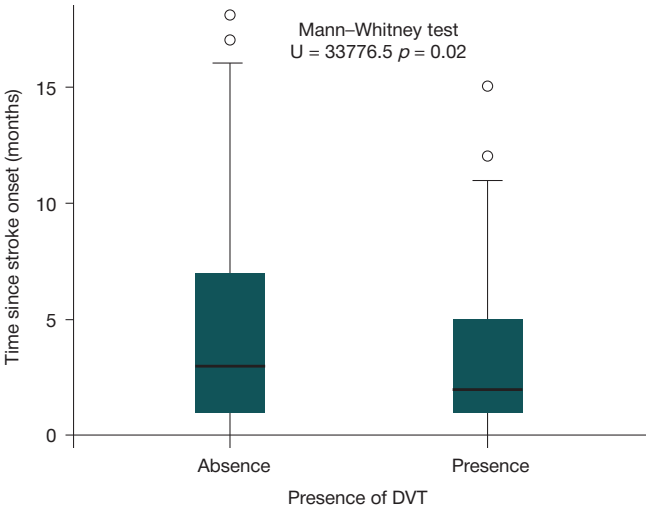

Fig. 3. Manifestations (signs) of DVT depending on time elapsed since the onset of stroke

damage to the lower extremity veins significantly more frequently ($p < 0.05$) (Fig. 5 and 6).

The mean BMI values associated with registered signs of DVT were 25.68 for men and 28.44 for women, those associated with SVT — 27.01 and 29.08, respectively. The differences in BMI of male and female patients not significant ($p > 0.05$). Two-way analysis that factored in gender and BMI with signs of acute SVT/DVT has also revealed no significant differences ($p > 0.05$), however, as a trend ($p = 0.095$), it was established that female patients tend to suffer both SVT and DVT more often as their BMI increases, while for male patients this relationship is inverse.

In the study cohort, echocardiography (and CT) revealed a single DVT case with signs of subclinical pulmonary artery thromboembolia (PATE) that did not result in death.

After registration of signs of acute thrombosis of the lower extremity veins, patients were prescribed anticoagulant therapy or its course was adjusted, the part of the active rehabilitation program associated with movement of lower extremities was limited, intermittent pneumatic compression (if it was done before results of the study have become available) that could contribute to thrombus migration — canceled.

DISCUSSION

The conducted study contains information about an artificial sample of people admitted to the specialized rehabilitation departments for recovery treatment. At the selection stage, the vast majority of patients had to meet a number of requirements (see the study's inclusion criteria). Thus, the studied cohort, despite being large, does not reflect the entire post-stroke population, i.e., it represents such population to a limited extent only.

The presence of SVT/DVT was one of the contraindications for admission. Nevertheless, lower extremity venous duplex scanning done at the Department of Ultrasound and Functional Diagnostics of the Federal Center of Brain Research and Neurotechnologies of the Federal Medical Biological Agency upon admission has revealed a significant number of acute thrombosis manifestation cases, which justifies the need for lower extremity venous ultrasound examination during the stroke recovery period, especially when a patient is admitted for active rehabilitation procedures, which may raise objections due to their economic "inefficiency."

The analysis of cost-effectiveness of routine lower extremity veins ultrasound examination as a thrombosis diagnosing effort [5] upon admission to inpatient rehabilitation has shown that ECVT were established in 6.6% of patients, and for asymptomatic patients that were diagnosed with DVT at ultrasound screening the inpatient rehabilitation period was shorter ($p = 0.045$), PATE incidence smaller ($p < 0.001$) and emergency admissions less frequent ($p = 0.002$) than for those who were diagnosed with thrombosis after the development of clinical symptoms. The authors concluded that routine ultrasound examination of the lower extremity veins upon admission to the inpatient rehabilitation department improves treatment outcomes while having no effect on the cost thereof, which makes such examination a justified measure.

There is no homogeneity in the published data on the frequency of DVT in acute stroke survivors, as well as in the relevant information concerning early and late stages of the stroke recovery period. On average, 12–15% of ACC patients had clinical signs of DVT [2, 16]. There is evidence that less then 10% of acute stroke survivors developed DVT, which had no significant effect on the outcome at the 3 months mark [7]. In another study, DVT was a frequent complication of acute stroke, and hemorrhagic stroke was associated with a higher incidence of damage to the deep veins (DVT was diagnosed in 21.1% of ischemic stroke cases and in 28.5% of hemorrhagic stroke cases) [12] . The frequency of lower extremity DVT in patients with intracerebral hemorrhage was higher than in ischemic stroke survivors, although not significantly [6].

