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# GENETIC ASPECTS OF BILIARY ATRESIA ETIOLOGY

Isaeva MKh[1] ✉, Belova VA[2], Korostin DO[2], Degtyareva AV[1,3]

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Biliary atresia (BA) is a cholestatic disorder of infancy that is fatal if untreated. Despite years of study the etiology of BA remains unknown. Three etiopathogenic mechanisms may be involved, such as immune dysregulation, environmental factors and genetic susceptibility. Genetic predisposition is being actively studied. Candidate genes associated with BA in certain populations, genes affecting the cholangiocyte cilia function, as well as genes involved in stress responses have been identified. However, the long-term follow-up of twins with BA suggests that genotype is not of paramount importance for the disease development. Both epigenetic patterns and postzygotic somatic mutations may contribute to etiology of the disease. Recently, some evidence is being accumulated on the possible genetic predisposition to certain outcome of Kasai portoenterostomy performed in patients with BA. However, the presence of a number of factors contributing to the development of the disease makes it difficult to identify the genetic markers.

**Keywords:** biliary atresia, biliary atresia etiology, cholestasis, liver disease, genetic factors

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# ГЕНЕТИЧЕСКИЕ АСПЕКТЫ ЭТИОЛОГИИ БИЛИАРНОЙ АТРЕЗИИ

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Билиарная атрезия (БА) — это холестатическое заболевание печени, которое дебютирует в младенческом возрасте и при отсутствии своевременного лечения приводит к циррозу печени с прогрессирующей печеночной недостаточностью. Несмотря на многолетние исследования, этиология билиарной атрезии остается не до конца изученной. Среди факторов, вовлеченных в этиопатогенез БА, выделяют иммунную дисрегуляцию, факторы окружающей среды и генетическую предрасположенность. Роль генотипа пациента в развитии заболевания активно изучается, выявлены гены-кандидаты, ассоциированные с БА в определенных популяциях, гены, влияющие на функционирование ресничек холангиоцитов, а также гены, участвующие в стресс-реакциях. Однако, исходя из многолетних результатов наблюдений близнецов с БА, можно сделать вывод о том, что роль генотипа не является первостепенной в развитии заболевания. Предположительно, в этиологию заболевания могут вносить вклад как эпигенетические механизмы, так и постзиготические соматические мутации. В последнее время накапливаются также данные о возможной генетической предрасположенности к исходу портоэнтеростомии по Касаи, проводимой при БА. Вместе с тем, наличие многих факторов, играющих роль в развитии заболевания, создает трудности в выявлении точных генетических маркеров.

**Ключевые слова:** билиарная атрезия, этиология билиарной атрезии, холестаз, заболевание печени, генетические факторы

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Biliary atresia (BA) is an inflammatory fibrosing obliteration of the extrahepatic biliary tree gradually involving the intrahepatic biliary system and leading to cirrhosis. The isolated (nonsyndromic) forms of the disease are more common (85% of cases) compared to syndromic (embryonal, 10–15% of cases) and cystic forms (5–8% of cases). The incidence of BA varies across countries from 1 : 8,000 (Asia and Africa) to 1 : 18,000 (Europe), and more common for female infants [1, 2]. The clinical presentation of the disease is neonatal cholestasis. However, the differential diagnosis is carried out with a large group of congenital and hereditary disorders, many of which have manifestations similar to BA during the first months of life [3, 4]. The diagnosis is confirmed by morphological examination of liver and bile duct biopsies during surgery. Surgical correction (Kasai portoenterostomy, KPE) and liver transplantation make it possible the overall infants survival with BA to 90% [4–6]. However, the causes of BA, as well as factors determining the treatment outcome remain poorly understood.

### Etiology of the disorder

Prior to becoming familiar with genetic patterns associated with BA and contributing to the treatment outcome, the other factors involved in the BA pathogenesis should be mentioned briefly: immune dysregulation and environmental factors (viruses, toxins).

There is a lot of data on etiological factors, among which the immune dysregulation plays an essential role. BA is a fibro-inflammatory disease characterized by presence of inflammatory cell infiltration and cytokines and/or chemokines overexpression in patients' liver biopsies. The key element in the BA immunopathogenesis is the innate immune response involving activation of NK cells and Th1cells subpopulation, also known as Th1 adaptive immune response involving the effector T cells, which results in inflammation and obstruction [7]. At the same time, there is a decrease in the number of Treg cells, which are able to suppress inflammation. After the onset of obstruction the immune-mediated bile duct damage persists due to activation of Th2 and Th17 responses regardless of the bile outflow restoration [8]. However, no post-transplant disease recurrence is observed, in contrast to other immune-mediated bile duct diseases [9].

The viral or toxic effect on the bile duct epithelium is likely to give rise to new epitopes contributing to initiation and exacerbation of autoimmune inflammation [8, 10]. The following pathogenic viruses are considered: CMV, HBV, human herpesvirus 6, EBV, reovirus and rotavirus [11]. Numerous studies using the PCR technique (detection of viral DNA/RNA) or viral $IgM^+$ and Mx protein immunostaining revealed traces of viral infection in some, but not all, liver tissue specimen [12, 13]. Currently, there is no clear evidence of BA resulting from viral infection due to inconsistent results of studies using no reference samples, methodological inaccuracies and ambiguous data interpretation [10, 11, 14]. Paradoxically, BA does not affect adults infected with these viruses. But viral infection aggravates the course of BA and increases the risk of adverse outcome [12, 13, 15].

Of exogenous toxins able to trigger the outbreak of BA in animals, the previously undescribed isoflavonoid biliatresone was isolated in Australia from plants consumed by livestock during the drought [16]. In zebrafish (*Danio rerio*) larvae, being a common animal model, biliatresone induces the damage to extrahepatic, but not to intrahepatic bile ducts [17]. Despite the fact that humans are not exposed to biliatresone, recognition of bile ducts damage patterns may contribute to uncovering toxins capable of affecting humans.

### Genetic factors and BA

In contemporary literature more data on the patients' genetic susceptibility to BA becomes available. The inheritance of biliary atresia is not Mendelian. As a result, the disorder is not inherited, although several such cases have been reported [18]. High incidence of BA in parts of Asia suggests the greater distribution of genetic variants associated with BA in these populations. However, the environmental factors (nutritional status, viral load, etc.), as well as the differences in diagnostic criteria used by Asian specialists should also be considered [19].

### Twins with BA

Genes that are likely to be involved in biliary atresia are discussed below. The intriguing results of meta-analysis reported in 2020 are worth noting: the authors reviewed the previously reported global cases of twins with BA and discovered that among 35 prominent pairs (19 monozygotic, 15 dizygotic, and one undefined) only one pair (dizygotic) demonstrated concordance for BA, and the other pairs were discordant, i. e. only one twin out of two was diagnosed with BA (97.1%) [20]. A retrospective study performed by Chinese researchers also reported 19 twin sets with BA, all of them demonstrated discordance for BA (8 were monozygotic and 11 were dizygotic) [21].

The twins' discordance for BA suggests that hereditary factors are not of paramount importance for the disease development, since monozygotic twins possess the same genotype. Meanwhile, contamination by infectious or toxic agent should rather have affected both twins in utero leading to the development of the disease. Among all reported cases of toxic or infectious embryopathy, the twins' concordance (especially monozygotic) is about 80% [22]. It is known that in addition to gene nucleotide sequence alterations, the epigenetic modifications possessing nonclassic genetic inheritance pattern may affect the phenotype. Thus, the monozygotic discordant twins with BA had different phenotypes, which increased the likelihood of the epigenetic factors contribution to the BA pathogenesis [20].

The post zygotic somatic pathogenic variant may also occur in one of the twins, within genes, regulatory regions, etc. being the potential triggers of obliterative cholangiopathy. The hypothesis about BA resulting from somatic mutation (it is better to use the term "pathogenic variant") was proposed in 2016. The authors suggest to analyze DNA from liver tissue and biliary tract of the patients, as well as to perform analysis of parental genomes in order to justify the hypothesis [22].

### Candidate genes

Genetic approaches to BA include the analysis of candidate genes, CNV (copy number variation), genome-wide association studies (GWAS), and whole exome sequencing (WES). GWAS are focused on evaluation of associations between the disorder and the common genetic variants in various populations of the treatment group of patients and the control group of healthy individuals. The table presents some candidate genes associated with BA identified in a number of GWAS.

### Genes *GPC1* and *AGXT*

During one of the GWAS, in two of 35 unrelated children with verified BA (2,026 healthy individuals served as controls) the heterozygous deletion of 2q37.3 was identified [23]. The overlapping deletion (1.76 Mb) contained 30 genes, of which the authors selected the candidate gene *AGXT* expressed in liver. Alanine-glyoxylate aminotransferase (AGXT), the hepatic peroxisomal enzyme involved in metabolism of toxic substances and lipid cleavage, is encoded by this gene. It can be assumed that the reduced activity of AGXT involved in detoxication is essential under the context of the toxin-mediated damage to biliary tract involvement in the pathogenesis of BA. The authors presented a detailed description of patients carrying a deletion 2q37.3. In the first case, the woman worked for a house cleaning service and was often exposed to potentially toxic cleaning agents during pregnancy. In the second case, the woman had a varicella infection at 15 weeks gestation, and was treated with acyclovir, which crossed the placental barrier and was metabolized in hepatocytes. However, in one of cases the patient's father also carried the deletion 2q37.3, but had no liver disease. Therefore, the deletion 2q37.3 may be considered

**Table.** Candidate genes associated with BA according to GWAS

| Locus | Ethnic group | Variant | Candidate gene | GWAS |
|---|---|---|---|---|
| 2q37.3 | Caucasian | Heterozygous deletion | *GPC1/AGXT* | 23/24 |
| 14q21.3 | Caucasian | Non-coding SNP | *ARF6* | 25 |
| 10q24.2 | Han Chinese | Non-coding SNP | *ADD3/XPNPEP1* | 26/27/28 |
| 2p.16.1 | Caucasian | Non-coding SNP | *EFEMP1* | 29 |

**Note:** SNP — single nucleotide polymorphism.

a factor contributing to susceptibility to BA, possibly due to biotransformation of xenobiotics.

In the other study the same researchers increased the sample size to 61 patients with BA vs. 5,088 healthy controls [24]. Deletions 2q37.3 of various lengths were identified in six patients (9.84%) and four healthy individuals (0.08%). However the region of interest contained the deletion of one *GPC1* gene copy. The gene encodes glypican ivolved in regulation of Hedgehog signaling and inflammation. Knockdown of *gpc1*in zebrafish (*Danio rerio*) overactivated the Hedgehog signaling resulting in the developmental biliary defects, smaller gallbladder and poor bile excretion. In the specimens obtained from patients, reduced GPC1 staining was observed on the apical surface of cholangiocytes. The authors concluded that gene *GPC1* appeared to be a BA susceptibility gene.

The case-control study performed by Chinese researchers revealed a possible association with reduced BA risk in 50% of patients with the following *GPC1* haplotypes: $C_{rs2292832}$-$C_{rs3828336}$&$T_{rs3828336}$ or $T_{rs2292832}$-$T_{rs3828336}$ [30].

### Genes *ADD3* and *XPNPEP1*

During the very first GWAS carried out in Chinese population the 500,000 single-nucleotide polymorphisms (SNP) were genotyped in 200 patients with BA and 481 healthy controls [26]. The strongest overall association was found for rs17095355 located on 10q24.2 between genes *XPNPEP1* (X-prolyl aminopeptidase) and *ADD3* (adducin 3). However, the authors failed to determine how the intergenic variant could affect the susceptibility to BA, but suggested that regulation of neighboring genes was involved.

ADD3 encodes adducin 3, the protein belonging to a family of membrane skeletal proteins involved in the spectrin-actin network assembly in erythrocytes, and at sites of cell-cell contact in epithelial tissues, including organs of the gastrointestinal tract, liver and biliary tract [31, 32]. The highest, compared to adults, expression of *ADD3* is observed in fetal hepatocytes and cells of biliary ducts [32]. The contraction of intrahepatic bile canaliculi facilitating the bile drainage is controlled by the actin–myosin interaction. It is important to note that during the experiment with medications the impaired interaction resulted in severe cholestasis [33]. Increased accumulation of actin and myosin around bile canaliculi was observed in patients with BA who did not exhibit bile flow after surgery [34]. Moreover, the α-smooth-muscle actin expression intensity correlated with the degree of fibrosis in patients with BA [35].

*XPNPEP1* is expressed in the biliary epithelial cells [36], it encodes the soluble X-prolyl aminopeptidase or the soluble aminopeptidase P (APP1). The APP1 enzyme contributes to degradation of bradykinin (BK) and substance P (SP) [37]. Bradykinin is involved in vasodilation and vascular permeability, the expression of bradykinin is regulated directly by the nuclear bile acid receptor, farnesoid X receptor (FXR), which plays a part in regulation of bile acid synthesis and secretion, and is involved in inflammation [38–39]. The inflammatory mediator SP is also involved in regulation of bile secretion, hepatobiliary transport and innervation of the liver. The role of hepatobiliary transporters (particularly the FXR) was studied in murine models [40].

This study attracted attention to locus 10q24.2. Several studies of the locus were carried out in different populations [41–46]. It's worth mentioning that the significant association between the described polymorphism rs17095355 and BA was also revealed in Thai population [42].

However, the study of the North American patients' cohort revealed no association to rs17095355, but revealed the association of BA and the other SNP (rs7099604), located in intron 1 of the *ADD3* gene [41]. The quantitative PCR detected significant differences in the expression of *ADD3*, not *XPNPEP1*, in liver tissue between diseased and healthy individuals, but there were no differences in the *ADD3* nucleotide sequence between the groups. Therefore, the diseased individuals may have alterations in the non-coding regulatory DNA regions or epigenetic modifications.

In 2020, the association of three SNPs in *ADD3* (rs17095355, rs10509906 and rs2501577) and two SNPs in *GPC1* (rs6750380 and rs6707262) with BA in the group of Chinese patients ($n$ = 340) was validated [59].

The first model of BA using the induced pluripotent stem cells (iPSCs) was developed in 2019 [60]. The iPSCs obtained from the BA patients' blood differentiated *in vitro* into pathological cholangiocytes with signs of fibrosis. The researchers integrated the BA-associated SNP in *GPC1* and *ADD3* in healthy iPSCs using the CRISPR/Cas9 system and induced the biliary differentiation. These cells reproduced the pathological development of cholangiocytes as BA-specific iPSCs. The iPSC-based models of BA hold great promise for further study of the BA pathogenesis.

### Gene *ARF6*

The BA-associated SNPs rs3126184 and rs10140366 in *ARF* (ADP-ribosylation factor) located in14q21.3 were identified in 2015 [25]. The minor alleles of these polymorphisms were associated with reduced expression of *ARF6*.

Genes *ARF6*, *GPC1* and *ADD3* have similar functions in the formation and development of the biliary tract. *GPC1* and *ARF6* are involved in FGF (fibroblast growth factor) and EGF (epidermal growth factor) signaling playing an important role in organogenesis. Along with *ADD3*, *ARF6* regulates the actin cytoskeleton remodeling, affects the cellular motility and intercellular junctions. ARF6 is activated by binding to the EGF receptor (EGFR) with its activator GEP100, which in turn sequentially triggers other reactions. The further activation of EGFR-GEP100-ARF6 results in activation of MAPK/ERK-CREB signaling cascade, which ultimately affects normal cell development and proliferation [49–52].

Currently, there is no single point of view whether changes in the intrahepatic bile ducts are secondary or independently formed. Thus, four infants diagnosed with BA during their first days of life underwent a number of biopsies, which revealed the paucity of intrahepatic bile ducts with no signs of fibrosis

and cirrhosis always found during Kasai portoenterostomy or liver transplantation [53]. As early as in 1974 Landing proposed that the entire biliary tree was involved in obstructive cholangiopathies: the involvement of intra- and extrahepatic bile ducts hinged of the duration and type of noci-influence [54].

The *arf6* knockdown in zebrafish (*Danio rerio*) larvae resulted in reduced liver size, lower number of biliary epithelial cells, poor bile excretion and impaired development of extra- and intrahepatic bile ducts. Similar effects were observed upon injection of EGFR inhibitor. The authors note that the EGFR-Arf6 signaling pathway may contribute to the intrahepatic bile ducts morphogenesis [25].

Thus, in two of 29 biopsy specimens taken from patients with BA the weak ARF6 immunostaining was detected, as well as the reduced number of intrahepatic bile ducts with signs of fibrosis. Based on these data the authors suggested that the *ARF6* expression downregulation facilitated the defective formation of both extra- and intrahepatic biliary network [25].

### Gene *EFEMP1*

The new susceptibility locus for BA in 2p.16.1 was revealed both in infants with isolated form of BA and with BA combined with other abnormalities [55]. The three BA-associated SNPs, rs10865291, rs6761893 and rs727878B, were detected within intron 5 of *EFEMP1* gene of the described locus. More *EFEMP1* transcripts in the liver tissue (mainly in cholangiocytes and portal fibroblasts) were observed in patients with either BA or other cholestatic diseases compared to control group.

*EFEMP1* gene encodes fibulin-3 involved in the extracellular matrix remodeling, tissue regeneration and organogenesis [56, 57]. Furthermore, *EFEMP1* is an activator of the Notch signaling *in vitro*, although is less efficient than *JAG1* [58]. It is known that fibulins interact closely with laminins and other extracellular proteins. Considering the close contact and proximity of the developing bile ducts with the portal mesenchymal tissue extracellular matrix, we can assume that the EFEMP1 protein is involved in the biliary tract development.

However, the study of Chinese patients with BA performed in 2020 revealed no associations to genes *EFEMP1* and *ARF6* [59].

### Genes *STIP1* and *REV1*

In 2020, the trio analysis of exomes in 30 families with BA in children revealed 66 de novo variants in 66 genes including potentially deleterious variants in *STIP1* and *REV1* [61]. The proteins encoded by these genes interact with the heat-shock protein HSP90 and are involved in stress-responses. The other group of researchers introduced mutations to genes *stip1* and *rev1* of zebrafish (*Danio rerio*) using the CRISPR/Cas9 system and exposed the fish to biliatresone: in contrast to wild-type fish, the mutant fish were sensitive to low doses of biliatresone [62]. The in vitro knockdown of these genes in cholangiocytes and exposure to biliatresone disrupted the cytoskeleton. These results support the hypothesis of the environmental contribution to BA in people with genetic susceptibility.