The analysis of results of this study has revealed the incidence of DVT (both acute and chronic) to be at 8.9%, incidence of SVT — at 4.5% (totaling to 13.4%), while the incidence of acute lower extremity vein thrombosis was at 7.8% (103 cases), these value generally being lower that those reported earlier [2, 7, 12, 16] but consistent with data from a study investigating incidence of DVT in patients with acute stroke [7], where 8.7% of ischemic stroke patients also had DVT. Repeated duplex scanning with the aim to detect acute thrombosis of the lower extremity veins may be associated with long-term transportation and forced

Table 3. Localization of lower extremity deep vein lesions

Localization	Frequency, people	Share, %
CFV / IIV / EIV	1	0.8
Combination of CFV/IV/EIV lesions with popliteal and femoral vein lesions	2	1.7
Popliteal and femoral vein	17	14.5
Combination of popliteal and femoral vein lesions and lesions of the deep veins of the lower leg	16	13.7
Isolated lesions of the deep veins of the lower leg (posterior tibial, peroneal and sural veins)	58	49.6
Combination of CFV/IV/EIV lesions and popliteal, femoral vein lesions and lesions of the deep veins of the lower leg	9	7,7
Lesion localization unclear	14	12
Total	117	100

Note: CFV — common femoral vein; IV — iliac veins; IIV — inferior iliac vein; IVC — inferior vena cava.

immobilization of patients at the pre-admission stage. Moreover, the prevalence (and suddenness of occurrence) of venous thrombosis in the inferior vena cava system may be affected by the SARS-CoV-2 pandemic factors [10, 11]. In addition, the possibility of diagnostic errors made at the medical establishments where patients underwent examination for the first time should not be disregarded. All acute thrombosis cases registered in our study were associated with potentially elevated risk of development of PATE, however, after adjustment of the rehabilitation tactics treatment regimens, out of 103 acute thrombosis cases, signs of subclinical PATE (as uncovered with echocardiography and CT) were evident in one case only, with no fatalities.

Patients with longer hospital stays and more severe varieties of stroke are known to be at greater risk of DVT [9, 17]. This is especially important in connection with the peculiarities of our sample, which included patients scoring 3 points on the Rankin scale for neurologic disability, a factor potentially capable of affecting the incidence of lower extremity vein thrombosis.

According to the literature, asymptomatic DVT was found in 11.5% of stroke patients, with 85.9% of thrombi detected in the distal segments of the lower extremity veins [9, 12, 13]. In the vast majority of patients (81%), thrombosis is localized in the lower leg veins, isolated [6]. The data obtained in our study also indicate a relatively higher incidence of isolated lower leg deep vein lesions (49.6%) (combined with lesions in other segments — additional 7.7%) compared with DVT of other localizations (0.8–14.5%).

Ischemic stroke patients develop DVT more often. It typically localizes in the paralyzed leg, however, with prolonged immobilization, there is a risk of bilateral damage [2, 4]. According to some reports, ascending thrombosis and flotation are registered mainly in paretic limbs [6]. Our study has also revealed a significant relationship between the side of lower limb paresis and the side of deep vein thrombosis.

There was no significant relationship discovered between the pathogenetic variant of stroke and the incidence of lower extremity vein damage, which is consistent with previously reported data [6].

As the time elapsed from the onset of stroke increased, the incidence of DVT and SVT in the study cohort decreased, which is probably because of the peculiarities of the sample: there were more individuals at the early stage of stroke recovery period there. This specific feature could also have been affected by the greater immobility of patients in the first weeks and months after stroke before start of the active rehabilitation phase.

According to a number of recent studies, long-term use of direct oral anticoagulants (DOAC) for preventive purposes, compared with combined low molecular weight heparin and an oral vitamin K antagonist, did not cause recurrence of ECVT and decompensated forms of venous insufficiency

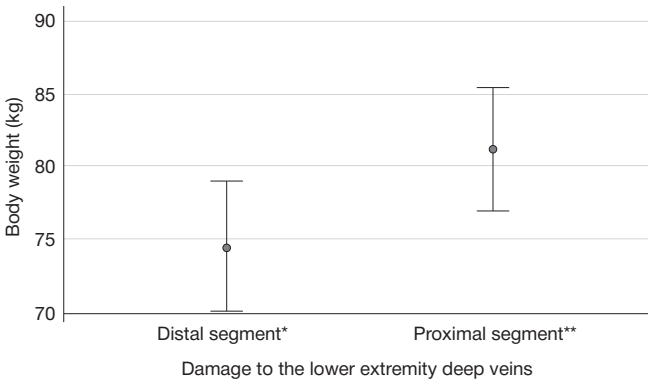

Fig. 4. Body weight of patients depending on the lesion location in proximal and distal segments of the lower extremity deep veins. Distal segment* — lesion of the lower leg deep veins, proximal segment** — lesion of the popliteal, femoral and/or iliac veins with damage (without damage) to the lower leg veins

[18–20]. However, there were described isolated cases of DOAC treatment ineffectiveness, mainly due to the individual differences in concentration of the drug in blood plasma [21]. The results of this study show that there is no relationship between development of lower extremity vein thrombosis and use of anticoagulants and antiplatelet agents. Most likely, this is due to the fact that the majority of study participants have already been taking antiplatelet agents after the stroke as part of the secondary prevention routine, and also received anticoagulants with low molecular weight heparins as part of the standard ischemic stroke therapy, i.e., the patients were in relatively equal conditions or started anticoagulant therapy, including with DOACs, after manifestation of the lower extremity vein thrombosis signs.

The list of established stroke-associated vein thrombosis risk factors includes immobilization, advanced age, obesity, diabetes mellitus, a history of DVT, hereditary coagulopathy [9, 22, 23]. This study has found that patients with a larger body weight were significantly more likely to have proximal segments of the veins affected by the disease. Probably, in such cases, thrombus formation is additionally affected by the factors associated with the increased intra-abdominal pressure conditioned by visceral fat hypertrophy. These factors can lead to compression of the ileofemoral segment with the development of hypertension in the femoral veins and impaired venous blood outflow from the lower extremity [22, 23].

The data in the reports describing incidence of lower extremity vein thrombosis in men and women are conflicting. For example, in 2020 it was shown that, disregarding factors associated with the reproductive function of women, the risk of the first vein thrombosis was twice as high in men than in women [24]. Despite the efforts aimed at studying various factors, the paradox of sex differences as they affect risk of both new and recurrent vein thrombosis remained unexplained.

Table 4. Frequency of acute thrombosis and post-thrombotic changes detection in the main lower extremity superficial veins (various localizations)

Localization	Frequency, people	Share, %
Great saphenous vein (unilateral lesion)	19	32.2
Small saphenous vein (unilateral lesion)	18	30.5
Great saphenous vein (bilateral lesion)	3	5
Small saphenous vein (bilateral lesion)	8	13,6
Great and small saphenous vein	2	3.4
Great saphenous vein (bilateral lesion) and small saphenous vein	1	1.7
Lesion of superficial veins (unspecified localization) combined with damage to deep veins	8	13.6
Total	59	100

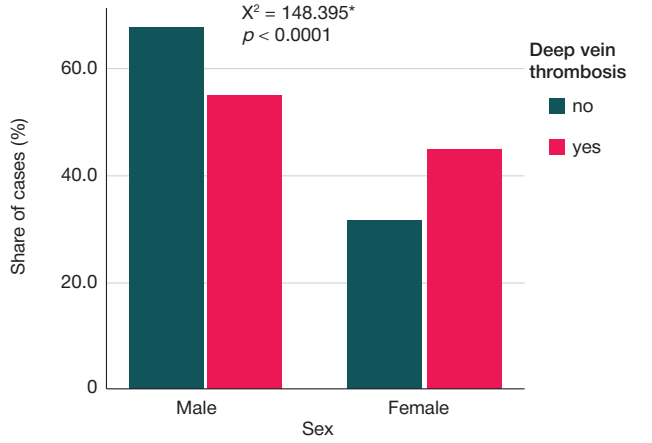

Fig. 5. Frequency of acute DVT depending on gender

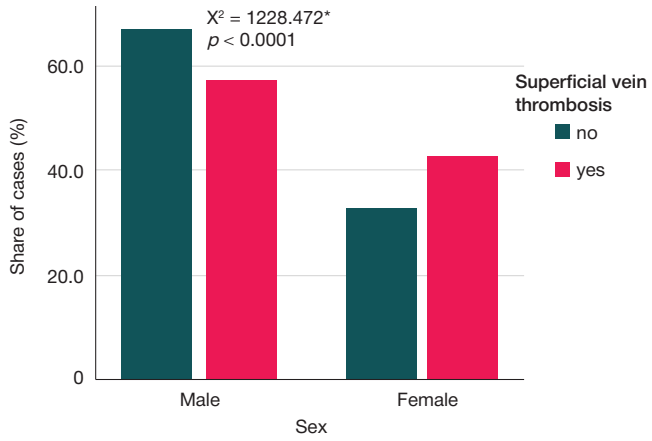

Fig. 6. Frequency of acute SVT depending on gender

There is another study that shows that men run higher risk of first and recurrent vein thrombosis than women [25]. There were suggested several explanations for the sex-associated differences. Body height was the main factor explaining about 20% of the differences in the population-attributable share. The alternative explanations suggested for the said differences hypothesized about presence of X- or Y-linked mutations or a gene mutation with a sex-specific effect [25]. However, in a 2012 study of 323 patients with acute stroke, DVT was significantly more common in women (71.4% versus 49.5%) [7]. According to the results of a 2021 study, female gender and high levels of D-dimer in stroke patients were independent significant factors altering incidence of DVT [26].

In our cohort, women suffered lesion of the lower extremity veins significantly more often, and, given the average age of the patients (59.23 ± 13.7 years), the reproductive system factors can be ignored. One of the probable explanations of this fact is the women's BMI, which, according to the two-way analysis, tended to be higher among those who had signs of the lower extremity vein thrombosis. It can be assumed that increased body weight could play a role in sex-associated distribution. Another probable cause that cannot be ruled is the possible association of higher incidence of thrombosis and increased estrogen levels, especially overweight women, since in such conditions venous tone deteriorates and the circulating blood volume may grow larger [27]. In addition, greater incidence of damage to the lower extremity veins in females may be associated with May-Thurner syndrome (compression of the common iliac vein (usually left) by the common iliac artery). The incidence of this syndrome in the population is up to 20%, and women suffer it more often [28].