### Genes of cholangiocyte cilia

The trio-WES of 89 families with BA in children was carried out in 2020 [63]. The researchers detected rare deleterious *de novo* variants in ciliary genes of 31.5% patients and identified three candidate genes: *KIF3B*, *PCNT* and *TTC17*. The patients carrying mutations in these genes had low levels of KIF3B and TTC17 proteins in liver tissue. The knockout of *kif3b*, *pcnt* and *ttc17* using the CRISPR/Cas9 system resulted in impaired bile outflow in zebrafish (*Danio rerio*) larvae. The researchers suggest that the defective cilia may cause hyperactivation of Hedgehog signaling. Abnormal cholangiocytes may also be damaged by bile, which facilitates Hh signaling and leads to inflammation and fibrosis.

### Genetic factors contributing to the outcome of BA

Specific genetic characteristic of the patient may significantly affect the disease severity. Despite the BA phenotypic heterogeneity, there are relatively few studies of genetic factors contributing to the surgical treatment (Kasai portoenterostomy) outcome. According to some authors, such genes as A1AT, *JAG1* and *CFTR* may contribute to surgical outcome [64–68].

The α1 antitrypsin deficiency is a monogenic autosomal recessive disorder (*A1AT ZZ* genotype) leading to liver disease in children. It has been shown that pathological alleles like Z, S, etc. (heterozygous) are more common in children with chronic liver disease ($n$ = 241), including those with BA ($n$ = 67), than in general population [69]. The BA children with such genotypes had lower age of liver transplant compared to BA children with normal MM genotype.

The WES analysis of DNA extracted from liver biopsies of 20 patients with BA obtained during portoenterostomy was carried out in Thailand [70]. After surgery, only seven patients showed signs of jaundice clearance, three patients improved partially, and in 10 patients, surgery had no effect. The 13 rare variants in nine genes responsible for known diseases, including cholestatic disorders, were identified in BA patients (no clinical manifestations of these disorders were observed): *JAG1* (Alagille syndrome, AGS); *MYO5B* (congenital microvillus atrophy progressive familial intrahepatic cholestasis type 6); *ABCB11* (familial intrahepatic cholestasis type 2); *ABCC2* (Dubin–Johnson syndrome); *ERCC4* (Fanconi anemia); KCNH1 (Zimmermann–Laband syndrome); *MLL2* (Kabuki syndrome); *RFX6* (Mitchell–Riley syndrome) and *UG1A1* (Crigler–Najjar syndrome type I). The authors suggest that BA and other liver diseases may have shared etiopathogenesis. Such associations are responsible for disease severity and poor prognosis in BA patients with native liver.

The missense *JAG1* mutations were identified in nine of 102 BA patients with no typical Alagille syndrome phenotype [71]. According to the authors, children with such variants had worse prognosis and portoenterostomy outcome. However, recent studies have shown that Alagille syndrome (AGS) may mimic BA: five children carrying the pathogenic variant in *JAG1* diagnosed with BA in early infancy have developed the typical for AGS clinical signs by 3 years, of age [72].

### Contribution of hepatocellular transporters and nuclear bile acid receptors to portoenterostomy outcome

Over the past decade the large amount of data was obtained describing the liver regeneration due to regulation of hepatocellular transporters (BSEP, MDR1, MDR3, OSTb) and nuclear bile acid receptors (FXR, PXR, CAR) activity in the context of cholestasis [73–75].

Liver adapts well to bile acids accumulation. It has been found that in healthy children the genetic deficiency of these receptors is of no clinical significance due to mechanisms of compensation. However, in patients with cholestatic disorders, including BA, the described alterations may become additional factors affecting the pathologic process. In case of hepatocellular transporters normal functioning

hepatocytes are protected from the toxic effect of bile acids due to their elimination by the BSEP transporter, and the biliary epithelium is protected due to major transporters FIC1 and MDR3 [76].

Based on these data, the studies has been carried out aimed at assessment of hepatic nuclear factors and hepatocellular transporters expression levels as predictors of KPE outcome in children with BA.

It was found that the expression of PXR and/or CAR receptor genes in the liver tissue of patients with poor KPE outcomes was significantly lower compared to patients with favourable outcomes. Five of six patients with low expression of both genes required liver transplant before one year of age (7–11 months) [75]. It had been previously shown that in Pxr knockout rats the liver damage caused by bile acids accumulation was significantly higher compared to healthy animals [77, 78]. It is believed that low levels of CAR and PXR may be associated both with genetic factors and inflammation. It has been determined that these nuclear bile acid receptors mediate the bile acid homeostasis by binding bile acids, are transported to nucleus and downregulate genes encoding the enzymes involved in the bile acids synthesis and reabsorption. At the same time they upregulate genes encoding transporters BSEP, MRP4 and OSTα-OSTβ responsible for export of bile acids from hepatocytes [79–90].

WES analysis was carried out aimed at searching for genetic variants more common in BA patients who required the liver transplant at early age due to no KPE effect than in children with native liver [91]. Among 98 children who required the liver transplant at early age the nonsynonimous variant p.A934T in *ABCB4* was more common compared to a group of children ($n$ = 97) who survived portoenterostomy with their native liver. Downregulation of *ABCB4* encoding MDR3 leads to the decrease in biliary phospholipids level, thus leading to bile acids damaging cholangiocytes.

The whole transcriptome mRNA sequencing of 29 liver samples obtained from patients with BA and differential expression analysis carried out in 2020 [92] identified the potential determinants of the KPE outcome: matrix metalloproteinase 7 (MMP7) and phosphoenolpyruvate carboxykinase (PCK1). MMP7 enzyme is involved in extracellular matrix remodeling in liver fibrosis; PCK1 is involved in gluconeogenesis, and its role in BA remains unclear. MMP7 expression was significantly elevated in patients who failed to clear jaundice after KPE as well as in patients with end stage liver disease. In contrast, PCK1 level was upregulated in patients who had successful KPE, while there was a significant downregulation in patients who failed KPE.

Thus, the expression patterns of various genes in liver tissue and bile ducts may be used as biomarkers for prediction of KPE outcome allowing the healthcare specialists to develop new BA treatment strategies.

### Epigenetic factors

The BA pathogenesis may be based on epigenetic modifications (for example, DNA methylation, histone modifications, expression of non-coding RNA. etc.). DNA methylation was significantly reduced in bile duct cells from BA patients compared to patients with other cholestatic disorders [93]. That could lead to IFNγ-sugnaling activation and inflammation. Various epigenetic modifications were identified in peripheral blood leukocytes (for example, $CD4^+$ T, Treg) of a number of BA patients [94–97].

It has been shown that hypomethylated *PDGFA* gene (platelet derived growth factor subunit A) is upregulated in the affected liver biopsy specimens possibly contributing to the BA pathogenesis [98]. The PDGF family proteins induce proliferative and fibrotic disorders in many organs. Thus, the variant rs9690350 (G>C) in *PDGFA* was associated with increased risk of BA in 506 BA patients when compared to 1473 healthy individuals [99].

The expression level of some miRNAs in the liver of BA patients was different compared to a group of healthy individuals. For example, mir-29b and mir-142-5p were overexpressed in the liver, despite the fact they targeted genes encoding key enzymes DNMT1 and DNMT3 involved in DNA methylation [100]. The other miRNA, mir-145-5p, targeted *ADD3* gene, it was downregulated in liver tissue of some BA patients [101].

## CONCLUSION

Despite years of study the etiology of BA remains poorly understood. Genetic research revealed no specific alterations. The disorder appears to have multifactorial etiology, which includes genetic alterations (inherited or somatic mutations), and epigenetic modifications due to genetic alterations or environmental factors (toxins, viruses).

The BA surgical correction (Kasai portoenterostomy) proposed as early as in 1955 makes it possible to preserve the liver function and to postpone transplantation. The factors affecting the surgical intervention effect and overall survival in patients with native liver remain understudied. Specific genetic characteristic of the patient as well as expression patterns of various genes in liver tissue and bile ducts are considered as prognostic biomarkers. However, further research is required.

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# INTERACTIONS BETWEEN GENE POOLS OF RUSSIAN AND FINNISH-SPEAKING POPULATIONS FROM TVER REGION: ANALYSIS OF 4 MILLION SNP MARKERS

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This study explored the gene pools of Russian and Karelian populations of Tver region. Forty-one samples representing Tver Karels ($n$ = 11) and Russians residing in the Western, Central and Eastern districts of Tver region ($n$ = 30) were genotyped using a genome-wide panel of 4,559,465 SNPs. In order to investigate the phenomenon of genetic admixture between Slavic and Finnish-speaking populations, the obtained results were compared to the data on the Russian populations inhabiting the neighboring territories, Karels from Karelia and other North Eastern Europeans. Studying the gene pools of Russian populations with a genome-wide SNP panel is essential for cataloging their genetic diversity and identifying the distinct features of regional gene pools; in addition, it provides valuable data for practical pharmacogenomics and forensics. Using the principal component analysis, the ADMIXTURE method and D- and f3-statistics, we demonstrated that the gene pool of Tver Karels is closest to the gene pool of Karelian Karels, despite a long (300 to 500 years) history of living among the larger Russian population and the twentyfold population decline during the 20th century. At the same time, the gene pool of Tver Karels exhibits more pronounced similarity to the gene pool of the studied Russian populations than does any other Karelian population. The genetic admixture between Tver Russians and Tver Karels occurred due to a more intense gene flow from Russians to Karels whereas the gene flow from Karels to Russians was much weaker: Tver Russians turned out to be as genetically different from Karels as Pskov Russians. The genetic similarity of Tver Karels to Karelian Karels assessed with the autosomal SNP panel exhibits a slight shift towards the Russian gene pool and is consistent with the previously published analysis of Y-chromosome lineages in these populations that detected no admixture between Tver Karels and Russians.

**Keywords:** genome-wide genotyping, SNP, Illumina array, gene pool, Karelians, Russians, Tver region, Central Russia

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# ВЗАИМОДЕЙСТВИЕ ГЕНОФОНДОВ РУССКОГО И ФИННОЯЗЫЧНОГО НАСЕЛЕНИЯ ТВЕРСКОЙ ОБЛАСТИ: АНАЛИЗ 4 МЛН SNP-МАРКЕРОВ

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Генофонды популяций Тверской области (русских и карел) изучены по широкогеномной панели из 4 млн аутосомных SNP-маркеров, типированной на суммарной выборке из 41 образца. Эти данные по популяции тверских карел ($n$ = 11) и русских из западных, центральных и восточных районов Тверской области ($n$ = 30) проанализированы на широком фоне русских популяций соседних областей, карел Карелии и других популяций Северо-Восточной Европы с целью изучения феномена взаимопроникновения генофондов славянского и финноязычного населения. Такое изучение генофондов населения России по наиболее обширной из существующих широкогеномных панелей важно для каталогизации геномного разнообразия населения России и характеристики региональных генофондов и имеет практическое применение в фармакогеномике и судебной медицине. Методами главных компонент, ADMIXTURE, d- и f3-статистик показано, что генофонд тверских карел, несмотря на их проживание среди преобладающего русского населения в течение 3–5 веков и 20-кратное сокращение численности в течение последнего столетия, сохраняет наибольшую близость к генофонду карел Карелии. Но при этом генофонд тверских карел более сходен с русским генофондом, чем генофонд других карельских популяций. Сближение генофондов русских и карел Тверской области происходит за счет более интенсивного потока генов от русских к карелам и при малозаметном потоке генов от карел к русским: тверские русские оказались столь же генетически отличны от карел, как, например, псковские. Сходство тверских карел с карелами Карелии по аутосомным маркерам (при небольшом смещении в сторону русского генофонда) согласуется с опубликованными данными по Y-хромосоме (отсутствие детектированного смешения тверских карел с русскими).

**Ключевые слова:** широкогеномные панели, SNP-маркер, чип Illumina, генофонд, карелы, русские, Тверская область, центральная Россия

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The city of Tver and the adjacent territories situated at the border between Central and Northwest Russia played an important role in the country's history in general and the interactions between Russian and Western Finnish-speaking populations in particular. Before Slavic colonization, which started around the middle of the 1st millennium, this area was inhabited by Finno-Ugric tribes, predominantly by the Merya. In the early 12th century, a settlement of merchants and craftsmen, which came to be known as Tver, emerged in the estuary of the Tvertsa river. In the middle of the 13th century, Tver rose as one of the 3 Grand Russian Principalities of the Mongol invasion period. For two centuries, Tver was vying with Moscow for the right to unify Russian lands under its rule, maintaining its status as a center of attraction for human resources.

The 15–16th centuries marked the beginning of Karelian migration from the Karelian Isthmus and the areas adjacent to Lake Ladoga lying to the North-East of today's Tver region. In the wake of the Russo-Swedish war, the migration intensified dramatically. By 1670, as many as 25,000 to 30,000 Orthodox Karelians had fled to the lands of Tver. The refugees settled in the areas devastated by famine and chaos during the Time of Troubles. They started their own closely built settlements away from Russian villages. The subsequent waves of Karelian migration were not so massive [1, 2]. Thus, the exodus of Karels from their homeland produced an ethnographic group of Tver Karels who maintained their native Karelian language (the Finnic subgroup of Finno-Ugric languages) throughout centuries. In 1937, the Karelian national district was established with an administrative center in Likhoslavl. Two years later, in 1939, it was abolished, and the activists of the Karelian movement were arrested. This might have driven some Tver Karels to rethink their ethnic self-identification. According to the censuses, the Karelian population shrank in half during the 20th century, declining from 150,000 people in 1930 (of whom 95% spoke Karelian) to 7,000 in 2010 [3]; still, Karels remained within the borders of their habitat [4].

The fact that 2 ethnic groups, Tver Russians and Tver Karels have been living side by side for over 3 centuries raises the question of possible genetic admixture between these two populations. This question was partially answered in our previous publication on the analysis of the Tver Karelian gene pool, which we conducted using a panel of 49 lineage-informative Y-chromosome SNPs for Eastern European populations [5]. We convincingly demonstrated the genetic similarity of Y-chromosomes between Tver Karels and the indigenous populations of Northeast Europe, especially South Karels and Karelian Veps. The study showed that Tver Karels retained their ancestral Y-chromosomal gene pool throughout more than 10 generations in spite of the dramatic twentyfold population decline and years of mingling with the Russian population. The massive population decline might be explained by a change in the self-identification of Tver Karels and their assimilation by the Russian population. If this explanation is valid, it would be natural to expect that the genome of today's Tver Russians will contain an increased proportion of Y-chromosomal variants typical of Northeastern European populations in general and Karels in particular. It is known that interethnic marriages between neighboring ethnic groups produce a more stable Y-chromosomal gene pool compared to the autosomal gene pool because the majority of such marriages are patrilocal (a woman moves into her husband's home village), i.e. resulting in the geographical migration of mitochondrial DNA and autosomes and no geographical migration of Y chromosomes. Both of these factors might be the reason why the autosomal gene pools of Tver Karels and Tver Russians were hugely mutually influential and became more homogenous than the Y gene pools.

Studying the gene pools of indigenous peoples with a genome-wide SNP panel is essential for cataloging the genetic diversity of the Russian population and identifying the distinct features of regional gene pools. These data are important for pharmacogenomics and forensics. The majority of existing pharmacogenetic protocols have been designed for European populations and may not produce a satisfactory result for the Russian populations which carry other allelic variants; besides, the frequencies of well-studied alleles differ significantly between the ethnic groups living in Russia, similarly to the populations of East Asia and Africa [6, 7]. The studies investigating the frequencies of pharmacogenetic markers in Russian populations have been summarized in a recent review [8]. Data on the gene pools are instrumental in forensic analysis in cases when there is a need to identify the origin of a person using only trace amounts of DNA. Currently, there are a few systems for DNA-based identification, and a few others are still in development, but the key thing is the availability of genetic data on ancestral populations [9, 10].

The aim of this study was to characterize the gene pools of Tver Karels and Tver Russians using a genome-wide panel of 4 million autosomal SNPs and to analyze the gene flow between these 2 populations. Conducted on a large dataset of samples from European Russia, the analysis will serve a more general purpose of exploring the interactions between Slavic and Finnish-speaking populations.

## METHODS

This field study of the Russian and Karelian populations inhabiting Tver region followed the method detailed in [11]. The study included only unrelated individuals who did not share a common grandparent (according to the information they provided in the questionnaire) and whose ancestors from at least 2 previous generations had been born in Tver region, self-identified as Russian or Karelian and had no memories of other ethnicities in their ancestry.

Participants were eligible for the study if 1) both of their grandmothers and both of their grandfathers identified as Russian or Karelian; 2) they were willing to give informed consent to participate.

The following exclusion criteria were applied: poor DNA quality or insufficient DNA amount for whole-genome genotyping.

The population of Tver Karels was represented by 11 individuals whose ancestors came from the central part of Tver Karels' habitat, including Likhoslavl district ($n$ = 4), Maksatikha district ($n$ = 1), Spirovo district ($n$ = 2), and Rameshki ($n$ = 4) district. In 1930, the Karelian population of these 4 regions numbered 88,000, amounting to 58% of the total population of Tver Karels (the distribution was as follows: 15% resided in Likhoslavl, 19% in Maksatikha, 8% in Spirovo, and 16% in Rameshki districts). In 2010, there were only 5,000 Karels living in these 4 districts, constituting 78% of the total population of Tver Karels (36% in Likhoslavl, 13% in Maksatikha, 15% in Spirovo, and 14% in Rameshki districts).

Tver Russians were represented by 30 individuals. Since we aimed to study interactions between the Russian and Karelian gene pools, the plan was to compile the Russian subset in such a way that it would represent the areas that did not overlap geographically with the habitat of Tver Karels but were in the vicinity to it. This strategy appears to be optimal for determining the intensity of gene flow from Russians to

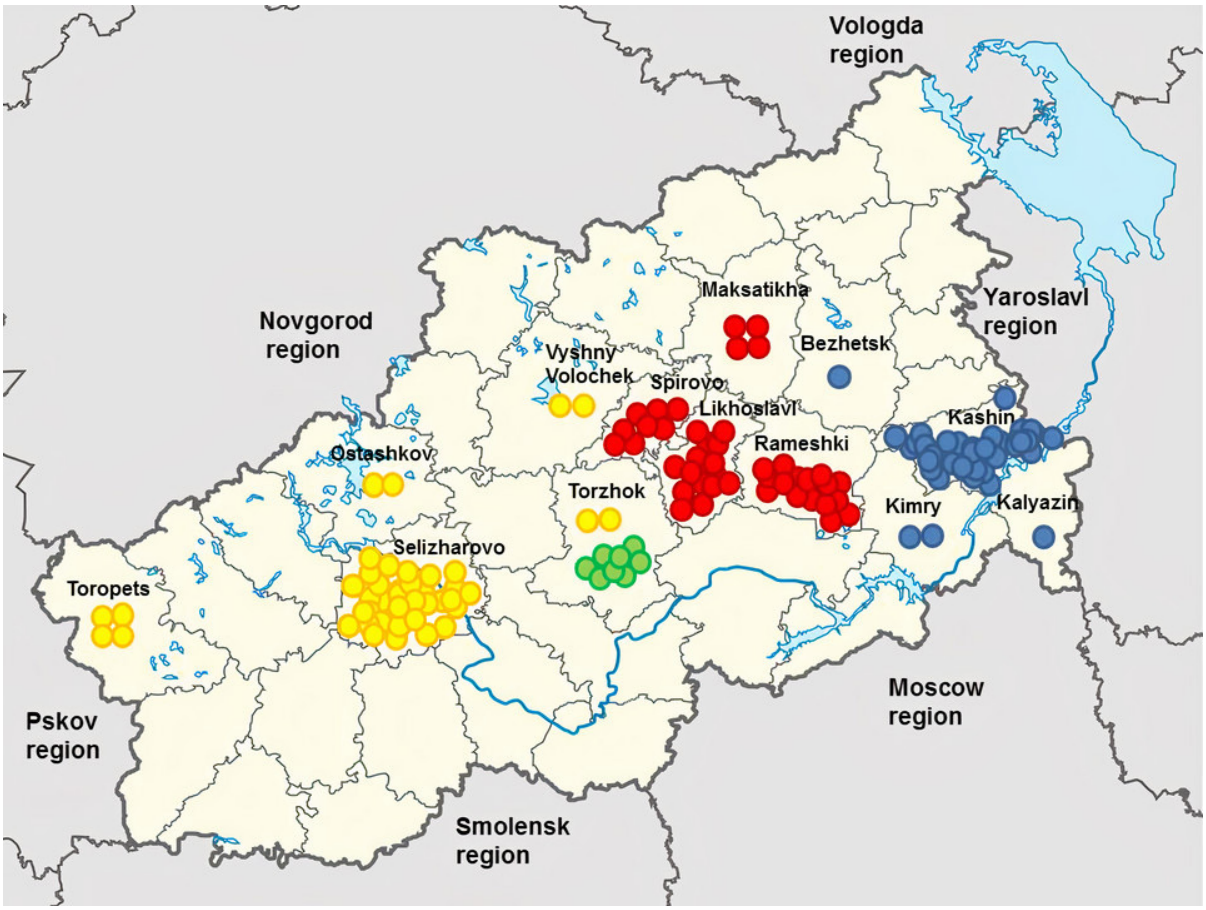


**Fig. 1.** The geographical map of the studied Tver region populations. Circles represent places of birth for each of 4 ancestors (2 grandmothers and 2 grandfathers) of each participant; Tver Karels are shown in red; East Tver Russians are shown in blue; South Tver Russians are shown in green; West Tver Russians are shown in yellow

Karels because the degree of genetic variation between Tver Karels and the populations of remote Russian settlements without a past history of direct contact with Karelians might turn out to be too high due to the genetic differences existing between Russian populations, whereas the degree of genetic variation between Karels and Russians living in Karelian villages might be too low as the Russian villagers might be actually the descendants of the Karels who once started to self-identify as a different ethnicity. For extra control, we studied several Russian populations (instead of one) living at various distances from the habitat of Tver Karels. The Eastern population of Tver Russians occupies the area neighboring the habitat of Tver Karels (Fig. 1). In the autosomal gene pool analysis, this population was represented by 13 individuals, all born in the Kashin district of Tver region. The Western population of Tver Russians was selected in such a way that geographically it was at a greater distance from the habitat of Tver Karels than the Eastern Russian population. The Western subset comprised 15 individuals born in the Selizharovo district of Tver region. Two individuals from the Torzhok district to the south of Likhoslavl, the administrative center of Tver Karels, were allocated to a separate Southern group. Thus, a total of 41 samples collected from the residents of Tver region were genotyped using a genome-wide SNP panel. Fig. 1 shows the places of origin for each of 4 grandparents of every participant.

The gene pool of Tver Russians and Karels was compared to the gene pools of the Russian populations from neighboring territories (Archangelsk, Vologda, Voronezh, Kursk, Pskov, Novgorod, Smolensk, and Yaroslavl) and South and North Karels residing in Karelia ($n$ = 16). It total, 27 Karelian, 100 Russian and a number of other East European genomes (Belarusian, Vepsian, Votian, Izhora Ingrian, Lithuanian, and Ukrainian) were analyzed using the same genome-wide SNP panel. The majority of the listed populations were previously studied using the panels of Y-chromosome markers [5, 12, 13].

All DNA samples, including those collected in Tver region and those representing the group of comparison, were genotyped using a panel by Illumina consisting of 4.5 million SNPs, an Infinium Omni5Exome-4 v1.3 BeadChip Kit (Illumina; USA) and an iScan genotyping system (Illumina; USA). Primary data analysis and quality control were carried out in GenomeStudio v2011.1 (Illumina; USA). For all the studied samples, the CallRate value was at least 0.99. Thus, genotypes were generated for 4, 559 465, SNP markers.

The obtained genotypes were uploaded to the GG-base [14] and are now available for downloading (RussiansTverKashin, RussiansTverSelizharovo, RussiansTverTorzhok, TverKarelians).

Primary data analysis was performed using the classic principal component analysis, which allowed us to identify the overall structure of the studied gene pools. Genetic drift between the studied populations was measured with f3-statistics. The D-statistic was employed to identify the direction of gene flow between the studied populations.

Data filtering was done in PLINK 1.9 [15, 16]. The applied filters are described below.

Prior to PCA, we filtered out SNPs with the genotyping rate of < 95% (geno 0.05) and the minor allele frequency of < 1% (maf 0.01); we also excluded samples with > 10% missing genotype rates (mind 0.1); SNPs that were in high linkage disequilibrium with each other ($r^2$ > 0.2) were pruned using a sliding window of 1,500 SNPs shifting 150 SNPs at a time (indep-pairwise 1500 150 0.2). The output files contained

274,036 SNPs and 126 (of the initial 131) samples. Principal components were computed in EIGENSTRAT smartpca [17, 18] with 5 outlier removal iterations. The results generated by smartpca were visualized using Python 3, pandas [19, 20], matplotlib [21] and seaborn [22] libraries.

To prepare the data for ADMIXTURE analysis, the same filters were applied (mind 0.1, geno 0.05, maf 0.01). After that, SNPs pairs with $r^2 > 0.2$ were pruned. The resultant dataset was analyzed in ADMIXTURE v1.3.0 [23]; cross validation errors were calculated for each k.

F3 statistics measure the genetic drift between two populations, i.e. the degree of their genetic ancestry relative to an outgroup. F3 statistics were computed in qp3Pop (AdmixTools) [24] using a Yoruba population from the 1000 Genomes Project as an outgroup [25]. Apart from the Yoruba dataset, the analysis covered 668 samples genotyped for 3,757,004 markers. The following filters were applied: mind 0.1, geno 0.05, maf 0.01; SNP pairs with $r^2 > 0.5$ were excluded from the dataset. The resultant dataset included 1,144,136 SNPs in a total of 635 samples.

The D-statistic is a tool for detecting genetic admixtures between 4 populations. In its classic version, the most genetically distant population (an African one) serves as an outgroup, and the test identifies the direction of gene flow between 3 remaining populations. The calculations were performed in qpDstat (AdmixTools) using a Yoruba population as an outgroup. In total, 748 samples and 3,757,004 SNPs were analyzed. The following filters were applied: mind 0.05; geno 0.2; maf 0.01; $r^2 > 0.6$. The resultant dataset included 1,355,253 SNPs in 633 samples.

## RESULTS

The position of Tver Russians and Tver Karels in the PCA space which was constructed based on the genome-wide panel of 4,500,000 SNPs is shown in Fig. 3. The Tver Karelian sample is closer to the samples of Karelian Karels and at some distance from the analyzed Russian populations (Tver, Novgorod, Vologda and Yaroslavl). Only one sample of Tver Karels is genetically close to Vologda Russians. All other samples of Tver Karels cluster together, demonstrating little genetic variation. This clustering is consistent with the results of our previous study which analyzed Y-chromosome lineages [5] and concluded that the community of Tver Karels retained its ancestral gene pool.

At the same time, the analysis of the autosomal markers included in the panel reveals a shorter genetic distance between Tver Karels and Russians. Fig. 2 shows that samples of North Karels, South Karels, Tver Karels, and Russians together form a clinal gradient. The closest to Karels are Russians from Vologda region; Tver, Pskov and Central Russian populations constitute a single genetic "cloud". Genetic differences between the Western and Eastern groups of Tver Russians are slight yet pronounced and consistent with their geography: the Western population of Tver Russians shares its genetic space with Pskov samples, which is seen in the PC plot, whereas the Eastern population of Tver Russians (Kashin district) remains on the periphery. Remarkably, two samples from the Eastern population join the Novgorod-Yaroslavl group, which the second principal component differentiates from the rest of the Russian populations (Fig. 2.)

According to PCA, the highest degree of similarity exists between Tver and Karelian Karels but not between Tver Karels and the studied Russian populations. Still, PCA results encourage a hypothesis that the genetic pools of Russian and Tver Karels might be characterized by a slight degree of admixture between each other. Of 3 studied Karelian populations, only Tver Karels shifted towards Russians, whereas Tver Russians, similarly to other Russian populations analyzed in this paper, keep their genetic distance from any of the studied Karelian populations. This suggests that the most intense gene flow occured from Russians to Karels and not the other way around. F3 statistics clarify the degree of genetic similarity between Tver Karels and Eastern European populations: the closest to Tver Karels are Baltic populations, including the Izhora (Inger), the Vote, South Karels, Veps, Lithuanians, and North Karels (listed in the descending order). Russian populations are more genetically distant from Tver Karels and can be arranged in the following descending order based on the degree of similarity: Pskov, Novgorod, West Tver, Smolensk, Kursk, East Tver, Yaroslavl, Vologda, Voronezh, and North East Arkhangelsk. Notably, the genetic similarity between Tver Russians and Tver Karels is far from being pronounced.

The ADMIXTURE analysis can qualitatively and quantitively assess the contribution of ancestral populations to a studied genetic pool. With ADMIXTURE, it is possible to vary the number of populations k to detect common ancestral components with various degree of fractionality.

At $k = 5$ (Fig. 3; Table), significant contribution is visible for only 2 components. Component A is shown in blue; its contribution is the greatest in the speakers of Uralic languages. Component B (Lithuanians, Belarusians, Ukrainians and most Russian populations) is shown in ochre. Component A prevails in Karelian Karels (85%; see Table). By contrast, component B is observed in a few individual samples representing this group but found in every sample of Tver Karels, comprising 41% of their genomes (see Table). Component B occurs twice as frequently in Tver Russians, making up 80% of their genomes. Thus, the results of the ADMIXTURE analysis at $k = 5$ do not contradict the hypothesis about partial gene flow from Russians to Tver Karels.

At $k = 6$ (Fig. 3) the picture becomes more detailed and complex, now showing the contribution of the bright yellow

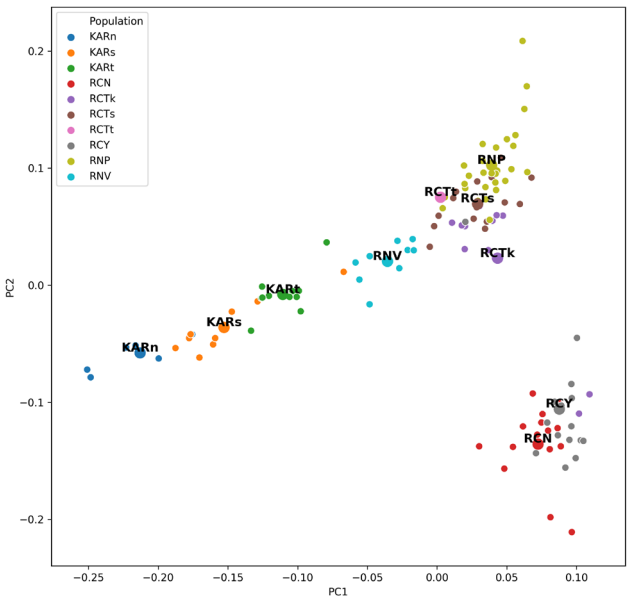


**Fig. 2.** The principal component plot showing genetic variance in the studied populations. KARn — North Karels, KARs — South Karels, KARt — Tver Karels, RCN — Novgorod Russians, RCTk — Tver Russians of Kashin (East), RCTt — Tver Russians of Torzhok (South), RCTs — Tver Russians of Selizharovo (West), RCY — Yaroslavl Russians, RNP — Pskov Russian, RNV — Vologda Russians. Individual samples are marked by small circles; centroids (centers of gravity for each population) are marked by larger circles of the same color

component C (Karelian genomes; see Table). In Karelian Karels, its contribution reaches 100%; it is twice as rare (52%) in Tver Karels and very rare in Tver Russians (8%) and Pskov Russians (4%), indicating that gene flow from these two groups to Karels was either insignificant or zero. Component C is present in Russians because all Eastern European populations share common ancestry. Surprisingly, component C is detected in other populations inhabiting the territories that neighbor Tver region, including the Russians of Novgorod (39%), Yaroslavl (30%) and Vologda (20%).

At $k$ = 8 (see Fig. 3), the chart reflects the differentiation of component C. The bright yellow component (arbitrary termed "West Finnish") still looks influential in Karels and Vologda Russians (96% in Karelian Karels, 53% in Tver Karels, 20% in Vologda Russians; this contribution is designated as component E). However, its contribution to the genome of other Russian populations is minimal. Perhaps, the presence of this component in the genomes of Russian populations does not reflect their recent intermixing with Karels, but is the evidence of historically distant events like the origin of Russian populations from Slavs mingling with authochtonous Finnish-speaking tribes.

Thus, at $k$ = 8 only Vologda Russians are characterized by a prominent (one-fifth of the genome) contribution of the Western Finnish component E. In other Russian populations, where component E is absent, a different component (shown in light gray, I) is observed, Its contribution is the greatest in Novgorod (91%) and Yaroslavl (90%) Russians, accounting for almost entire genome. Component I also constitutes over one-third of the genome in Tver (39%), Pskov (36%) and Vologda (34%) Russians. This "Novgorod" component also occurs in other studied populations of the Central and Southern Russia, making up at least 38% of their genomes.

Based on what proportion of the genome is represented by components I and K (arbitrarily termed "South Russian"), 2 groups of Tver Russians can be identified. Interestingly, these groups are not in accord with their geography genetically: component K is dominant relative to component I in the Western part of Tver region bordering on Novgorod region (K/I = 63/27), whereas in the Eastern population of Tver Russians the contributions of both components are equal (K/I = 42/42); in Central Tver, the "Novgorod" component I comprises the entire gene pool (100%).

## DISCUSSION

This study was conducted using a genome-wide panel of autosomal SNPs. Its findings support the conclusion of our previous study, in which we used a panel of Y-chromosome markers: the genetic distance between Tver Karels and Karelian Karels is closer than between Tver Karels and their Russian neighbors residing in Tver region [5]. Importantly, it was not only descriptive statistics (PCA, ADMIXTURE) but also D-statistics that underpinned this conclusion. Classically, the D-statistic (the f4-statistic) employs one African population as an outgroup. The method helps to understand the direction of gene flow between the 3 remaining populations; its results are considered reliable at $|Z| > 3$. The Z scores generated by the D-statistic (Yoruba, TverKarelians; SouthKarelians, TverRussians) for the Eastern and Western populations of Tver Russians were –6.9 and –5.0, respectively. This proves that the gene pool of Tver Karels is closer to the gene pool of Karelian Karels than to the gene pool of Tver Russians. At the same time, there is more pronounced genetic similarity between Tver Karels and the studied Russian populations than between the studied Russian populations and Karels from South (and certainly North) Karelia. Z scores for Tver Karels become statistically significant if the analysis includes more southern (relative to Tver) populations of Russians. For example, the D statistic (Yoruba, RussiansSmolensk; TverKarelians, Karelians) produces Z = –3.4 for the Russian populations inhabiting the South of Smolensk region. This indicates that the gene pool of Tver Karels, which, on the whole, is similar to that of Karelian Karels, is reliably close to the gene pools of Smolensk Russians and other Russian populations.

To sum up, assuming that initially the ancestors of Tver Karels and Karelian Karels existed as a single population [1, 2, 4], D-statistics prove that later the ancestors of Tver Karels accepted the genetic contribution of populations inhabiting the southern territories of the East European plain. Eastern Europe has witnessed a lot of complex migration patterns, so the source of southern admixture in Tver Karels cannot be identified with absolute certainty; however, history suggests that the best candidates here are the Russian populations of Tver and the neighboring regions.

## CONCLUSION

We have studied the gene pools of Tver Karels and Tver Russians using a panel of 4,500,000 autosomal SNPs and compared them to the samples of Karelian Karels and the inhabitants of Russian regions bordering on Tver (Pskov, Novgorod, Vologda, Yaroslavl). The applied statistical methods (PCA, ADMIXTURE, D- and f3-statistics) generated consistent results.