CONCLUSIONS

The conducted research allows stating the following. The incidence of acute lower extremity vein thrombosis in the study cohort was 7.8%, post-thrombotic changes — 5.6%. Detection of the signs of thrombosis and (or) its consequences did not correlate with the pathogenetic variant of ischemic stroke, and the incidence of acute thrombosis decreased with the time elapsed from the onset of stroke. Isolated deep vein lesions were more common in lower legs than in other locations. Women have lower extremity vein thrombosis significantly more often. Excess body weight is associated with lesion of the proximal lower extremity veins. Repeated lower extremity venous duplex scanning upon admission to the rehabilitation hospital allowed learning the specifics that altered treatment, prevention and rehabilitation tactics, the alterations, inter alia, aimed at mitigation of the ECVT occurrence risk during the stroke recovery period.

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INFLUENCE OF NEUROPSYCHOLOGICAL STATUS ON BODY SCHEMA IN EATING DISORDERS

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The study of neuropsychological features that cause eating disorders may provide a starting point for planning complex studies that allow for integral assessment of the internal and external mechanisms and patterns of eating disorders. The work aims to evaluate the influence of the neuropsychological status on features of the body schema in eating disorders. We conducted an analysis of the subjective and objective indicators of the body image on 51 women aged 20–35 years using face-relative hand position reproduction tests, the "Silhouette" method, measurement of the right hand index finger diameter and of the foot length, and a self-image questionnaire. We carried out qualitative and quantitative assessment of the neuropsychological status using the Luriev test battery. For the analysis of control functions, we used the Wisconsin sorting card test, Cantidad-Numér interference task (Canum), and "Block Span". We found that women with atypical eating behaviors noted the following features associated with a subjective attitude towards their own body: prevalence of dissatisfaction in one's emotional evaluation due to the perception of one's own appearance, stemming from the beliefs and ideas about one's ideal appearance, despite the absence of the abnormalities associated with the objectified ideas of one's own body (weight, size, body proportions). We identified modal-nonspecific control function deficiencies characteristic of different types of eating disorders.

Keywords: neuropsychological status, eating disorder, body schema

Author contribution: all authors contributed equally to study design, literature review, data collection, analysis, and interpretation.

Compliance with ethical standards: the study was approved by the ethics review board of the Federal State Autonomous Educational Institution of Higher Education Pirogov Russian National Research Medical University of the Ministry of Health of Russia (protocol # 211 of 18 October 2021) and carried out in accordance with the requirements of the Fundamentals of Legislation "On the protection of the health of citizens"; All participants signed a voluntary informed consent for the examination.

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ВЛИЯНИЕ НЕЙРОПСИХОЛОГИЧЕСКОГО СТАТУСА НА ОСОБЕННОСТИ СХЕМЫ ТЕЛА ПРИ НАРУШЕНИИ ПИЩЕВОГО ПОВЕДЕНИЯ

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Исследование нейропсихологических особенностей, обусловливающих нарушение пищевого поведения, может дать отправную точку для построения комплексных исследований, позволяющих осуществить интегральную оценку внутренних и внешних механизмов и закономерностей нарушения пищевого поведения. Целью работы было выявить влияние нейропсихологического статуса на особенности схемы тела при нарушении пищевого поведения. Обследовали 51 женщину в возрасте 20–35 лет. Оценку субъективных и объективированных показателей образа тела проводили с помощью проб на воспроизведение положения руки по отношению к лицу, методики «Силуэт» и изображения диаметра указательного пальца правой руки и длины стопы, опросника образа собственного тела. Качественную и количественную оценку нейропсихологического статуса осуществляли с помощью Луриевской тестовой батареи. Для анализа управляющих функций использовали Висконсинский тест сортировочных карточек, «Cantidad-Numer interference task» (Canum), «Block span». По результатам исследования было установлено, что у женщин с выраженными типами пищевого поведения, рассматриваемыми как граница нормы, отмечены особенности, связанные с субъективным отношением к собственному телу: с одной стороны, преобладает неудовлетворенность, которая включает в себя эмоциональную оценку, чувства, связанные с внешностью, и убеждения и представления об идеальной внешности; с другой стороны, отсутствуют нарушения, связанные с объективирующими представлениями о собственном теле (вес, размер, пропорции). Выявлены модально-неспецифичные дефициты управляющих функций, характерные для разных типов нарушений пищевого поведения.

Ключевые слова: нейропсихологический статус, нарушение пищевого поведения, схема тела

Вклад авторов: все авторы внесли одинаковый вклад в планирование исследования, анализ литературы, сбор, анализ и интерпретацию данных.

Соблюдение этических стандартов: исследование одобрено этическим комитетом ФГАОУ ВО РНИМУ им. Н. И. Пирогова Минздрава России (протокол № 211 от 18 октября 2021 г.), проведено в соответствии с требованиями основ законодательства «Об охране здоровья граждан»; все участники подписали добровольное информированное согласие на обследование.