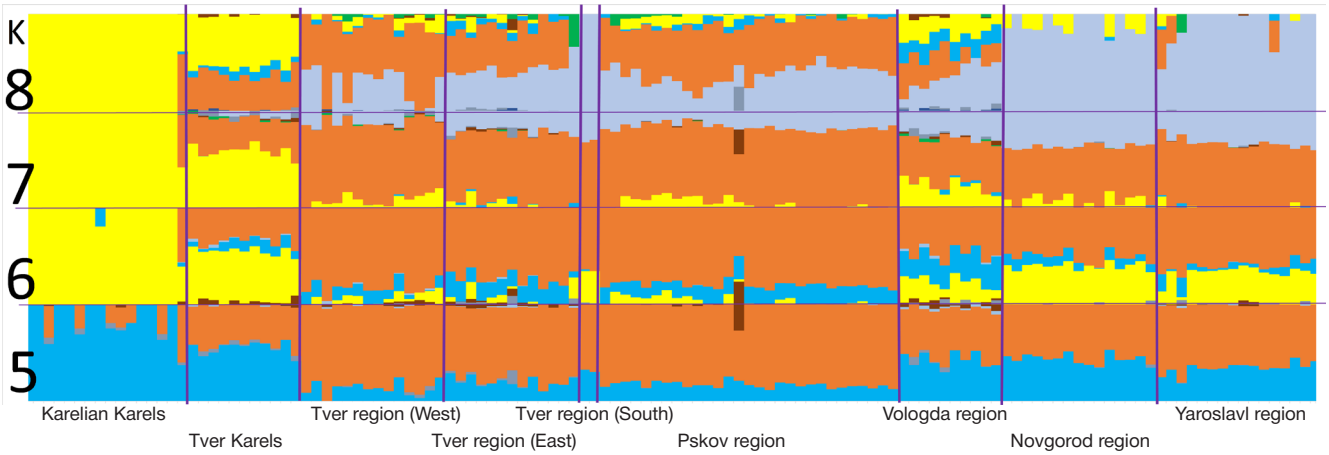


**Fig. 3.** The ADMIXTURE chart (contributions of ancestral components to the studied populations (at different $k$ values). Profiles of individual samples are provided in separate columns; individual samples representing different populations are separated by vertical bars

**Table.** The contributions of ancestral ADMIXTURE components to the studied populations (at different *k* values)

| Fractionality level | Component. | Ethnicity | Karels | | | | | | Russians | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Populations | Karelia | | | Tver | | | Tver (west) | | | Tver (east) | | | Tver (south) | | | Pskov | | | Vologda | | | Novgorod | | | Yaroslavl | | |
| | | Sample size | *n* = 16 | | | *n* = 11 | | | *n* = 14 | | | *n* = 13 | | | *n* = 2 | | | *n* = 29 | | | *n* = 10 | | | *n* = 15 | | | *n* = 16 | | |
| | | Color code for Fig.3 | MEAN | MIN | MAX | MEAN | MIN | MAX | MEAN | MIN | MAX | MEAN | MIN | MAX | MEAN | MIN | MAX | MEAN | MIN | MAX | MEAN | MIN | MAX | MEAN | MIN | MAX | MEAN | MIN | MAX |
| K | | | % | % | % | % | % | % | % | % | % | % | % | % | % | % | % | % | % | % | % | % | % | % | % | % | % | % | % | % |
| 5 | A | Blue | 85 | 37 | 100 | 55 | 41 | 63 | 13 | 0 | 24 | 20 | 10 | 29 | 31 | 30 | 33 | 17 | 11 | 25 | 42 | 30 | 52 | 43 | 34 | 53 | 34 | 18 | 45 |
| | B | Ochre | 13 | 0 | 60 | 41 | 35 | 50 | 85 | 71 | 96 | 75 | 65 | 90 | 69 | 67 | 70 | 83 | 52 | 89 | 51 | 42 | 61 | 56 | 47 | 65 | 65 | 55 | 77 |
| 6 | C | Bright yellow | 95 | 36 | 100 | 52 | 38 | 60 | 6 | 0 | 17 | 5 | 0 | 22 | 33 | 32 | 33 | 4 | 0 | 15 | 20 | 8 | 31 | 39 | 29 | 46 | 30 | 3 | 37 |
| | D | Blue | 2 | 0 | 20 | 9 | 3 | 14 | 10 | 0 | 18 | 18 | 6 | 27 | 1 | 1 | 2 | 16 | 8 | 24 | 28 | 20 | 35 | 9 | 0 | 14 | 8 | 2 | 20 |
| | E | Ochre | 3 | 0 | 56 | 35 | 27 | 45 | 81 | 68 | 97 | 73 | 62 | 84 | 65 | 64 | 66 | 79 | 50 | 86 | 47 | 37 | 60 | 51 | 41 | 62 | 60 | 49 | 72 |
| 8 | F | Bright yellow | 96 | 38 | 100 | 53 | 40 | 59 | 7 | 0 | 19 | 3 | 0 | 16 | 0 | 0 | 0 | 5 | 0 | 13 | 20 | 10 | 30 | 9 | 0 | 23 | 1 | 0 | 13 |
| | G | Blue | 0 | 0 | 3 | 8 | 2 | 13 | 2 | 0 | 7 | 6 | 0 | 15 | 0 | 0 | 0 | 3 | 0 | 11 | 18 | 12 | 22 | 0 | 0 | 4 | 1 | 0 | 7 |
| | H | Light gray | 0 | 0 | 0 | 0 | 0 | 0 | 27 | 0 | 47 | 42 | 31 | 66 | 100 | 100 | 100 | 36 | 13 | 57 | 34 | 8 | 55 | 91 | 72 | 100 | 90 | 43 | 100 |
| | K | Ochre | 3 | 0 | 58 | 37 | 29 | 47 | 63 | 36 | 98 | 42 | 0 | 54 | 0 | 0 | 0 | 54 | 36 | 70 | 24 | 0 | 38 | 0 | 0 | 0 | 6 | 0 | 41 |

The gene pool of Tver Karles retains its similarity to the gene pool of Karelian Karels despite a long (300 to 500 years) history of living among the larger Russian population and the twentyfold population decline during the 20th century. At the same time, the gene pool of Tver Karels exhibits greater similarity to the Russian gene pool, in comparison with other analyzed Karelian populations. Having compared the findings of the analysis of autosomal SNP markers (a partial shift towards the Russian gene pool) and the previously obtained results of genotyping for Y-chromosome markers (no detected admixture between Tver Karels and Russians), we conclude that gene flow between Russians and Tver Karels was predominantly determined by marriages between Karelian men and Russian women.

Demographic data (the sharp decline in the Tver Karelian population) and historical events suggest that Tver Karels changed their ethnic self-identification and were assimilated by the Russian population. Therefore, it would be logical to hypothesize that the genome of Tver Russian descendants of Karels who once changed their ethnic self-identification contains a greater Karelian genetic component. This, however, is not the case: Tver Russians are as genetically distant from Karels as Pskov Russians.

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# SELECTIVE CHANGES IN EXPRESSION OF INTEGRIN α-SUBUNITS IN THE INTESTINAL EPITHELIAL CACO-2 CELLS UNDER CONDITIONS OF HYPOXIA AND MICROCIRCULATION

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Intestinal epithelial cells are constantly exposed to physiologically hypoxic environment. The further reduction of tissue oxygen delivery may result in the intestinal epithelial cells function impairment, being a sign of active inflammation. The cell culture conditions are important when performing *in vitro* studies, since those may affect the cells' properties. The study was aimed to assess the integrin receptor expression in the human colon adenocarcinoma Caco-2 cell line when simulating both the cobalt (II) chloride-induced hypoxia and microcirculation. Transcriptome analysis revealed the significantly increased expression of *ITGA2* and *ITGA5* genes, encoding α2 and α5 integrin subunits, under hypoxic conditions, as well as the reduction of *ITGA5* during incubation in the microfluidic chip. The expression of β-subunits did not change. Analysis of microRNA transcriptomes revealed the decreased expression of hsa-miR-766-3p and hsa-miR-23b-5p microRNA. One of the validated targets for both microRNAs is the *ITGA5* mRNA. It has been shown that microcirculation makes it possible to bring the intestinal epithelial cell culture conditions closer to physiological conditions. The possible biological significance of the detected integrin expression profile alterations and the role of microcirculation have been discussed.

**Keywords:** hypoxia, integrins, microfluidic chip, miRNA, mRNA, proteome, gut microbiota, Caco-2

**Author contribution:** Maltseva DV — working with cultured cells, molecular biology research, data analysis, manuscript writing; Poloznikov AA — proteomic and transcriptomic analysis data processing, bioinformatics analysis, functional analysis of genes, statistical analysis, manuscript writing, study management; Artyushenko VG — interpreting the study results, manuscript reviewing.

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# ИЗБИРАТЕЛЬНОЕ ИЗМЕНЕНИЕ ЭКСПРЕССИИ α-СУБЪЕДИНИЦ ИНТЕГРИНОВ В КЛЕТКАХ КИШЕЧНОГО ЭПИТЕЛИЯ CACO-2 ПРИ ГИПОКСИИ В УСЛОВИЯХ МИКРОЦИРКУЛЯЦИИ

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Кишечный эпителий постоянно находится в условиях физиологической гипоксии. Дополнительная тканевая гипоксия способна приводить к нарушению его функций и является признаком активного воспалительного процесса. В исследованиях *in vitro* значительную роль играют условия культивирования, поскольку в свою очередь могут влиять на свойства клеток. Целью работы было провести оценку экспрессии интегриновых рецепторов в клетках аденокарциномы толстого кишечника человека Caco-2 при моделировании условий гипоксии хлоридом кобальта (II) и микроциркуляции. С помощью транскриптомного анализа обнаружено значимое увеличение экспрессии генов α2- и α5-субъединиц интегриновых рецепторов *ITGA2* и *ITGA5* в условиях гипоксии и уменьшение *ITGA5* при инкубировании в микрофлюидном чипе. Экспрессия β-субъединиц при этом не изменилась. При анализе микроРНК-транскриптомов выявлено уменьшение экспрессии микроРНК hsa-miR-766-3p и hsa-miR-23b-5p. В число валидированных мишеней обеих микроРНК входит мРНК гена *ITGA5*. Показано, что микроциркуляция позволяет создать для энтероцитов кишечника условия культивирования, более близкие к физиологическим. Представлено обсуждение возможного биологического значения выявленных изменений экспрессии профиля интегринов и роли микроциркуляции.

**Ключевые слова:** гипоксия, интегрины, микроРНК, мРНК, микрофлюидный чип, протеом, кишечная микробиота, Caco-2

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The intestinal epithelium normally exists in a state of physiological hypoxia due to the commensal bacteria metabolism [1]. However, pathological processes, including inflammatory diseases and tumors, result in additional tissue hypoxia [2]. Yet the dramatic alterations in transcriptome and structure of the intestinal epithelial cell surface could be observed [3], which may result in the most important intestinal epithelium functions impairment, including barrier function and differential absorption, as well as in impaired interaction with microbiota. Thus, the interest to studying the effect of hypoxia on the intestine function have rised constantly in recent years.

There are several approaches to experimental models of hypoxia *in vitro*: the use of gas mixtures with low oxygen concentration [4], or the use of hypoxia mimetic agents, which increase the intracellular concentration of HIF-1α, the hypoxia-inducible transcription factor. Such hypoxia mimetic agents include oxyquinoline derivatives [5], $Fe^{2+}$ chelators and dimethyloxalylglycine (DMOG) [4]. However, the most widely used hypoxia mimetic agent is cobalt chloride ($CoCl_2$) [6]. It is also used in the experimental models of intestines in vitro [7]. Cobalt chloride induces stabilization of factors HIF-1α and HIF-2α, which are normally rapidly degraded.

MicroRNA (miRNA) is a class of small (of about 22 nucleotides in length) non-coding RNAs responsible for post-transcriptional gene expression regulation [8]. About one half of mammalian miRNA genes are encoded in introns of other genes. Together with the Argonaute family proteins, miRNAs interact with complementary sites in the target mRNAs, usually by partial binding [8]. Generally, the effective interaction with mRNA requires complementarity to a minimal seed region (matching nucleotides 2–7/8 of the miRNA), although often there are some other matching nucleotides. The Argonaute proteins attract other protein complexes which promote translational repression and the target mRNA degradation. About 60% of all protein-coding genes are regulated by miRNAs. A good deal of evidence suggests the key role of miRNA in the broad range of pathophysiological processes, including the intracellular and intercellular interactions [9], cancer, bacterial and viral infections [10]. Thus, the human miRNA families hsa-let-7e / hsa-mir-125a и hsa-mir-141 / hsa-miR-200 have recently been reported, responsible for regulation of genes *ACE2* and *TMPRSS2* being a gateway for coronavirus infection [10].

The Caco-2 cell line is widely used in the experimental models of intestinal epithelial barrier *in vitro* [11]. This cell line was first derived from patient with colon adenocarcinoma. However, when cultured, the Caco-2 cells become differentiated and form the monolayer of polarized cells with prismatic phenotype, expressing a number of intestinal brush border enzymes and membrane transporters specific to small intestine epithelial cells [12]. The proteome analysis data indicate that the differentiated Caco-2 cells resemble the native intestinal epithelium [13]. Our study is focused on the effect of hypoxia on the intestinal epithelial cells' adhesive properties. Integrins play a vital part in adhesion. Integrins are a superfamily of transmembrane receptors responsible for interaction with the extracellular matrix components and the other cell's surface proteins (including those of bacterial cells). All integrins are heterodymers consisting of α- and β-subunits. The 18α- and 8β-subunits have been identified in mammals. In the Caco-2 cells the major subunits include α-2, 5, V and β-1, 3, 4 subunits [14], which is consistent with the integrin subunit expression profile of the human primary enterocytes [15]. The study was aimed to assess the Caco-2 cells' transcriptome and proteome alterations under the normal and hypoxic conditions, as well as to compare the results with alterations in the transcriptome of Caco-2 cells cultured in the microfluidic chip.

METHODS

**Caco-2 cell culture**

The immortalized human colorectal adenocarcinoma Caco-2 cells (Institute of Cytology RAS; St. Petersburg) were grown in the MEM culture medium (Gibco; USA) with the following supplements: 20% fetal bovine serum (FBS, Gibco; USA), 1% (v/v) non-essential amino acids solution (Gibco; USA), penicillin (100 U/mL) and streptomycin (100 μg/mL) (Gibco; USA). The Caco-2 cells were cultured in the 6-well plates (Corning; USA) for 23 days up to the state of fully differentiated enterocytes. The culture medium was changed every 2–3 days. In order to simulate hypoxia, $CoCl_2$ (Sigma-Aldrich; USA) was added to the culture media (Sigma-Aldrich; USA) to concentration of 300 μM and incubated for 24 h in accordance with the previously reported algorithm [7]. After incubation, the cells were washed with the 1× DPBS (Gibco; USA), and lysed for transcriptome and proteome analysis using the previously reported method [16].

The the effect of hypoxia on the cell monolayer was assessed by impedance spectroscopy. Prior to plating of Caco-2 cells on the 96-well plates with membrane inserts (Corning; USA), all the wells were filled with culture medium (50 μl into the upper chamber, 235 μl into the lower chamber) and incubated for 1 h at a temperature of 37 °C in the 5% $CO_2$ atmosphere. Then the Caco-2 cells were plated, approximately 5,600 cells per membrane insert, in the volume of 50 μL, and cultured for 23 days up to the state of fully differentiated enterocytes. The culture medium was changed every 2–3 days. $CoCl_2$ was added to the culture medium in order to induce hypoxia as described above.

In order to assess the influence of perfusion on the transcriptome, the Caco-2 cells were cultured in the microfluidic chip, which was designed during execution of the project "Study of Bacterial Adhesion in a Microfluidic Model of the Human Intestinal Barrier" supported by the Ministry of Science and Higher Education of the Russian Federation. The microfluidic chip is the flow system containing two cell culture wells, each of the wells is separated by membrane with the 0.4 μm pore size.

First, the wells of microfluidic chip were coated with laminin 332 (Biolamina; Sweden). For that, 57 μL of laminin 332 solution in DPBS (0.01 mg/mL) was added to the well and incubated for 24 h in the fridge at a temperature of 4 °C. Then

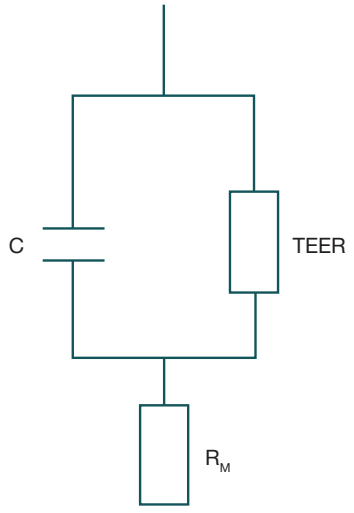


**Fig. 1.** Equivalent circuit used for calculation of the cell monolayer basic electrical parameters

**Table 1.** Primers for qPCR. Nucleotide sequences are read from 5' to 3' end

| Gene | Upstream primer | Downstream primer |
|---|---|---|
| *DDIT4* | gtttgaccgctccacgagcc | cgaagtcgggcaacgacacc |
| *EGLN1* | gacctgatacgccactgtaac | cggataacaagcaaccatg |
| *ITGA2* | gccaataatccaagagttgtgtt | tattttcttgcatattgaattgcttc |
| *ITGA5* | agagctacgggccaagctaa | ttccccataaagtttggtccac |
| *LDHA* | atgggtgggtccttggggaa | tagcccaggatgtgtagcctttga |
| *PFKFB3* | acgcctgtcgcttatggctg | ggctttttggtgggttcgggg |
| *SLC2A1* | gcagatgatgcgggagaagaaggt | atgaggatgggctggcggtag |
| *SLC2A3* | tggtttattgtggccgaactc | ggtaatgaggaagccggtga |
| *VEGFA* | tggcagaaggaggagggcag | aggggcacacaggatggctt |
| *GAPDH* | gaaggtgaaggtcggagtc | gaagatggtgatgggatttc |
| *ACTB* | ctggaacggtgaaggtgaca | aagggacttcctgtaacaacgca |

the Caco-2 cells were detached from the substrate using the 0.25% trypsin-EDTA and the Hank's Balanced Salt Solution. After that the cells were resuspended in the culture medium and counted with the Countess Automated Cell Counter (Invitrogen; Germany). The cell suspension containing 20,000 Caco-2 cells in the volume of 50 µL was added to each well of the chip, then the microfluidic chips were incubated in the cell incubator (5% $CO_2$, 37 °C) for 2 h. After the incubation the chip was connected to peristaltic pump to ensure perfusion in the 50 µL/h mode. The cells were cultured for 2 days in the cell incubator (5% $CO_2$, 37 °C) with regular (at least twice a day) measurement of transepithelial electrical resistance in order to obtain the value of 350 Ohm·cm$^2$. The cells being in state of fully differentiated enterocytes were washed with the 1× DPBS (Gibco; USA) and lysed for transcriptome analysis using the previously reported method [16].

### Study of the collagen IV and laminin 332 membrane coating impact on the Caco-2 cell proliferation

The surface of the Transwell membrane inserts of the Corning 96-well plate was coated with collagen IV and laminin 332. For that, 50 µL of collagen IV solution in DPBS (0.1 mg/mL) or 57 µL of laminin 332 solution in DPBS (0.01 mg/mL) was added to the appropriate inserts and incubated for 24 h at a temperature of 4 °C. Then the inserts were washed to remove the unbound extracellular matrix components, and the plate wells were filled with culture medium (50 µl into the upper chamber, 235 µl into the lower chamber). The plate was put in the cell incubator (5% $CO_2$, 37 °C) and incubated for 1 h. After incubation, the culture medium was removed from membrane inserts; the cell suspension containing 5,000, 10,000 or 20,000 cells was added to the membrane inserts and incubated in the cell incubator (5% $CO_2$, 37 °C) for 96 h. After cultivation, MTS reagent with a concentration of 0.5 mg/mL was added to the culture medium and incubated at a temperature of 37 °C for 4 h. Then the optical density was measured at a wavelength of 590 nm.

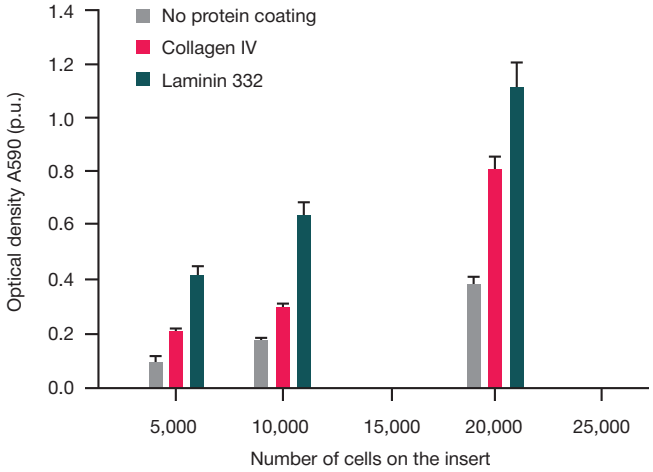


**Fig. 2.** Assessment of Caco-2 cells viability after 96 h of growth on the membrane inserts with no coating, coated with collagen IV and coated with laminin 332

### Impedance spectra measurement and calculation of electrical parameters

The impedance spectra were measured in the frequency range of 40–20,000 Hz using the impedance spectroscopy measurement system (Bioclinicum; Russia) and the STX100C96 electrode (World Precision Instruments; USA) at room temperature. In order to obtain the average values of the electrical parameters, three distinct membrane inserts with cells were used. Calculation of the cell monolayer electrical parameters (transepithelial electrical resistance (TEER), capacity C, and monolayer resistance $R_M$) was performed using the CEISA impedance fitting software (Bioclinicum; Russia) and the cell monolayer equivalent circuit (Fig. 1). The further statistical analysis was carried out using the R 3.5 programming language and the RStudio graphical user interface. The statistical significance of the observed differences in the TEER values was evaluated by the two-way analysis of variance (ANOVA). The P values of less than 0.05 were regarded as statistically significant.

### Transcriptome analysis

Transcriptome analysis was carried out using the Gene Chip Human Transcriptome Array 2.0 microarray for mRNA and the Gene Chip miRNA 4.0 microarray for miRNA (TermoFisher Scientific-Affymetrix; USA). RNA isolation, quality control and quantification were performed in accordance with the previously reported method [16]. The RNA integrity number (RIN) values exceeded 9.5 in all studied samples. The cDNAs were synthesized from 500 ng of isolated total RNA. Sample preparation, hybridization, microarray washing, staining and scanning were carried out in accordance with the manufacturer's instructions. The CEL files obtained by the microarray scanning were processed using the Transcriptome Analysis Console 2.0 software package (TermoFisher Scientific-Affymetrix; USA). Microarrays contained multiple probes designed to hybridize to different gene regions for each gene, which comprised the multiple probe set (probe set). The probe sets not corresponding to any of the known genes (un-annotated probe sets) were

excluded from analysis. When assessing the gene expression, the threshold signal intensity was set at 6.0 on the Affymetrix logarithmic scale.

### Real-time PCR (qPCR)

The qPCR was performed in accordance with the previously reported method [16]. The real-time PCR product accumulation detection was based on SYBR-Green I fluorescence. The *ACTB* and *GAPDH* transcripts were used as reference genes (average threshold cycle values: 25.3 and 23.4 respecively). The nucleotide sequences of primers (Syntol; Russia) are presented in Table 1.

### Caco-2 cells proteome analysis

The Caco-2 cells sample preparation, total protein extraction, hydrolysis and the subsequent procedures were carried out in accordance with the previously reported method [16]. After trypsinolysis, the supernatant fraction was analysed using the Q Exactive HF mass spectrometer with nano-electrospray ionization (nESI) source operated in the positive ionization mode (Thermo Fisher Scientific; USA), with the emitter voltage of 2.1 kV and the capillary temperature of 240 °C. The protein level quantification was performed using the Progenesis IQ software (Waters; USA) with default settings. The proteins were identified using the SearchGUI v.3.3.1 software and the HumanDB database (UniProt Release 2018_05) with the following search parameters: digestive enzyme trypsin, monoisotopic mass accuracy ±5 ppm, mass measurement accuracy on tandem MS/MS data (tandem mass spectrometry) ±25 ppm, and the chance to skip one cleavage site. In order to assess the differentially expressed proteins, the raw data were analyzed with the MaxQuant 1.6 software (Max-Planck-Institute of Biochemistry; Germany) (iBAQ algorithm). The further data processing was carried out using the Perseus software and the R 3.5 programming language with the integrated development environment RStudio 1.1 (R-Tools Technology; USA). Student's *t*-test was used to determine the statistical significance of the differences observed.

### Statistical data processing

The raw microarray data were normalized using the oligo package for the R programming language [17]. The data obtained were log2-transformed. Student's *t*-test was used for analysis of differential expression of genes and miRNA. The false discovery rate (FDR) was controlled using the Benjamini–Hochberg method developed for the multiple hypotheses testing [18]. The miRNA false positive rate was evaluated using the previously reported algorithm [19].

Functional gene annotation was performed using the DAVID version 6.8 databases and algorithms [20]. The experimentally supported miRNA-target interactions were exported from the DIANA-TarBase version 8 database [21]. The miRNA-binding sites in the 3'-untranslated regions (3'-UTR) of miRNA targets were predicted with the miRWalk tool [22]. The miRIAD database was used to explore the intragenic miRNA and their host genes [23].

## RESULTS

### Exposure to $CoCl_2$ mimics hypoxia in Caco-2 cells and upregulates the integrin α-subunits

According to literary sources, coating of the membrane used for Caco-2 cell culture with extracellular matrix proteins, such as collagens and laminins [24], can greatly affect the rate of monolayer formation and cell differentiation. Therefore, when optimizing the microfluidic intestinal barrier model culture conditions, we assessed the effect of the collagen IV and laminin 332 membrane coating on the Caco-2 cell proliferation. The study was carried out using the Transwell Corning membrane inserts. Coating of membrane with laminin 332 more than doubled the Caco-2 cell proliferation (Fig. 2). Therefore, cell culture on the laminin 332-coated membrane was selected for further investigation.

In order to mimic hypoxia, the Caco-2 cells were exposed to $CoCl_2$ (see "Methods"). The impact of hypoxia on the cell monolayer was assessed by impedance spectroscopy. Impedance spectroscopy allows one to measure the electrical impedance (total resistance to sinusoidal alternating current) as a function of the frequency of applied electrical current (see "Methods") [25]. The efficiency of impedance spectroscopy for such kind of research has been previously reported [25]. The average TEER values, both for hypoxia and normoxia, are above 3,000 Ohm (corresponds to resistivity of 429 Ohm·cm$^2$). The TEER values depend both on the intracellular resistance resulting from the state of transmembrane channels responsible for transport of ions, and the state of tight junctions responsible for paracellular resistance. For example, it is known that the TEER values rapidly decrease with decreasing concentration of calcium in the medium, since the calcium ions are essential for maintenance of the tight junctions' normal structure. On the other hand, cell death results in the broken integrity of the membrane and the significantly decreased intracellular resistance. High TEER values observed throughout the experiment are the indication of intact tight junctions and lack of severe cytotoxicity resulting in cell death (Fig. 3).

Analysis of transcriptome for the treated and reference cells was carried out using the Affymetrix microarrays (TermoFisher Scientific-Affymetrix; USA). The significantly increased expression of genes involved in response to hypoxia in accordance with [26] was detected in samples exposed to CoCl2, the alterations revealed were validated using qPCR (Table 2). The significant alterations of expression (twofold or more; FDR < 0.05) were detected in 165 genes. The functional annotation of genes using the DAVID databases and algorithms [20] showed the significant enrichment of the HIF-1 signalling pathway (KEGG-pathway hsa04066), mediating the response to hypoxia (Table 3). Functional annotation revealed no enrichment of antioxidant

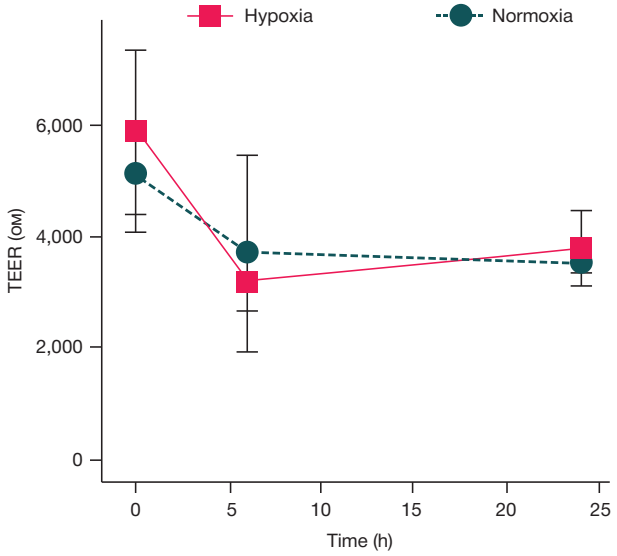


**Fig. 3.** Transepithelial electrical resistance (TEER) vs. time for normoxia and hypoxia

**Table 2.** Differential expression of genes involved in response to hypoxia*

| Gene | qPCR | Microarrays |
|---|---|---|
| | Expression change** | Expression change** |
| *DDIT4* | 2.7 | 2.4 |
| *EGLN1* | 3.4 | 2.7 |
| *LDHA* | 4 | 2.0 |
| *PFKFB3* | 4.3 | 2.1 |
| *SLC2A1* | 2.1 | 3.1 |
| *SLC2A3* | 6.1 | 3.0 |
| *VEGFA* | 2.4 | 1.4 |

**Note:** * — the list of genes involved in response to hypoxia was formed based on the previously reported results [26]; ** — the ratio of expression levels (expressed as arbitrary units) under conditions of hypoxia simulation to reference samples ($p < 0.05$ for qPCR and microarrays). The expression of all listed genes increased under hypoxia-mimicking conditions compared to reference samples.

defence genes (GO: 0016209, antioxidant activity). Mass spectrometry of proteome for the treated and reference cells failed to detect the significant uncrease in the level of the following four proteins encoded by the HIF-1 signalling pathway genes: *ENO2* by 19.6, *HMOX1* by 29.1, *PDK1* by 2.8, and *SLC2A1* by 2.2 times respectively. Proteome analysis revealed the total of 120 proteins, the expression of which significantly changed under hypoxia-mimicking conditions.

Comparison of the $CoCl_2$-treated and reference cells' transcriptomes revealed the significantly increased expression of two genes encoding the integrin α-subunits: under hypoxia-mimicking conditions the expression of *ITGA2* increased by 3.2 times ($p = 0.02$), and the expression of *ITGA5* increased by 1.9 times ($p = 0.0088$). The alterations were validated using qPCR: the expression of *ITGA2* and *ITGA5* increased by 3.5 and 2.0 times respectively. No expression increase was observed for genes encoding β-subunits. During the proteome analysis, among all β-subunits only the β1- and β4-subunits were detected, and there were no alterations in expression of those under hypoxia-mimicking conditions. In Caco-2 cells, these two subunits made up more than 90% of the total β-subunits number [14]. No α-subunits were detected in the proteome, which could be due to insufficient analysis sensitivity. Furthermore, it had been shown before that the levels of α2- and α5-subunits in the Caco-2 cells were 10 and 100 times lower than the level of β1-subunits [14]. It's important that when growing the Caco-2 cells in the microfluidic chip, the expression of *ITGA5*, on the contrary, decreased by 2.1 times. The 1.6-fold decrease in the expression of gene *LAMA1* encoding the normally abscent in the healthy gut laminin α1 chain was observed. This is evidence of the conditions closer to physiological established in the microfluidic chip. It should be noted that no significant alterations in genes involved in response to hypoxia and HIF-1 signalling pathway compared to static condition have been revealed.

### Altered *ITGA5* expression is associated with alterations in the regulatory miRNAs' expression

To explore the possible cause of the *ITGA2* and *ITGA5* integrin expression alteration in response to hypoxia simulation, we analyzed miRNAs involved in regulation of these genes. Using the Affymetrix miRNA microarrays, the miRNA-transcriptomes of the $CoCl_2$-treated and reference cells were assayed. Expression of nine miRNAs significantly changed under hypoxia-mimicking conditions. Of those two miRNAs, hsa-miR-23b-5p and hsa-miR-766-3p, turned out to be the experimentally validated *ITGA5* gene expression regulators [27, 28]. In the hypoxic state the expression of those decreased by 2.2 and 2.1 times respectively ($p = 0.046$), which was opposite to the expression alteration of their target gene *ITGA5*.

Analysis of the discussed miRNAs binding to 3'-UTR of the *ITGA5* mRNA showed that both miRNAs were perfectly complementary to the target mRNA in the seed region (nucleotides 2-7/8 of miRNA) (Fig. 4). Perfect complementarity to the seed region is essential for efficient interaction between miRNA and mRNA. Moreover, both miRNAs are able to pair with the *ITGA5* mRNA outside the seed region, which further increases the strength of binding (see Fig. 4).

Genes of both miRNAs are located in introns: hsa-miR-23b-5p is located in the intron of gene *AOPEP*, and hsa-miR-766-3p is located in the intron of gene SEPTIN6. It should be noted that under conditions of hypoxia simulation no significant alterations in the listed hosting genes' expression have been detected. This may indicate that these miRNAs have independent transcription start sites, which have been previously found in many intronic miRNAs [29].

**Table 3.** Differential expression of genes involved in HIF-1 signalling pathway

| Gene | Expression change* | *p*-value | FDR |
|---|---|---|---|
| *PFKFB3* | 2.1 | 0.00016 | 0.0038 |
| *ENO2* | 3.0 | 0.00028 | 0.0043 |
| *HMOX1* | 2.5 | 0.0053 | 0.012 |
| *HK2* | 3.8 | 0.0077 | 0.013 |
| *EGLN1* | 2.7 | 0.014 | 0.018 |
| *PDK1* | 3.9 | 0.015 | 0.019 |
| *ENO1* | 2.3 | 0.018 | 0.022 |
| *SLC2A1* | 3.1 | 0.019 | 0.022 |

**Note:** * — the ratio of expression levels (expressed as arbitrary units) under conditions of hypoxia simulation to reference samples; the results have been obtained using the microarray transcriptome analysis.

## DISCUSSION

The increased expression of genes involved in the HIF-1 signalling pathway was detected under hypoxic conditions simulated by exposure of colon adenocarcinoma Caco-2 cells to cobalt (II) chloride. The results obtained were consistent with literary data on the transcriprome alterations due to treatment of Caco-2 [30] and Caki-1 [31] cell lines with cobalt (II) chloride.

Hypoxia modeling has revealed the significantly increased expression of genes *ITGA2* and *ITGA5*. These genes encode the integrin receptor α2- and α5-subunits. Since no significant alterations of β-subunits have been detected, it can be assumed that the proportion of receptors containing α2- and α5-subunits have increased under hypoxic conditions. We discovered that in the static Caco-2 cell culture the expression of *ITGA5* was 2.1 times higher compared to cell culture in the microfluidic chip. Microcirculation brings the cell culture conditions closer to physiological conditions and increases the nutrient supply. Thus, alterations in the integrin receptor expression profile (higher proportion of α2- and α5-subunits) may be the Caco-2 cells' response to the growth conditions deterioration. During the study we have discovered the potential regulators of *ITGA2* and *ITGA5* expression, the hsa-miR-23b-5p and hsa-miR-766-3p miRNAs. The expression of those decreased under hypoxic conditions. In should be noted that in addition to the listed miRNA activity there may be cofactors promoting the expression of *ITGA2* and *ITGA5*, for example, transcription factors involved in regulation of the discussed genes, which are also found to have altered expression levels.

The existing literature reports little information on the two identified miRNAs. Thus, it is known that the hsa-miR-766-3p downregulation results in increased proliferative activity of renal cell carcinoma cells [32] and hepatocellular carcinoma aggression [33]. Downregulation of hsa-miR-23b-5p results in increased proliferation and migration of the lung adenocarcinoma cells [34].

In order to become a receptor, integrin chains must form an αβ-heterodymer. In the intestinal epithelial cells, one of the major partners for α2- and α5-chains is β1-chain. The main ligands of the α2β1 integrin receptor are laminin, collagen and epithelial cadherin, and the receptor α5β1 binds to fibronectin [35]. These proteins, in turn, function as ligands for bacterial adhesins. For example, the YadA adhesin found on the outer membrane of the gram-negative bacteria *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* (zoonotic bacterial pathogens causing pseudotuberculosis and yersiniosis) binds to collagen, laminin and fibronectin [36]. Furthermore, invasin, the protein of the outer membrane of the *Yersenia* genus pathogenic bacteria,

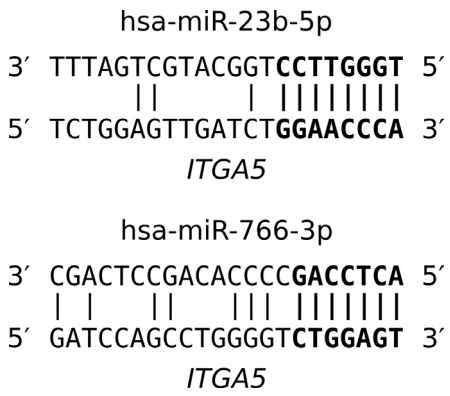


**Fig. 4.** Hsa-miR-23b-5p and hsa-miR-766-3p miRNA binding sites for 3'-UTR of *ITGA5* mRNA

selectively binds the β1 family integrin receptors, which results in translocation of bacteria across the epithelial layer [37]. It should be noted that in one of the studies simulation of hypoxia using DMOG was associated with a lower sensitivity of Caco-2 cells grown on plastic to *Yersinia enterocolitica* [38], which, according to the authors, was due to lower expression of integrin β1-chain. In our study, no alterations in the integrin β1-chain expression in cells grown on the permeable membrane insert were observed. This seems to indicate the importance of cell culture conditions used in such experiments. However, the influence of the specific hypoxia mimetic agents cannot be excluded.

Thus, the intestinal epithelial cells hypoxia, insufficient nutrient supply and excess metabolic waste result in altered integrin receptor expression profile, which may contribute to increased susceptibility to a number of bacterial pathogens. Hsa-miR-23b-5p and hsa-miR-766-3p miRNAs may function as regulators of the discussed process.

## CONCLUSION

The use of cobalt (II) chloride made it possible to simulate hypoxia in the Caco-2 cell line. Transcriptome analysis revealed activation of key HIF-1 signalling pathway components, as well as alterations of the integrin expression profile in the intestinal epithelial cells. The discussed process could be regulated by the hsa-miR-23b-5p and hsa-miR-766-3p miRNAs, the expression of which inder hypoxic conditions decreased by 2.2 and 2.1 times respectively. Microcirculation has the opposite effect on the expression of *ITGA5* and has no effect on the HIF-1 signalling pathway genes' expression.