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To study eating disorders most qualitatively, one must consider the phenomenon in the continuum of norm and pathology. The most blatant manifestation of pathology is summarized by the concepts of "eating disorder" (F50, ICD-10) and "nutrition and eating disorders" (6B8, ICD-11), understood as a class of psychogenic behavioral syndromes characterised by abnormalities in the eating behavior and associated with physiological symptoms. Determining the boundary of the norm is associated with much greater difficulties due to the need to define the concepts of “violation”, “pre-disease”, “donosology” and correlate them with the qualitative and quantitative features manifested in the eating behavior. Aside from the natural and

obvious research- and practice-related reasons for such an approach, we identify one more: in borderline-normal conditions, success of the clinical and psychological assistance will rely on whether the intervention is based on seeing the psychological factors and determinants that underlie the psychogenic nature of the eating behavior as a developing system of phenomena on psychophysiological, neuropsychological, personal, and socio-psychological levels. Thus, by investigating the neuropsychological status on the margins of various types of eating behavior, we attempt to find deficiencies in higher mental functions and to carry out qualitative and quantitative analysis in order to identify systemic characteristics of eating disorders, allowing, in the future, to implement factor analysis that incorporates the neuropsychological point of view.

In research on the degree of disorder severity that allows the affected to remain within the norm, a number of authors identify intermediate states, called "donosology" [1]. Donosology is understood as a change in immunological resistance under the influence of low intensity industrial and domestic factors. Such people are not sick, although assignment to groups with prepathological conditions is recommended [2].

Specialists must timely identify the state of donosology to assist in increasing the body's resistance to adverse conditions [3].

Brekhman singles out the third state as incomplete health, which can last for a very long time and which cannot be identified with premorbid states [4]. Up to half of the entire human population exists in this state [3].

Impaired eating behavior is a broad term, and its characteristics are both substantial and structural. Substantial manifestations include overeating and compensatory energy-consuming forms of behavior (vomiting, abuse of laxatives, diet pills, diuretics, and compulsive weight loss exercises) [1]. These manifestations are habits, which does not allow them to be classified as real disorders, such as anorexia nervosa, bulimia nervosa, atypical eating disorders (or eating disorders not classified elsewhere) [5].

Traditionally, eating disorders are classified as disorders exhibiting the following features:

– a clear change in eating habits or behavior associated with weight control;

– behavior change leading to clinically significant damage to physical health or psychosocial functioning (cardinal symptoms of impaired eating behavior include malnutrition and concomitant overestimation of shape or body weight);

– the aforementioned changes in behavior not being the consequence of any somatic or other mental disorders [6].

The structure of eating behavior as a complex system from the standpoint of determining the safety-violation of the system can be analysed employing the criteria of divergence, coherence, and organization, as proposed in the framework of the metasystem approach to understanding the features of the development and functioning of complex systems, as well as their structural-level organization [7]. Seeing specific forms of behavior, behavior strategies, behavioral patterns as elements of the system at different levels, low divergence is defined as a rigid fixation on a limited number of behavioral food types and strategies, and low coherence is defined as a decrease in the interconnections of both horizontal and vertical elements of the system, which leads to difficulty in flexible switching between them or even complete lack thereof. The disorganization of eating behavior as a system is manifested in the difficulties associated with planning, control and arbitrary regulation of eating behavior [7].

As a rule, three main types of eating behavior are believed to exist: external eating behavior as a reaction to external stimuli, emotional eating behavior as a hyperphagic reaction to stressful situations, and restrained eating behavior as excessive self-restraint and overcontrol [8], the analysis of the severity and stability of which is usually considered as basis for diagnosing the severity of violations. In this framework, disorder is believed to manifest when one of the types of eating behavior [9] begins to noticeably predominate compared to others [6].

In this study we accept the definition of the body scheme as an unconscious internal representation, a complex of information about the structural organization of the body, its dynamic characteristics, the current and changing position of its parts relative to each other, as well as in the horizontal plane [10]. The body scheme is a dynamic subjective entity, since it is formed by the person themselves in the course of their vigorous activity [11]. A person creates a body schema from various manifestations of body awareness in various life situations [12]. The basis of the body schema is a complex of organized information about the dynamic system [13].

Neuropsychologically, the body schema can be considered a functional system, which consists of proprioceptive, gnostic, antipathy-prognostic and control functions.

At the proprioceptive level, the body schema is represented by a joint-muscular feeling and is understood as a complex type of sensitivity that creates the basis for a sense of the relative position and ratio of the sizes of parts of one's own body; the structure of the psyche, reflecting the structure of a person's own body; a flexible, dynamic representation of the subject about his own body, which is continuously created and changed by a person during his life [14]. The gnostic level of the body schema is represented by tactile, muscular, and visual images. Some authors give the following definition:

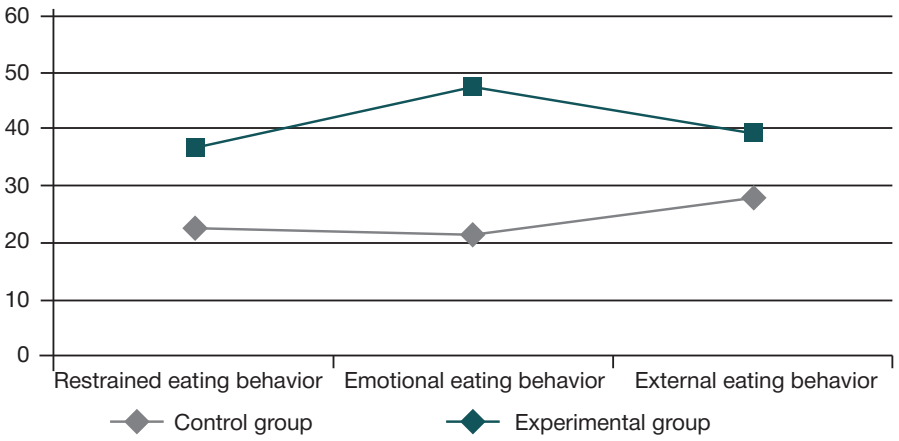

Fig. 1. Diagram of average values of severity of eating behaviors in empirical groups. * — significant difference (i.e. $p \leq 0.05$).

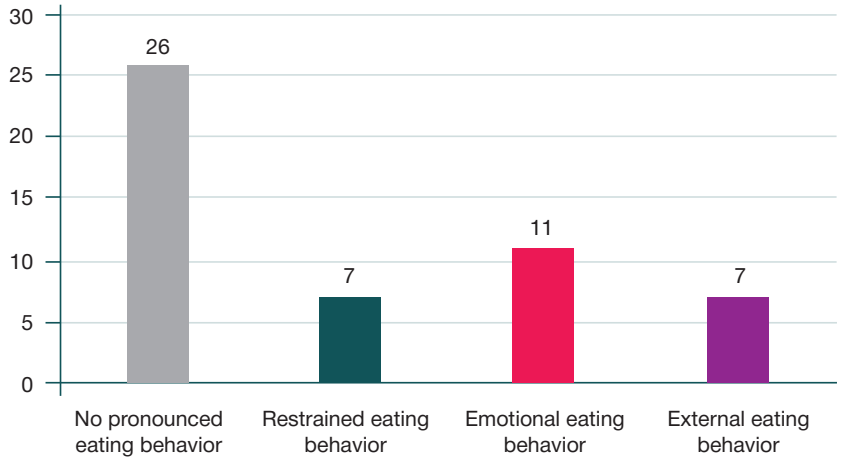

Fig. 2. Diagram of the distribution of the empirical sample in groups

"actively organizing and modifying the impressions produced by incoming sensory signals / stimuli in such a way that the final experience of the position of the body or its location enters consciousness, connecting with what happened before" [15].

Anticipation is understood as the ability of a person to anticipate the course of events with a high probability, predict the development of situations and their own reactions to them, and act with a temporal-spatial lead [16]. Control functions provide arbitrary ways to control behavior: programming, regulation and control [17]. The described level allows for the widest possible analysis and synthesis of ideas about one's own body in the context of the current physical and social situation.

To summarise, we aim to investigate the neuropsychological status on the margins of various types of eating behavior, search for deficiencies in higher mental functions and carry out qualitative and quantitative analysis in order to identify systemic characteristics of eating disorders, allowing to develop a type of factor analysis that incorporates the neuropsychological point of view.

METHODS

The following methods were used: the Dutch Eating Behavior Questionnaire [8]; a test to reproduce the position of the hand in relation to the face [18]; the "Silhouette" technique [18]; image of the diameter of the index finger of the right hand and the length of the foot [19]; self-image questionnaire [20]; Luriev test battery [10]; Wisconsin sorting card test [21]; "Cantidad-Numer interference task" (Canum) [22]; "Block span" [23].

An empirical study on a sample of 51 people (female, age 20–35 years, considered healthy) was carried out from September to December 2021. All study subjects participated voluntarily with informed consent. Experimental group inclusion criteria: >35 points on at least one of the scales corresponding to three different eating behaviors according to the Dutch Eating Behavior Questionnaire: the average value was 36.7 points for restrained eating behavior, 47.7 points for emotional eating behavior, 39.5 points for external eating behavior. In the control group (n = 26), the average values do not exceed 27 points (Fig. 1).

Thus, the total sample was distributed as follows: experimental group 1 (n = 7) — restrained eating behavior; experimental group 2 (n = 11) — emotional eating behavior; experimental group 3 (n = 7) — external eating behavior; control group (n = 26) — no pronounced type of eating behavior (Fig. 2).

RESULTS

As a result of the study of the features of the body schema in eating disorders, statistically significant differences were revealed in the empirical groups in terms of the image of one's own body.