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# ANALYSIS OF THE POLYMORPHIC VARIANTS OF *ADRB2* GENE ASSOCIATION WITH THE $\beta_2$-AGONISTS RESPONSE IN PATIENTS WITH A RARE THERATYPE OF ASTHMA

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Standard asthma therapy includes prescription of $\beta_2$-agonists. Changes in the functional activity of $\beta_2$-adrenergic receptor are associated with *ADRB2* gene polymorphism and related to the low therapeutic response to β2-agonists. Identification of carriers of the clinically significant gene variants will help to avoid ineffective treatment and prescribe an alternative therapy. This study aimed to assess clinical significance of the *ADRB2* gene polymorphisms (Arg16Gly and Gln27Glu) associated with the therapeutic response to $\beta_2$-agonists in the group of asthma patients. We subjected a small group of adult nonsmoking patients ($n$ = 21) with moderate asthma (III–IV stage of GINA) to clinical and genetic examination. The group included patients with the new theratype, those that poorly respond to $\beta_2$-adrenergic drugs but significantly to M-cholinergic agonists. The first group included patients responding well to both salbutamol and ipratropium bromide. The second group was comprised of the patients for whom salbutamol was not effective but who tested positive for response to ipratropium bromide. The analysis of distribution of polymorphic variants of Arg16Gly and Gln27Glu revealed no significant relationship between alleles and genotypes and the efficacy of $\beta_2$-agonists (0.52 for the rs1042713 variant, $p$ = 1.0; 1.0 for the rs1042714 variant, $p$ = 0.74, respectively). The genotype of patients that did not respond to salbutamol was either Arg16Gly or Gly16Gly. Further studies are needed that would involve a larger number of patients and an expanded list of the tested polymorphic variants.

**Keywords:** asthma, asthma control, gene polymorphism, $\beta_2$-adrenergic receptors, *ADRB2*, Arg16, Gly16, bronchodilators, short-acting $\beta_2$-agonists, SABA, short-acting anticholinergics, SAMA, long-acting anticholinergics, LABA

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**Compliance with ethical standards:** the study was approved by the ethics committee of the Institute of Immunology of the Federal Medical-Biological Agency (Minutes #13 of October 16, 2017); all patients signed voluntary consent to participate in the study.

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# АНАЛИЗ АССОЦИАЦИИ ПОЛИМОРФНЫХ ВАРИАНТОВ ГЕНА *ADRB2* С ОТВЕТОМ НА $\beta_2$-АГОНИСТЫ У ПАЦИЕНТОВ С РЕДКИМ ТЕРАТИПОМ БРОНХИАЛЬНОЙ АСТМЫ

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Стандартная терапия бронхиальной астмы (БА) включает назначение $\beta_2$-агонистов. Изменение функциональной активности $\beta_2$-адренорецептора ассоциировано с полиморфизмом гена *ADRB2* и связано с низким терапевтическим ответом на $\beta_2$-агонисты. Выявление носителей клинически значимых вариантов гена поможет избежать неэффективного лечения и послужит основанием для назначения альтернативной терапии. Целью исследования было оценить клиническую значимость ассоциированных с терапевтическим ответом на $\beta_2$-агонисты полиморфных вариантов гена *ADRB2* (Arg16Gly и Gln27Glu) для группы пациентов с БА. Проведено клиническое и генетическое обследование небольшой группы взрослых некурящих пациентов ($n$ = 21) с БА средней степени тяжести (III–IV ступень по GINA), в том числе пациентов нового тератипа, для которых характерны плохой ответ на $\beta_2$-адренергические средства, но значимый ответ на М-холинергические средства. В первую группу были определены пациенты с подтвержденной эффективностью применения сальбутамола, которые в то же время имели хороший ответ на ипратропия бромид. Во вторую группу вошли пациенты с низкой эффективностью терапии сальбутамолом и положительным тестом с ипратропия бромидом. Анализ распределения полиморфных вариантов Arg16Gly и Gln27Glu показал отсутствие достоверной связи аллелей и генотипов с эффективностью применения $\beta_2$-агонистов (0,52 — для варианта rs1042713, $p$ = 1,0; и 1,0 — для варианта rs1042714, $p$ = 0,74 соответственно). При этом пациенты с отсутствием ответа на сальбутамол имели генотип либо Arg16Gly, либо Gly16Gly. Необходимы дальнейшие исследования с большим числом пациентов и расширением перечня тестируемых полиморфных вариантов.

**Ключевые слова:** бронхиальная астма, контроль астмы, полиморфизм генов, $\beta_2$-адренорецепторы, *ADRB2*, Arg16, Gly16, бронхолитические средства, короткодействующие $\beta_2$-агонисты, КДБА, короткодействующие антихолинергические препараты, КДХП, длительно действующие антихолинергические препараты.

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Personalized medical assistance employs current molecular genetics technologies (pharmacogenetic testing, identification of genomic and transcriptomic biomarkers) to individualize the choice of the drug [1]. In this context, asthma is of considerable interest, since both the pathology itself and the response to asthma therapy are largely shaped by the genes [2–5]. For example, a change in the functional activity of $\beta_2$-adrenergic receptor (ADRB2) associated with polymorphism of its encoding gene can worsen the pharmacological response to $\beta_2$-agonists, which asthma therapy mostly relies on [6, 7].

According to the Ensembl database [8], *ADRB2* is a highly polymorphic gene. Its coding part contains over 500 single nucleotide substitutions and insertion-deletion polymorphisms. Of these, 276 are missense mutations causing a shift in the reading frame or appearance of a stop codon. From the point of view of response to anti-asthma therapy, the most interesting are the Argl6Gly (rs1042713), Gln27Glu (rs1042714), and Thrl64Ile (rs1800888) polymorphic variants of the gene. Their association with the efficacy of response to $\beta_2$-agonists is clear. However, various studies [9–11] failed to reliably reproduce the associations established for these molecular genetic markers. In this connection, the question of the possibility of clinical application of the results of testing for *ADRB2* gene polymorphisms remains open [12–14].

Some of the reasons behind inability of some researchers to confirm the clinical effect of this or that polymorphic variant of the gene are population heterogeneity, small (insufficient) sample, incomplete description of characteristics of the control groups [15, 16]. Thus, it is necessary to further study the molecular mechanisms of asthma pathogenesis with the involvement of numerous cohorts from different populations. It should be noted that there are practically no efforts pursuing the mentioned purpose in Russia.

Despite the aforesaid, clinicians already have the experience and the necessary tools to use pharmacogenetic testing in practice. It seems interesting to approach the issue of establishing the clinical significance of genetic markers from the other side. We did not aim to establish the association of a marker with a sign; on the contrary, we investigated the applicability of markers with association already established for a limited cohort of patients we have clinically described previously [17]. The confirmation of significance of pharmacogenetic markers for this group would allow actual use of genetic testing results as an additional justification of management decisions made for patients torpid to standard therapy.

Thus, this study aimed to assess the clinical significance of ADRB2 gene polymorphisms associated with therapeutic response to $\beta_2$-agonists in a group of patients with a rare asthma theratype which we have described earlier.

## METHODS

### Patients

The inclusion criteria were: signed informed consent to participate in the study; 18 years of age and older (both genders); severe allergic asthma persisting for two years or more; the ability to adequately assess your symptoms and follow recommendations; confirmed reversibility of the bronchial obstruction (after inhalation of 400 μg of salbutamol FEV1 growth of 12% and 200 ml or more). It was considered acceptable when the patient had reversibility of bronchial obstruction confirmed with a document dated within 12 months before signing of the informed consent.

The exclusion criteria were: acute infectious disease (until recovery), exacerbation of concomitant chronic disease (until stabilization of the condition); any clinically significant, uncontrolled medical condition for which the patient is receiving or not receiving treatment and that would hinder adherence to the study schedule or procedures, efficacy data interpretation, or pose a threat to the safety of the patient; diagnosed malignant neoplasm; development of a serious adverse event during the course of the study.

The study involved non-smoking adult patients ($n$ = 21) of Russian ethnicity of both sexes (8 men and 13 women), the mean age was 53 years (minimum — 47, maximum — 63); they all suffered from moderate asthma (III–IV stage of GINA) for the mean period of 13 years (minimum — 1 year, maximum — 32 years). All patients were prescribed medium to high doses of inhaled corticosteroids as the main therapy in combination with long-acting anticholinergics (LABA). Asthma symptoms were either not controlled or the control was incomplete: the patients needed symptomatic therapy daily; they scored 15–20 points on the ACT scale; the 1 s forced expiration volume (FEV1) before administration of a bronchodilator reached 70.6 ± 5% of the normal values. The patients were divided into two groups. The first ($n$ = 14) included patients who responded well to salbutamol (400 μg of inhaled salbutamol causing the growth of FEV1 of over 12% and 200 ml), with that response confirmed clinically and instrumentally, and, at the same time, exhibited good response to 50 μg of ipratropium bromide (SABA+SAMA+). The second group ($n$ = 7) was comprised of the patients that had poor response to salbutamol (400 μg of inhaled salbutamol causing the growth of FEV1 of less than 12% and 200 ml) and tested positive for response to 50 μg of ipratropium bromide, inhaled (inhalation yielding the growth of FEV1 of over 12% and 200 ml in 30 minutes; SABA–SAMA+).

### Genetic markers

The *ADRB2* gene is located on the long arm of chromosome 5q32, next to a cluster of genes encoding cytokines and the glucocorticoid receptor. *ADRB2* belongs to the genes of receptor molecules that control bronchial lability [18].

The Arg16Gly polymorphism (international polymorphism code: rs1042713) is a single nucleotide substitution in the coding region of the *ADRB2* gene, where guanine nucleotide (G) is replaced with adenine nucleotide (A) (genetic marker G46A). This substitution changes the amino acid sequence of the ADRB2 protein at position 16: arginine is replaced by glycine (Arg16Gly). Thus, the following variants are possible: Arg16Arg, Arg16Gly, Gly16Gly. In vitro studies have shown a change in the functional activity of *ADRB2* [19]. Some researchers report that the patients homozygous for these gene variants quickly lose sensitivity to short-acting $\beta_2$-agonists (SABA) and need to corticosteroids prescribing [14].

The Glu27Gln polymorphism (international polymorphism code: rs1042714) is a single nucleotide substitution of cytosine for guanine (genetic marker C79G). As a result of this substitution, the amino acid sequence of the ADRB2 protein has glutamine replaced by glutamic acid (Glu27Gln) at position 27. Martinez et al have reported that Glu27 allele is associated with decreased sensitivity of asthma patients airways to methacholine [20].

### DNA purification and typing

Genomic DNA was isolated from peripheral blood lymphocytes through phenol-chloroform extraction. The obtained samples were immediately used for genotyping or stored at –20 °C. The DNA concentration was determined with the help of

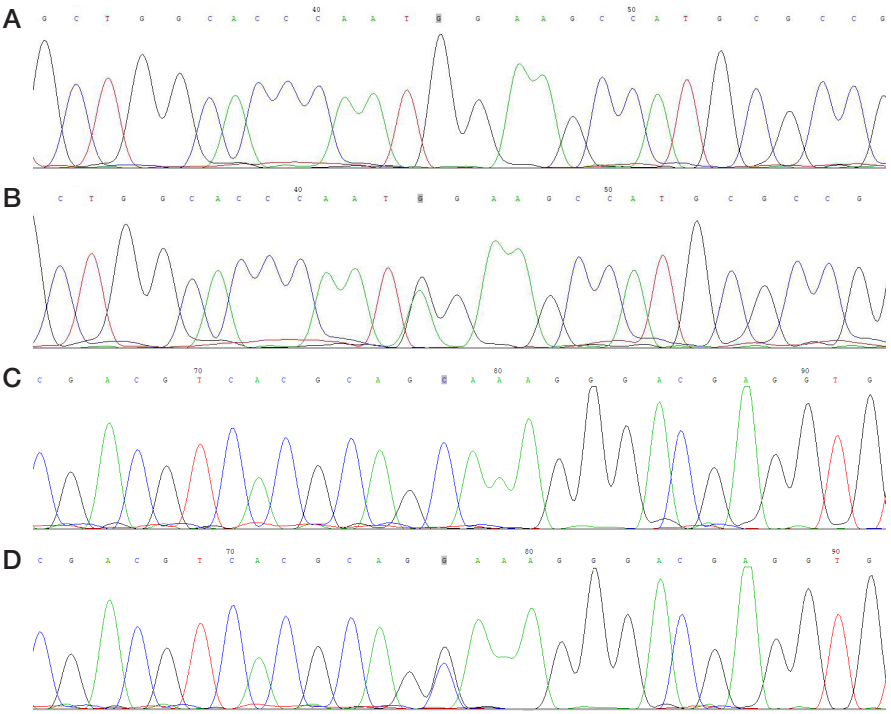


**Fig. 1.** The results of sequencing of homozygous and heterozygous samples. The varying nucleotides are shown in gray. Peculiar to the homozygotes is a single peak in the chromatogram at the position of the rs1042713 and 1042714 polymorphisms (**A** and **B**, respectively). Heterozygotes are characterized by a double peak at the position of the rs1042713 and 1042714 polymorphisms (**C** and **D**, respectively)

Qubit fluorimeter (Invitrogen; USA); it averaged at 50–100 μg/ml. *ADRB2* gene's polymorphisms rs1042713 and rs1042714 were PCR-analyzed (real-time PCR) in the DTprime amplifier (DNA-Technology LLC; Russia) with primers ADRB2-f: 5'-AGTGCGCTCACCTGCCAGACTG-3' and ADRB2': 5'-CCAAACACGATGGCCAGGACGA-3'. The primers were synthesized on a solid support using inverted (5') phosphoramidites and photodegradable linkers. The latter were used to take primers off the solid support, the process relying on the ultraviolet radiation.

To determine the genotypes, we resorted the modified adjacent probes method [21]. This approach compares favorably with the majority of molecular genetic methods enabling determination of single nucleotide polymorphisms, including those relying on the TaqMan technology. The genotype is determined twice, independently, using two fluorescence channels, which significantly increases the reliability of genotyping. No other approach allows this level of accuracy. For amplification, we used 35 μL of reaction mixture, which contained 2.5 μL of 10 — Taq buffer (67 mM Tris-HCl (pH 8.8), 16.6 mM (NH4) 2SO4, 2.5 mM MgCl2, 0.01% Tween-20), 0.1 μg of genomic DNA, dNTP mixture (dATP, dGTP, dCTP, dTTP, 200 μM each), 1 unit of DNA polymerases (DNA-Technology LLC; Russia) and 5–10 pM of locus-specific oligonucleotide primers and probes. The amplification temperature regime was as follows: 94 °C for 10 s, 64 °C for 30 s, for 50 cycles. When the amplification was complete, the reaction mixture was cooled to 25 °C at the rate of 2 °C/s. The melting curves were obtained as follows: the temperature of the reaction mixture was increased from 25 to 75 °C in 1 °C increments, with the fluorescence level measured at each increment.

MS Excel 2013 (Microsoft; USA) enabled statistical processing of the data, which employed Fisher's exact test to check the equivalence of the observed distribution of genotype frequencies [24, 25]. The differences between groups were considered significant at $p < 0.05$. The following formula was used to establish frequency of the alleles:

$$f = {}^{n}/_{2N} * 100\%,$$

where *n* is the occurrence of the allele.

## RESULTS

The study focused on the rs1042713 and rs1042714 polymorphisms (rs1800888 was not included because of its low occurrence [24, 25]). To accomplish the objective declared, we designed a new test system to analyze the *ADRB2* gene polymorphisms using real-time PCR, and confirmed its efficacy by direct sequencing of homozygous and heterozygous samples (Fig. 1).

In the course of the study, we formed control groups from patients at the Institute of Immunology of the FMBA of Russia. These patients had asthma of different theratypes; we genotyped them and analyzed the differences in the occurrence of alleles and genotypes. Table shows the results of genotyping.

## DISCUSSION

We subjected moderate BA patients (III–IV stage of GINA) with variable pharmacological response to $\beta_2$-agonists to clinical and genetic examination; SABA+SAMA+ are the patients with clinically and instrumentally confirmed positive response to salbutamol, and SABA–SAMA+ are patients that responded poorly to salbutamol but well to ipratropium bromide. In the previous paper, we provided detailed clinical characteristics of these groups of patients [17]. Alleles and genotypes of patients were determined for rs1042713 (Arg16Gly) and rs1042714 (Glu27Gln), polymorphisms of the *ADRB2* $\beta_2$-adrenergic receptor gene.

### Arg16Gly polymorphism (rs1042713)

Among the SABA+SAMA+/SABA–SAMA+ patients, those that exhibited poor response to salbutamol had the frequency of

**Table.** Distribution of the allele and genotype frequencies of *ADRB2* gene's rs1042713 (Arg16Gly) and rs1042714 (Gln27Glu) polymorphisms in asthma patients with various theratypes (SABA+SAMA+ — patients with clinically and instrumentally confirmed positive response to salbutamol)

| Patient group, *n* (%) | Arg16Gly | | | | | | |
|---|---|---|---|---|---|---|---|
| | Alleles | | | Genotypes | | | |
| | Arg | Gly | *p*-value | Arg16Arg | Arg16Gly | Gly16Gly | *p*-value |
| SABA+SAMA+ (*n* = 14) | 10 (36%) | 18 (64%) | 1 | 2 (14%) | 6 (43%) | 6 (43%) | 0.52 |
| SABA+SAMA+ (*n* = 7) | 5 (36%) | 9 (64%) | | 0 | 5 (71%) | 2 (29%) | |
| All patients (*n* = 21) | | | | 2 (10%) | 11 (52%) | 8 (38%) | |
| | Gln27Glu | | | | | | |
| | Alleles | | | Genotypes | | | |
| | Glu | Gln | *p*-value | Glu27Glu | Gln27Glu | Gln27Gln | *p*-value |
| SABA+SAMA+ (*n* = 14) | 17 (61%) | 9 (39%) | 0.74 | 4 (29%) | 9 (64%) | 1 (7%) | 1 |
| SABA+SAMA+ (*n* = 7) | 8 (57%) | 6 (43%) | | 2 (29%) | 4 (57%) | 1 (14%) | |
| All patients (*n* = 21) | | | | 6 (28%) | 13 (62%) | 2 (10%) | |

heterozygotes 1.5 greater than those that responded well to the drug. Both groups had the Arg16 and Gly16 alleles detected with the same frequency (see Table).

We have shown that the Arg16Gly polymorphism is associated with desensitization of the *ADRB2* receptor. A receptor with Gly16Gly is more susceptible to desensitization by endogenous catecholamines than a receptor with Arg16Arg or Arg16Gly in its structure [26]. As described in the published papers, a variability in response to $\beta_2$-agonists was revealed [27]. Our data partially agree with the data stating lack of therapeutic response to $\beta_2$-agonist inhalation therapy in moderate asthma patients that have the Gly allele (Arg16Gly and Gly16Gly genotypes) dominating [28]. In our study, the genotype of all patients showing no response to salbutamol was Arg16Gly or Gly16Gly. However, we could not confirm the association when assessing the effect the Gly allele has on poor response to $\beta_2$-agonists (odds ratio [OR], 1.00; 95% CI 0.26–3.81). The most pronounced response to a single administration of a $\beta_2$-adrenergic agonist was registered in the group of patients homozygous for Arg at position 16 (Arg16Arg) compared with homozygous for Gly at this position (genotype Gly16Gly) [20]. Another study also confirms that the Arg16Arg genotype is associated with mild asthma and a better response to salbutamol [29]. According to our data, 14% of patients that responded well to salbutamol in the SABA+SAMA+ group had the Arg16Arg genotype. No patient in the poor response group has this genotype.

Unfortunately, we only managed to recruit a small number of SABA–SAMA+ asthma patients, since this theratype is rare. Probably, further identification of such patients and a study on a larger sample will yield significant differences.

### Glu27Gln polymorphism (rs1042714)

The distribution of genotypes and alleles for the 27th position among SABA+SAMA+/SABA–SAMA+ patients was almost identical. The two groups did not differ significantly in this regard (see Table). However, in the SABA+SAMA+ group we revealed a number of Gln27Glu heterozygotes (64%) that is relatively larger than the frequency of 45.7% previously established for the Russian population [30], but this observation requires confirmation on a larger sample.

The studies focusing on the Gln27Glu polymorphism and variability of response to $\beta_2$-agonists are limited, and these results are inconsistent, which prevents us from correlating our data with those reported in the literature. The key subject for research was the distribution of genotype frequencies with asthma of various severity in the background. It was shown that the prevailing genotype in the cohort of severe asthma patients is Glu27Glu (55 and 75%, respectively) [31, 32]. Another study reported the following distribution of genotypes for the 27$^{th}$ position in asthma patients: Glu27Glu — 9.2%, Gln27Glu — 27.8%, Gln27Gln — 63%; there were no differences found in patients with different severity and response to $\beta_2$-agonists [29]. Thus, the data we obtained are consistent with the data reported in [29] that reports lack of relationship between the response to $\beta_2$-agonists and the rs1042714 (Gln27Glu) polymorphism.

In this study, we did not evaluate other polymorphisms of the *ADRB2* gene that could influence the response to $\beta_2$-agonists. It is possible that other, nongenetic reasons for desensitization of the ADRB2 gene underlie the poor response to salbutamol in patients with the rare SABA–SAMA+ theratype.

## CONCLUSION

The analysis of distribution of rs1042713 (Arg16Gly), *ADRB2* gene polymorphisms, showed that the genotype of all patients with no response to $\beta_2$-agonists (salbutamol) was either Arg16Gly or Gly16Gly, however, we could not confirm the association when assessing the effect the Gly allele has on poor response to $\beta_2$-agonists, with small sample size being the possible reason therefor. We established no differences in the distribution of rs1042714 (Gln27Glu) allele and genotype frequencies when comparing groups of patients with different clinical responses to $\beta_2$-agonists. A further study that would include a larger sample of asthma patients with the rare SABA–SAMA+ theratype may reveal statistically significant differences in the distribution of polymorphic rs1042713 (Arg16Gly) variants. In this study, we did not evaluate other polymorphisms of the *ADRB2* gene for their possible effect on the response to $\beta_2$-agonists. It is advisable to include in further research rare functional variants identified as a result of resequencing of polyethnic cohorts. In addition, other, nongenetic reasons for desensitization of the *ADRB2* gene may be associated with a poor response to salbutamol in patients with the rare SABA–SAMA+ theratype.

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# CARTOGRAPHIC ATLAS OF FREQUENCY VARIATION FOR 45 PHARMACOGENETIC MARKERS IN POPULATIONS OF RUSSIA AND ITS NEIGHBOR STATES

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The lack of information about the frequency of pharmacogenetic markers in Russia impedes the adoption of personalized treatment algorithms originally developed for West European populations. The aim of this paper was to study the distribution of some clinically significant pharmacogenetic markers across Russia. A total of 45 pharmacogenetic markers were selected from a few population genetic datasets, including ADME, drug target and hemostasis-controlling genes. The total number of donors genotyped for these markers was 2,197. The frequencies of these markers were determined for 50 different populations, comprised of 137 ethnic and subethnic groups. A comprehensive pharmacogenetic atlas was created, i.e. a systematic collection of gene geographic maps of frequency variation for 45 pharmacogenetic DNA markers in Russia and its neighbor states. The maps revealed 3 patterns of geographic variation. Clinal variation (a gradient change in frequency along the East-West axis) is observed in the pharmacogenetic markers that follow the main pattern of variation for North Eurasia (13% of the maps). Uniform distribution singles out a group of markers that occur at average frequency in most Russian regions (27% of the maps). Focal variation is observed in the markers that are specific to a certain group of populations and are absent in other regions (60% of the maps). The atlas reveals that the average frequency of the marker and its frequency in individual populations do not indicate the type of its distribution in Russia: a gene geographic map is needed to uncover the pattern of its variation.

**Keywords:** pharmacogenetics, cartographic atlas, populations, ethnic groups, gene pool, gene geography, Russia, North Eurasia

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**Compliance with ethical standards:** the study was approved by the Ethics Committee of the Research Center for Medical Genetics (Protocol № 3/1 dated September 5, 2018) and carried out on the samples obtained during the population genetic study of the gene pools of ethnic groups from Russia and its neighbor states. The donors gave voluntary informed consent to participate.

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# КАРТОГРАФИЧЕСКИЙ АТЛАС РАСПРОСТРАНЕНИЯ 45 ФАРМАКОГЕНЕТИЧЕСКИХ МАРКЕРОВ В НАРОДОНАСЕЛЕНИИ РОССИИ И СОПРЕДЕЛЬНЫХ СТРАН

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Нехватка информации о распространенности в РФ фармакогенетических маркеров приводит к невозможности внедрения алгоритмов персонализации, разработанных для Западной Европы. Целью работы было систематическое изучение распространенности ряда значимых фармакогенетических маркеров по всей территории России. Из нескольких массивов популяционно-генетических данных отобраны 45 маркеров (ADME-генов; генов, кодирующих фармакодинамические мишени лекарственных средств; генов, кодирующих компоненты системы гемостаза), генотипированных суммарно для 2197 индивидов. Определены частоты этих маркеров в 50 популяциях, включающих информацию о 137 этнических и субэтнических группах. В результате создан фармакогенетический атлас — систематическое собрание геногеографических карт распространенности фармакогенетических ДНК маркеров по всей территории России и сопредельных стран. Атлас выявил три паттерна пространственной изменчивости. Паттерн клинальной изменчивости (градиентного изменения частот по оси «восток–запад») объединяет маркеры, следующие основной закономерности всего генофонда населения Северной Евразии (13% карт атласа). Паттерн равномерного распределения выделяет маркеры, средняя частота которых характерна для большинства регионов России (27% карт атласа). Паттерн «очаговой» изменчивости объединяет фармакогенетические маркеры, характерные только для определенной группы этносов и отсутствующие в других регионах (60% карт атласа). Атлас показывает, что средняя частота маркера и информация о его встречаемости в отдельных популяциях не могут служить указанием на тип его распределения в пространстве РФ — для выявления паттерна изменчивости необходима геногеографическая карта.

**Ключевые слова:** фармакогенетика, картографический атлас, популяции, этносы, генофонд, геногеография, Россия, Северная Евразия

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The key genes involved in the absorption, distribution, metabolism, and elimination (ADME) of drugs have been described in multiple studies. Although ADME genes are relatively modest in number, each of them is represented by a few functionally relevant polymorphic variants [1, 2]. In addition to ADME genes, research has identified drug target genes and the genes encoding the components of the hemostatic system. Assays and protocols are being developed to guide drug selection and dosing based on the patient's genotype [3–5]. However, genetic differences between ethnicities remain the major hurdle impeding the widespread adoption of such assays: commercial panels designed to screen ADME genes for clinically significant polymorphisms do not account for their variation across races and ethnic groups and thus are poorly adapted for ethnically diverse regions.

Studies conducted in polyethnic regions across the world have demonstrated both quantitative (allele frequencies) and qualitative (population-specific alleles) differences in ADME genes and other pharmacogenetic markers between ethnic groups. Although interethnic differences have been well known since the inception of pharmacogenetics [6, 7], most pharmacogenetic studies are still carried out on Caucasian patients. Consequently, their results cannot be extrapolated to other ethnic groups. For example, it has been shown that standard algorithms for essential drug dosing are often ineffective for African and Hispanic patients, creating life-threatening situations or resulting in death or disability [8, 9].

Pharmacogenetic analysis can be applied not only to an individual but also to an entire population, in which case an average genotype based on the allele frequency in a studied population is analyzed. Indeed, individual genotypes may differ from the average population genotype. Even so, predictions based on the average genotype that reflects the characteristics of the population's gene pool will work better than one-size-fits-all recommendations on drug selection and dosing.

This problem has inspired a wealth of studies researching the possibility of optimizing pharmacogenetic recommendations for a given population based on its genetic characteristics. For example, the average effective warfarin dose was shown to be different between European and Caribbean populations [10, 11]. The situation is further compounded by the fact that many populations are genetically different from the "typical" representatives of their race: many African populations are genetically very different from African Americans; Siberian peoples are different from the Chinese; Eastern Slavs and the populations of the Caucasus differ from white Americans. A striking variation in the frequency of ADME genes is reported between the Han Chinese, who make up 98% of China's population, and other Mongoloid minorities, including Uyghurs, Kyrgyz and Kazakhs [12]. Likewise, black African populations are characterized by great genetic variation in the genes coding for P450 isoenzymes, compared to Caucasian populations [9]. Undescribed or rare alleles of ADME genes can substantially contribute to the efficacy and safety of pharmacotherapy. For example, a recent study has identified a novel allele responsible for up to 31% of interpatient warfarin dose variability in African Americans [8]. In another study of variability in warfarin dose response, rare alleles typically found in African Americans were detected in a Puerto Rican population [13]. Another genetic marker (HLAB*15:02) associated with severe allergic response to carbamazepine occurs in 8% of Asians and only 1% of Europeans; the prevalence of carbamazepine allergy differs five- to sixfold between these 2 populations [14]. This indicates that direct extrapolation of data generated by foreign studies to the Russian population is absolutely unacceptable.

Russia is a multiethnic state, hence significant variation in responses to pharmacotherapy and the frequency of ADME, drug target and hemostasis-controlling gene variants in the patients of different ethnic/regional descent. In 2019, a systematic review of publications looking into the geographic distribution of alleles of ADME genes in the Russian population [15] discovered that not all ethnic groups and not all studied polymorphisms were equally represented in the literature. Most studies included in the review focused on the major polymorphisms in the genes coding for cytochrome P450 isoenzymes and some factors of the ADME system [16–25]. Many Russian regions and ethnic groups remain understudied.

In view of the foregoing, we set a goal to explore the frequencies of a few significant ADME gene variants across Russia and the neighbor states and to create a draft version of the pharmacogenetic atlas of Russia. The goal implies a systematic approach that ensures the even, wide coverage of the studied territory and can be implemented owing to the data generated by population studies using genome-wide SNP panels.

## METHODS

We analyzed a total of 50 populations inhabiting the main regions of North Eurasia and its neighbor states. Samples that formed the basis for our study were provided by the Biobank of North Eurasia, the largest repository of biological specimens collected from indigenous Russian populations and the populations of Russia's neighbor states [26]. The Biobank provided 2011 samples representing 40 different populations; the average sample size was $n = 50$. The initially small datasets of dozens ethnic groups were pooled to form a larger population sample. By contrast, the initially large sample of Russians was divided into 3 geographically different populations. The resultant datasets were genotyped using an Omni-Exome genome-wide array of 4.5 million SNPs (Illumina; USA).

The list of pharmacogenetic markers was compiled from 2 major sources containing some overlapping data: Very Important Pharmacogene (VIP) summaries [27], which describe pharmacogenetic markers involved in pharmacodynamics, pharmacokinetics and, ultimately, response to pharmaceuticals, and a list of markers from the popular commercial OpenArray™ PGx Express Panel (Thermo Fisher Scientific; USA). The resultant list was extended to include a few promising pharmacogenetic markers characterized by distinct racial/ethnic variation in frequency (*CES1*, *PON1*, *IFNL3*, *ITGB3*) and, thus, comprised a total of 95 pharmacogenetic markers. Of them, 55 were present in the Omni-Exome Illumina panel [28].

This dataset was expanded with the published data on 10 populations from China and the European countries that share a border with Russia [29–44]. The total number of the samples representing those 10 populations was 186; the average sample size was $n = 19$. The samples had been previously genotyped using the genome-wide panels of 600 thousand to 1 million SNPs. In some cases, the data on Russia's ethnic groups generated by the published studies were pooled with the corresponding Biobank data. The analysis of this literature-based dataset revealed the presence of 53 SNPs of 95 pharmacogenetic markers included in our list.

The two datasets (data from the Biobank of North Eurasia and the published genome-wide sequencing data) were compared and found to share 45 pharmacogenetic SNPs polymorphic (frequency above 1%) in the populations of North Eurasia. This paper analyzes their geographic distribution. A total of 2,197 samples representing 50 North Eurasian

**Table 1.** Characteristics of the studied populations

| Arbitrary name of population | Ethnic groups constituting the population | Sample size *n* | Population coordinates | |
|---|---|---|---|---|
| | | | Latitude (degrees) | Longitude (degrees) |
| Altai | Altaian | 40 | 52 | 87 |
| Amur | Nanai, Negidal, Nivkh, Orochi, Udege, Ulchi | 52 | 51 | 139 |
| Armenian | Armenian | 102 | 40 | 45 |
| Bashkir | Bashkir | 47 | 54 | 57 |
| Buryat | Buryat | 47 | 53 | 110 |
| Upper Volga | Mari, Chuvash | 58 | 56 | 47 |
| Greece | Greek, Macedonian | 29 | 40 | 22 |
| North Dagestan | Avar, Dargyn, Kubachi, Kumyk, Lak | 52 | 43 | 47 |
| South Dagestan | Agul, Lezgin, Rutul, Tabasaran | 34 | 42 | 48 |
| Ashkenazi Jews | Ashkenazi Jews | 18 | 48 | 16 |
| Other Jews | Jews of Azerbaijan, Georgia, Sephardi Jews of Uzbekistan | 16 | 38 | 38 |
| Transcaucasia | Azerbaijani, Georgian | 40 | 42 | 46 |
| West Siberia | Mansi, Selkup, Khanty | 45 | 63 | 72 |
| West Caucasus 1 | Abazin, Abkhaz, Adyg, Sphapsug | 51 | 44 | 41 |
| West Caucasus 2 | Balkar, Kabardin, Karachai, Cherkess | 52 | 44 | 43 |
| Italy | Italian | 17 | 42 | 13 |
| Kamchatka | Itelmen, Koryak, Chukchi | 70 | 61 | 167 |
| Karakalpakstan | Karakalpak, Turkmen | 36 | 40 | 60 |
| Karelia | Veps, Vod, Izhora, Karel | 61 | 62 | 33 |
| Kirghizia | Kyrzyg | 38 | 41 | 75 |
| China | Chinese (Han) | 7 | 34 | 120 |
| Komi | Komi, Komi-Permiak | 54 | 60 | 54 |
| Moldova | Bulgarian, Gagauz, Moldovan | 36 | 45 | 27 |
| Mongolia | Mongol | 103 | 47 | 101 |
| Mordovia | Moksha, Shoksha, Erzya | 43 | 54 | 43 |
| Nogai | Karanogai, Astrakhan Nogai, Kuban Nogai, Stavropol Nogai | 34 | 44 | 43 |
| Ossetia | Iron, Digor | 41 | 43 | 44 |
| Baltics | Latvian, Lithuanian, Estonian | 19 | 58 | 24 |
| North Russia | Russians of Arkhangelsk and Vologda regions | 67 | 61 | 40 |
| Central Russia | Russians of Kostroma, Novgorod, Pskov, Tver, Yaroslavl regions | 97 | 57 | 38 |
| South Russia | Russians of Belgorod, Voronezh, Kaluga, Kursk, Nizhniy Novgorod, Orlov, Ryazan, Smolensk, Tambov regions, Kuban Cossack | 94 | 53 | 35 |
| North Siberia | Nganasan, Nenets, Ket | 25 | 67 | 83 |
| Northern Europe* | German, Polish, Swedish | 19 | 54,54,59 | 13,18,18 |
| North China | Yi, Nakhi, Oroqen, Xibo, Tu, Tujia, She | 8 | 48 | 126 |
| South Slavs | Bosnian, Serb, Slovene, Croat | 45 | 45 | 14 |
| Central Asia | Kazakh, Uzbek, Uighur | 53 | 46 | 69 |
| Tadzhikistan 1 | Tadzhik | 28 | 37 | 71 |
| Tadzhikistan 2 | Pamir peoples, Pushtun, Yaghnobi | 48 | 38 | 70 |
| Volga-Ural Tatars | Kazan Tatar, Kryashen Tatar, Mishar Tatar | 45 | 55 | 52 |
| Siberian Tatars | Siberian Tatar | 58 | 57 | 67 |
| Tuva | Tofalar, Tuvan | 59 | 52 | 94 |
| Udmurtia | Besermyan, Udmurt | 31 | 57 | 53 |
| Ukraine | East and West Ukrainian | 77 | 49 | 29 |
| Central Europe | Hungarian, Romanian, Slovak | 26 | 47 | 21 |
| Central Caucasus | Ingush, Chechen | 32 | 43 | 45 |
| Evenk | Evenk | 31 | 60 | 111 |
| Even | Even | 31 | 61 | 145 |
| South-west Europe | Basque, Spanish, French | 15 | 47 | 3 |
| South Siberia | Khakas, Shor | 48 | 54 | 89 |
| Yakutia | Dolgan, Yukagir, Yakut | 28 | 66 | 124 |
| Total 50 | Total 137 | | | |