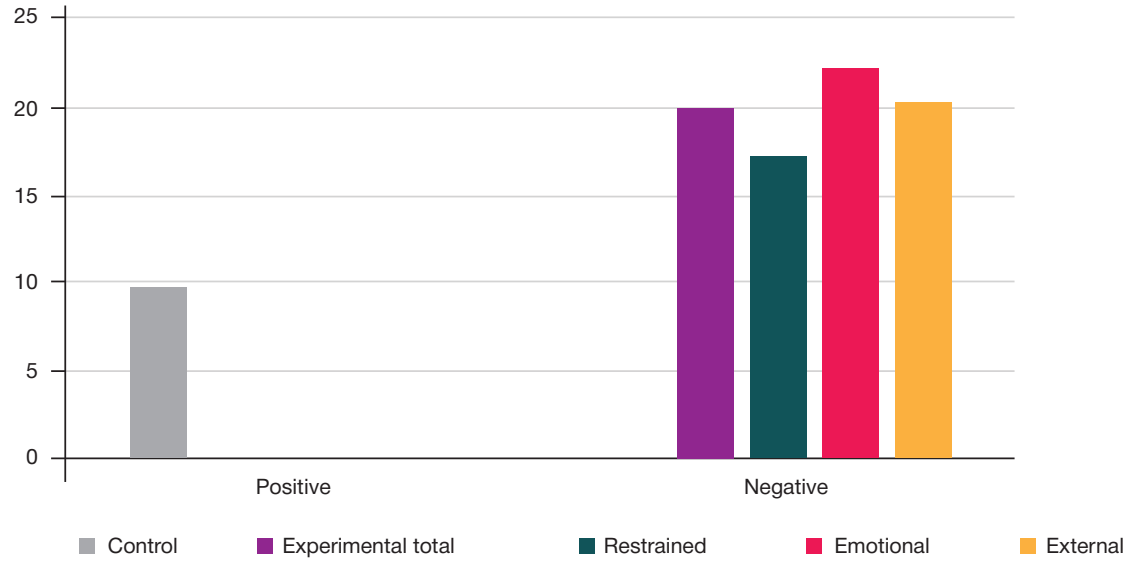

Fig. 3. Diagram of average values of measurements of the image of one's own body in empirical groups

Table 1. Mean values of neuropsychological status indicators (gnosis, praxis, auditory-speech memory) in the empirical groups

	Visual object gnosis			Stereognosis			"Fist-'Side'-Palm" dynamic praxis test			"Fence" graphic test			Oral praxis			Auditory memory	
	Speed	Accuracy	Differentiation	Speed	Accuracy	Differentiation	Speed	Accuracy	Differentiation	Speed	Accuracy	Differentiation	Speed	Accuracy	Differentiation	Speed	Accuracy
Control group (n = 26)	12.4	4	4	4	4	4	3.1	4	4	4.6	4	4	2.8	4	4	3.6	3.5
Restrained eating behavior (n = 7)	16.7	4	4	4	4	4	5.8	4	3.5	4.9	3	4	5.8	4	4	9.3	2.5
Emotional eating behavior (n = 11)	13.4	4	4	4	4	4	3.2	3	3	4.8	2.5	3	3.1	3	3	4.2	3
External eating (n = 7)	14.01	4	4	4	4	4	3.6	3	2.5	4.8	2.5	2.5	3.2	2.5	3	5.3	3

The attitude towards one's own body in subjects with eating disorders is primarily that of dissatisfaction, comprised of two components: an evaluation component includes emotional assessment and feelings associated with appearance, and the cognitive component is comprised of beliefs and ideas about the ideal appearance, a cognitive idea about the body scheme. Dissatisfaction with one's own body is associated with a real change in weight and the sensations generated by this process. Methodologically, the questionnaire aims to make a global assessment of the body, which includes satisfaction or dissatisfaction with one's weight, the shape of the body as a whole and its individual parts.

When comparing the results of the survey in the group without a pronounced type of eating behavior and in the combined group with different severity of types (Fig. 3), the following average values were obtained ($p \leq 0.05$): in the control group, $X_{av.}$ = 9.76 points, i.e., on average, in the group, there is an acceptance of the image of one's body and a positive attitude towards it; in the experimental group, $X_{av.}$ = 19.81 points, i.e. notable dissatisfaction with one's own body, which leads to a significantly low assessment of their appearance ($p \leq 0.05$).

When determining the differences in each of the empirical groups, a significant ($p \leq 0.01$) decrease in indicators associated with satisfaction with one's own body was revealed in groups with emotional ($X_{av.}$ = 22.08) and external ($X_{av.}$ = 20.2) eating behaviors; in the group with restrained eating behavior, a decrease in satisfaction was also noted ($X_{av.}$ = 17.6) at the trend level.

Results of application of methods that objectify the idea of one's own body showed no significant differences between the empirical groups.

To identify neuropsychological features that determine the features of the body scheme, a neuropsychological study was carried out, including an analysis of the state of basic higher mental functions (praxis, gnosis, memory, speech), as well as programming, regulation and control functions (visual working memory, cognitive flexibility, executive attention).

Analyzing in general the results of neuropsychological diagnostics of higher mental functions in the empirical groups, it should be noted that the functions were preserved for all subjects (Table 1). All indicators are within the normal range. Trendwise, however, a steady decrease in the speed in performing tests aimed at studying the features of dynamic

Table 2. Severity of mean values of neuropsychological status indicators (visual working memory, cognitive flexibility, executive attention) in empirical groups

	Visual working memory				Cognitive flexibility									Executive attention		
	Block size	Total score	Amount of correct	Memory size	Correct responses	Total errors	Perseverative responses	Perseverative errors	Corrected errors	Non-perseverative errors	Inability to maintain a set	Ability to learn	Concept-level responses	Mistakes	Speed	Concentration
Control group (n = 26)	6.5	48	9.6	7.2	36	8.1	0*	0	0.7*	0	0	1.29	49.3*	0	8.6	0.67
Restrained eating behavior (n = 7)	5.4	32	4.1	6.4	45	19	17.4*	6.1	16.4*	0	0	–9.01	26.7*	16.4*	19.2*	0.3
Emotional eating behavior (n = 11)	5.3	26	4.6	6.1	53	11	16.2*	7.4	11.8*	1	0	–8.17	21.6*	11.2*	11.4	0.12*
External eating (n = 7)	3.8	34	5.1	3.9	47	17	16.6*	5.8	13.5*	0	0	–14.9	18.8*	15.6*	13.1	0.19*

praxis ($X_{av.}$ = 5.8) and gnosis ($X_{av.}$ = 16.7) for subjects with restrained eating behavior should be noted, in addition to faster exhaustion in the study of auditory memory. It is also necessary to point out a number of cases of the influence of homogeneous interference during delayed reproduction. Subjects in groups with emotional and external eating behaviors characteristically made errors associated with accuracy and differentiation in the following tests aimed at studying serial reproduction: "Fence", oral praxis, and dynamic praxis.

According to the results of the study of visual working memory and executive attention, no statistically significant differences were found among the subjects in the empirical groups (Table 2). At the same time, the control group generally scored higher in total, which indicates greater size and stability of working memory. It should also be noted that in subjects with emotional eating behavior, there is a greater scatter in the average values of visual working memory indicators compared to other empirical groups. For subjects with restrained eating behavior, a monotonically decreasing performance curve is characteristic, which is consistent with previous results of a study of higher mental functions. The subjects of the group with external eating behavior are characterized by a smaller length of the chain of stimuli held in the visual field, indicating reduced visual memory.

The study of cognitive flexibility, based on the results of the Wisconsin sorting card test, showed statistically significant differences in the control group compared to the experimental ones in terms of "perseverative responses" ($p \leq 0.05$), "non-perseverative errors" ($p \leq 0.05$) and “concept-level responses” ($p \leq 0.05$). In the empirical groups, the number of perseverative responses is significantly higher than in the control group. The greatest number of perseverative responses is observed in the restrained eating behavior group. A characteristic feature of all empirical groups is negative learning ability.

The study of executive attention allows us to conclude that rate and attention productivity are significantly reduced in the restrained eating behavior group. Groups with emotional and external types of behavior are characterized by a significant decrease in concentration and productivity.

DISCUSSION

Subjects with pronounced types of eating behavior, seen as only marginally within the norm, noted features associated with a subjective attitude towards their own body: on the one hand, dissatisfaction prevails, which includes emotional assessment, feelings associated with appearance and beliefs and ideas about the ideal appearance, on the other hand, there are no anomalies associated with objectifying ideas about one's own body (weight, size, proportions). The neuropsychological status of subjects in the restrained eating behavior group is characterized by modally nonspecific deficits: test performance speed decrease, exhaustion and homogeneous interference during delayed reproduction, a steady working capacity decrease, inertia, and significantly reduced rate and productivity of attention. The revealed features allow us to conclude that the modal-nonspecific factor predominates, which is associated with a decrease in the dynamic characteristics of control functions with restrained eating behavior. In the group with emotional eating behavior, the features of the neuropsychological status are most often manifested, associated with the accuracy and differentiation of the performance of tests, as well as deficits associated with the retention of the motor program, and serial movements. Visual working memory is characterized by instability, executive attention is characterized by a decrease in concentration and productivity. The revealed features allow us to conclude that the modal-nonspecific factor predominates, associated with a decrease in the controlling characteristics of executive functions in the emotional type of eating behavior. In the group with an external type of eating disorder, along with a decrease in the accuracy and differentiation of the performance of tests, there is a pronounced decrease in the volume of visual working memory, as well as a significant number of perseverative errors and perseverative responses, which indicates a decrease in cognitive flexibility. Executive attention is characterized by decreased concentration and productivity. The identified features allow us to conclude that the modal-nonspecific factor predominates, associated with a decrease in the programming of mental activity in the external type of eating behavior.

CONCLUSIONS

The conducted study can be considered an exploratory stage in studying a broader problem: the construction of a neuropsychological model of control functions that determine the features of the body schema in eating disorders.

The study results allow us to identify the found neuropsychological features as subtle deficiencies in the functioning of the third functional block of the brain (which performs programming, regulation and control over ongoing activities) manifested in the work of a modal-non-specific factor. This can be used to determine the targets of clinical and psychological prevention and eating disorder correction programs.

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