**Note:** * — the population of Northern Europe is represented by 3 dots (Germany, Poland and Sweden).

**Table 2.** Genetic variation of 45 pharmacogenetic markers across North Eurasia and its neighbor states

| Gene | SNP | SNP frequency | | | | $F_{ST}$ | Variation pattern |
|---|---|---|---|---|---|---|---|
| | | MEAN | MIN | MAX | MAX-MIN | | |
| *ABCB1* | rs1045642-G | 0.48 | 0.36 | 0.68 | 0.32 | 0.0123 | Uniform |
| | rs4148738-G | 0.46 | 0.26 | 0.69 | 0.42 | 0.0122 | Uniform |
| *APOE* | rs429358-G | 0.00 | 0.00 | 0.02 | 0.02 | 0.0080 | Focal |
| *CES1* | rs2244613-C | 0.37 | 0.10 | 0.73 | 0.63 | 0.0598 | Clinal |
| *COMT* | rs4680-A | 0.46 | 0.21 | 0.66 | 0.44 | 0.0248 | Clinal |
| *CYP1A2* | rs12720461-A | 0.01 | 0.00 | 0.03 | 0.03 | 0.0070 | Focal |
| | rs2069526-C | 0.06 | 0.00 | 0.21 | 0.21 | 0.0129 | Uniform |
| | rs762551-C | 0.35 | 0.21 | 0.52 | 0.31 | 0.0100 | Uniform |
| *CYP2B6* | rs28399499-G | 0.00 | 0.00 | 0.02 | 0.02 | 0.0069 | Focal |
| *CYP2C19* | rs28399504-G | 0.00 | 0.00 | 0.05 | 0.05 | 0.0140 | Focal |
| | rs41291556-G | 0.00 | 0.00 | 0.02 | 0.02 | 0.0068 | Focal |
| | rs4244285-A | 0.14 | 0.04 | 0.25 | 0.21 | 0.0098 | Uniform |
| | rs4986893-A | 0.02 | 0.00 | 0.14 | 0.14 | 0.0249 | Focal |
| | rs56337013-A | 0.00 | 0.00 | 0.02 | 0.02 | 0.0060 | Focal |
| | rs6413438-A | 0.00 | 0.00 | 0.01 | 0.01 | 0.0056 | Focal |
| *CYP2C9* | rs1057910-C | 0.08 | 0.01 | 0.20 | 0.19 | 0.0138 | Uniform |
| | rs1799853-T | 0.08 | 0.00 | 0.23 | 0.23 | 0.0270 | Focal |
| | rs28371685-A | 0.00 | 0.00 | 0.04 | 0.04 | 0.0087 | Focal |
| | rs28371686-G | 0.00 | 0.00 | 0.07 | 0.07 | 0.0341 | Focal |
| | rs56165452-G | 0.00 | 0.00 | 0.01 | 0.01 | 0.0054 | Focal |
| *CYP2D6* | rs28371725-A | 0.02 | 0.00 | 0.17 | 0.17 | 0.0326 | Focal |
| | rs5030862-A | 0.00 | 0.00 | 0.03 | 0.03 | 0.0098 | Focal |
| | rs59421388-A | 0.00 | 0.00 | 0.02 | 0.02 | 0.0072 | Focal |
| *CYP3A4* | rs12721629-A | 0.00 | 0.00 | 0.01 | 0.01 | 0.0052 | Focal |
| | rs2242480-A | 0.11 | 0.05 | 0.23 | 0.18 | 0.0088 | Uniform |
| | rs4986910-G | 0.00 | 0.00 | 0.02 | 0.02 | 0.0054 | Focal |
| | rs55785340-G | 0.00 | 0.00 | 0.01 | 0.01 | 0.0036 | Focal |
| | rs62471956-A | 0.02 | 0.00 | 0.12 | 0.12 | 0.0192 | Focal |
| *CYP3A5* | rs10264272-A | 0.00 | 0.00 | 0.01 | 0.01 | 0.0047 | Focal |
| | rs28365083-A | 0.00 | 0.00 | 0.02 | 0.02 | 0.0050 | Focal |
| | rs41303343-AA | 0.00 | 0.00 | 0.03 | 0.03 | 0.0097 | Focal |
| | rs776746-A | 0.10 | 0.03 | 0.26 | 0.23 | 0.0131 | Uniform |
| *CYP4F2* | *3 rs2108622-A | 0.30 | 0.18 | 0.49 | 0.31 | 0.0154 | Uniform |
| *DPYD* | *2A rs3918290-A | 0.00 | 0.00 | 0.03 | 0.03 | 0.0094 | Focal |
| *Factor II* | rs1799963-A | 0.01 | 0.00 | 0.10 | 0.10 | 0.0176 | Focal |
| *Factor V Leiden* | rs6025-A | 0.02 | 0.00 | 0.13 | 0.13 | 0.0249 | Focal |
| *IFNL3* | rs8099917-C | 0.14 | 0.02 | 0.28 | 0.25 | 0.0165 | Clinal |
| *ITGB3* | rs5918-G | 0.11 | 0.02 | 0.28 | 0.26 | 0.0178 | Uniform |
| *MTFHR* | rs1801131-C | 0.31 | 0.16 | 0.49 | 0.32 | 0.0100 | Clinal |
| | rs1801133-A | 0.27 | 0.03 | 0.51 | 0.48 | 0.0253 | Uniform |
| *PON1* | rs662-G | 0.37 | 0.19 | 0.69 | 0.51 | 0.0264 | Clinal |
| *SLCO1B1* | rs4149056-G | 0.15 | 0.06 | 0.27 | 0.21 | 0.0100 | Uniform |
| *TPMT* | rs1142345-G | 0.01 | 0.00 | 0.05 | 0.05 | 0.0079 | Focal |
| | rs1800460-A | 0.01 | 0.00 | 0.04 | 0.04 | 0.0063 | Focal |
| *VKORC1* | rs9923231-T | 0.57 | 0.3 | 0.95 | 0.65 | 0.0699 | Clinal |

populations were genotyped for those 45 SNPs. Characteristics of the studied populations are provided in Table 1; the SNPs are listed in Table 2.

Genome-wide genotyping data were pooled and analyzed; allele frequencies were calculated in PLINK 1.9 [45, 46]. A frequency matrix was generated for 45 pharmacogenetic markers in 50 populations of Russia and its neighbor states based on the genotypes of 2,197 samples (average sample size $n$ = 44). Using the matrix, we constructed the gene geographic maps of allele frequencies in the populations of North Eurasia and bordering states. The maps were created in GeneGeo [47–49] using average weighted interpolation; the radius of influence was set to 2,000 km; the weight function power was set to 3 [48]. On the maps, the regions lying farther than 2,000 km

**Table 3.** Characteristics and pharmacogenetic significance of 12 ADME markers shown in Fig. 1–3

| Gene | SNP | Allele | Chromosome | Position in GRCh37 | Position in GRCh38 | Function and significance |
|---|---|---|---|---|---|---|
| *ABCB1* | rs4148738 | G | 7 | 87138645 | 87509329 | P-glycoprotein is an ATP-dependent transporter involved in transporting biological substrates across the cell membrane. Its primary function is to regulate the permeation of various compounds, including xenobiotics, across biological barriers. It is also responsible for drug transport across the blood-brain barrier. The rs4148738 polymorphism of the ABCB1 gene is associated with plasma concentrations of direct oral anticoagulants, which may affect the efficacy and safety of these drugs |
| *CES1* | rs2244613 | C | 16 | 55844609 | 55810697 | Carboxylesterases are enzymes that hydrolyze chemical compounds containing complex carboxylic acids, amide and thioester functional groups. They play an important role in hydrolyzing drugs into nonactive metabolites (dabigatran, capecitabin, etc.) |
| *COMT* | rs4680 | A | 22 | 19951271 | 19963748 | Catechol-O-methyltransferase is an enzyme involved in the regulation of dopamine activity in the prefrontal cortex. It participates in promoting sensitivity to neuroleptics and is associated with adverse effects. The rs4680 polymorphism in the COMT gene determines the efficacy of therapy with nicotine, opioids and some antipsychotic drugs |
| *CYP2C19* | rs4986893 | A | 10 | 96540410 | 94780653 | Enzymes of the P450 cytochrome family participate in the metabolism of drugs and xenobiotics. Reduced activity of these enzymes may affect the efficacy and safety of proton pump inhibitors, NSAID, clopidogrel, and other drugs |
| *CYP2C9* | rs1057910 | C | 10 | 96741053 | 94981296 | |
| *Factor II* | rs1799963 | A | 11 | 46761055 | 46739505 | Prothrombin (blood-clotting factor II) is a vitamin K-dependent glycoprotein synthesized in the liver as an inactive zymogen. It plays a significant role in hemostasis and thrombosis. The rs1799963 allele of the Factor II gene may increase the risk of venous thromboembolism in its carriers exposed to oral contraceptives |
| *Factor V Leiden* | rs6025 | A | 1 | 169519049 | 169549811 | Proaccelerin (blood-clotting factor V) is a soluble ß-globulin. Factor V Leiden increases the risk of primary and recurrent venous thromboembolism three-to-sixfold therefore should be accounted for when prescribing oral contraceptives to the carriers of this mutation |
| *IFNL3* | rs8099917 | C | 19 | 39743165 | 39252525 | Interferon lambda 3. The rs8099917 polymorphism in the *IFNL3* gene reduces the efficacy of therapy with interferons and ribavirin in patients with viral liver disease |
| *ITGB3* | rs5918 | G | 17 | 45360730 | 47283364 | Integrin beta 3 is a component of glycoprotein IIb/IIIa responsible for the aggregation of platelets. Mutations in the ITGB3 gene often lead to Glanzmann thrombasthenia, a common inherited blood clotting disorder. Carriers of the rs5918 polymorphism can develop moderate to severe mucosal bleeding. The mutation can determine the efficacy of antiplatelet therapy with clopidogrel and NSAID |
| *MTFHR* | rs1801131 | C | 1 | 11854476 | 11794419 | MTHFR is an enzyme essential for the metabolism of folates and methionine. Homozygous carriers of its mutant alleles produce only 30% of the normal MTHFR amount. The rs1801131 polymorphism determines the interpersonal variability in the efficacy and safety of methotrexate, capecitabin, fluorouracil and some other drugs |
| *TPMT* | rs1800460 | A | 6 | 18139228 | 18138997 | TPMT (thiopurine S-methyltransferase) is mostly known for its role in the metabolism of thiopurine derivatives, including azathioprine, 6-mercaptopurine and 6-thioguanine. TPMT catalyzes the S-methylation of thiopurine-based drugs. Mutations in the TPMT gene result in the reduced methylation and poor inactivation of 6-mercaptopurine and, therefore, increase its toxicity |
| *VKORC1* | rs9923231 | T (A) | 16 | 31107689 | 31096368 | The *VKORC1* genes encodes the subunit 1 of the vitamin K1 epoxide reductase complex. This enzymatic complex is responsible for the reduction of vitamin K1 2,3-epoxide to its active form, which is crucial for effective blood coagulation. Warfarin dosage should be lowered for the carriers of this allele. In the literature, the allele is described as C>T or G>A (based on the complementary strand) |

away from the studied populations are highlighted in white. In population genetics, it is imperative to study an indigenous population in any given territory; it is this population's habitat that is shown on a gene geographic map. The gene pools of immigrant populations can be easily reconstructed from the map using information about their migration sources.

To visualize the cartographic models, we applied a "universal" scale of frequency intervals developed for genetic markers with a broad variation in frequency [50]. Frequencies constituting the main body of the frequency "spectrum" (5–60%) are represented by equally sized intervals that follow each other at an eincrement of 5%; the space of low frequencies is represented by 3 intervals, while the space of high frequencies, by intervals with 10% increment. This grading approach allows keeping the number of intervals within a reasonable limit (it would be a challenge to distinguish between over 17 colors on a map). Frequencies below 1% (i.e., below the below the criterion of polymorphism 1%) are shown in the contrasting gray.

## RESULTS

The gene geographic maps showing allele frequency variation for 45 pharmacogenetic markers in the populations of North Eurasia and its neighbor states are available on the web site (see the Appendix). The main characteristics of their allele frequency variation are provided in Table 2. Although the geographic distribution of each marker is unique (there are no two identical maps), the analysis of the maps reveals 3 prominent patterns: clinal variation, uniform distribution and focal variation (see Fig. 1–3). Since it would be impossible to describe all the

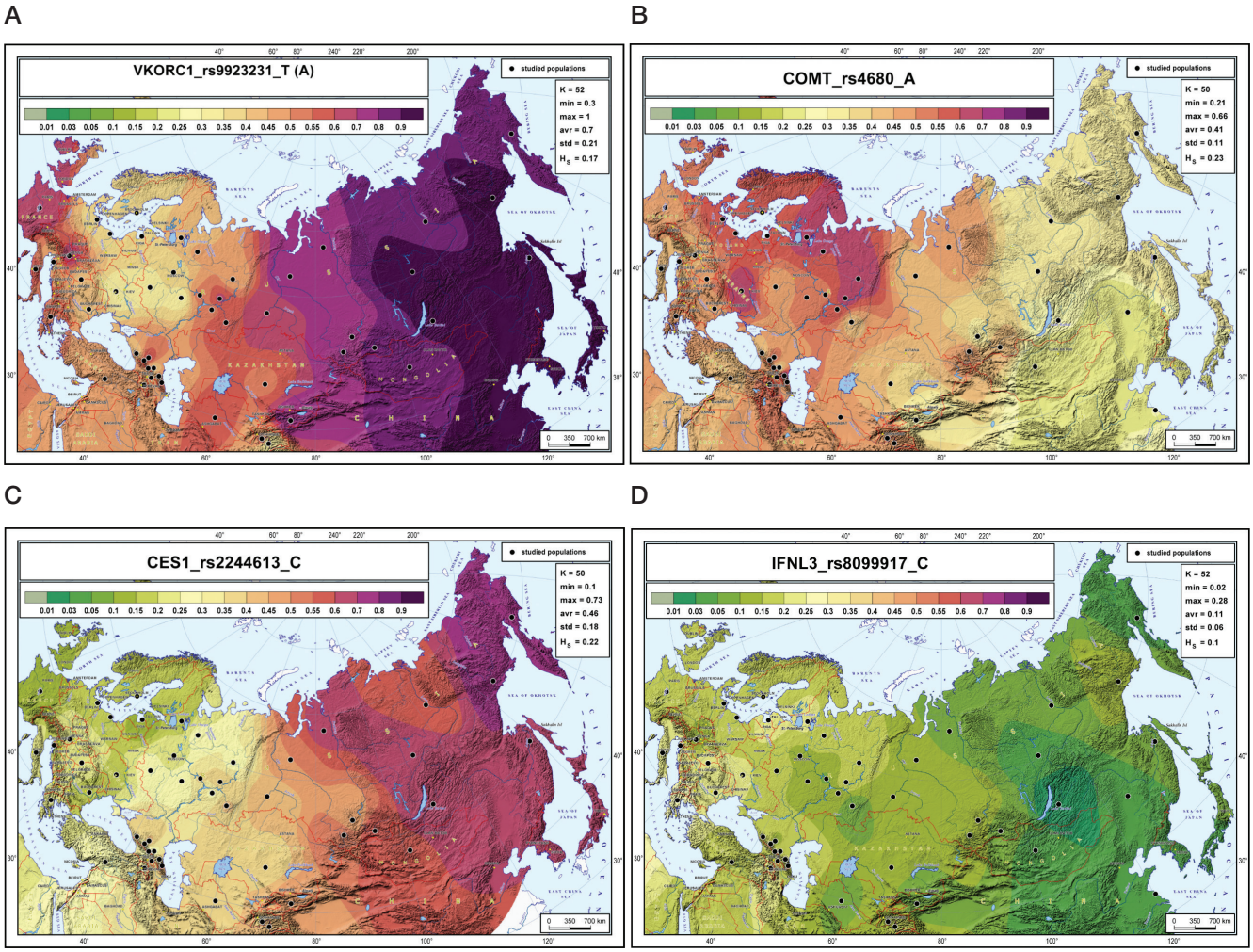


**Fig. 1.** Maps of frequency distribution for pharmacogenetic markers following the clinal variation pattern: *VKORC1* (rs9923231-T(A)) (**A**); *COMT* (rs4680-A) (**B**); *CES1* (rs2244613-C) (**C**); *IFNL3* (rs8099917-C) (**D**)

maps in this publication, we selected 12 markers to illustrate each pattern (4 maps per pattern). These markers are briefly described in Table 3.

## Clinal variation

The clinal (gradient) pattern of allele frequency variation along the East–West axis dominates the gene pool of North Eurasian populations. The pattern was identified during the analysis of classic markers [49, 51] and then corroborated using the data on DNA markers [49]. Moreover, this pattern is typical for the most ancient populations of North Eurasia [49, 52]. This may suggest that the clinal variation pattern emerged in the earliest days of the North Eurasian population and has persisted ever since. Therefore, it is only natural that some of the studied pharmacogenetic markers are characterized by a very distinct clinal variation pattern (see Fig. 1).

The distribution of *VKORC1* rs9923231-T(A) frequencies on the gene geographic map follows a very distinct clinal pattern (Fig. 1A): the lowest frequencies are observed in the West of European Russia and the Caucasus, increasing gradually toward Eastern Eurasia. Notably, the frequencies lying between the Western and Eastern "extremes" of the frequency spectrum are found in West Siberia (but not in the Ural region), similar to the first PCA component of the North Eurasian gene pool and the map of archeological Paleolithic sites [49, 52]. The lowest frequency is observed in the lands inhabited by Eastern Slavs and the populations of the Baltic region ($0.3 \le q < 0.4$; here and below frequency is designated by $q$). From there, the area of low frequencies stretches eastward to the Volga river and includes the habitat of the indigenous population of Mordovia. The frequency of the polymorphism gradually increases toward the West of Europe and southwards. The domain of high frequencies ($q > 0.80$) begins in the East of North Eurasia with Tuvan and Mongol populations; the polymorphism reaches its frequency peak (> 90%) on the Eastern coast of the continent in the indigenous peoples of the Amur region, Evens, Evenks and continental Buryats.

The map of *COMT* (rs4680-A) frequencies (Fig. 1B) demonstrates the same pattern of clinal variation, but the frequency range is narrower (0.44 vs 0.65; see Table 2). Peak values ($0.6 \le q < 0.7$) are observed in the West; however, in the East they fall by only 20–30%. Consequently, the genetic diversity there is much lower ($F_{ST} = 0.02$; see Table 2) than on the previous map ($F_{ST} = 0.07$; see Table 2). High frequencies ($0.5 \le q < 0.7$) are observed in the European part of the continent, but their domain expands to the East, covering the Mari, Chuvash, Udmurt, and Tatar populations of the Volga-Ural region. The region of average frequencies spreads further to the East, spanning the Yenisey River basin. But by and large, the area of high frequencies ($0.2 \le q < 0.35$) corresponds to the area of low frequencies on the previous map.

For *CES1* (rs2244613-C) (Fig. 1C), the pattern of clinal variation is almost a copy of the *VKORC1* (rs9923231-T)

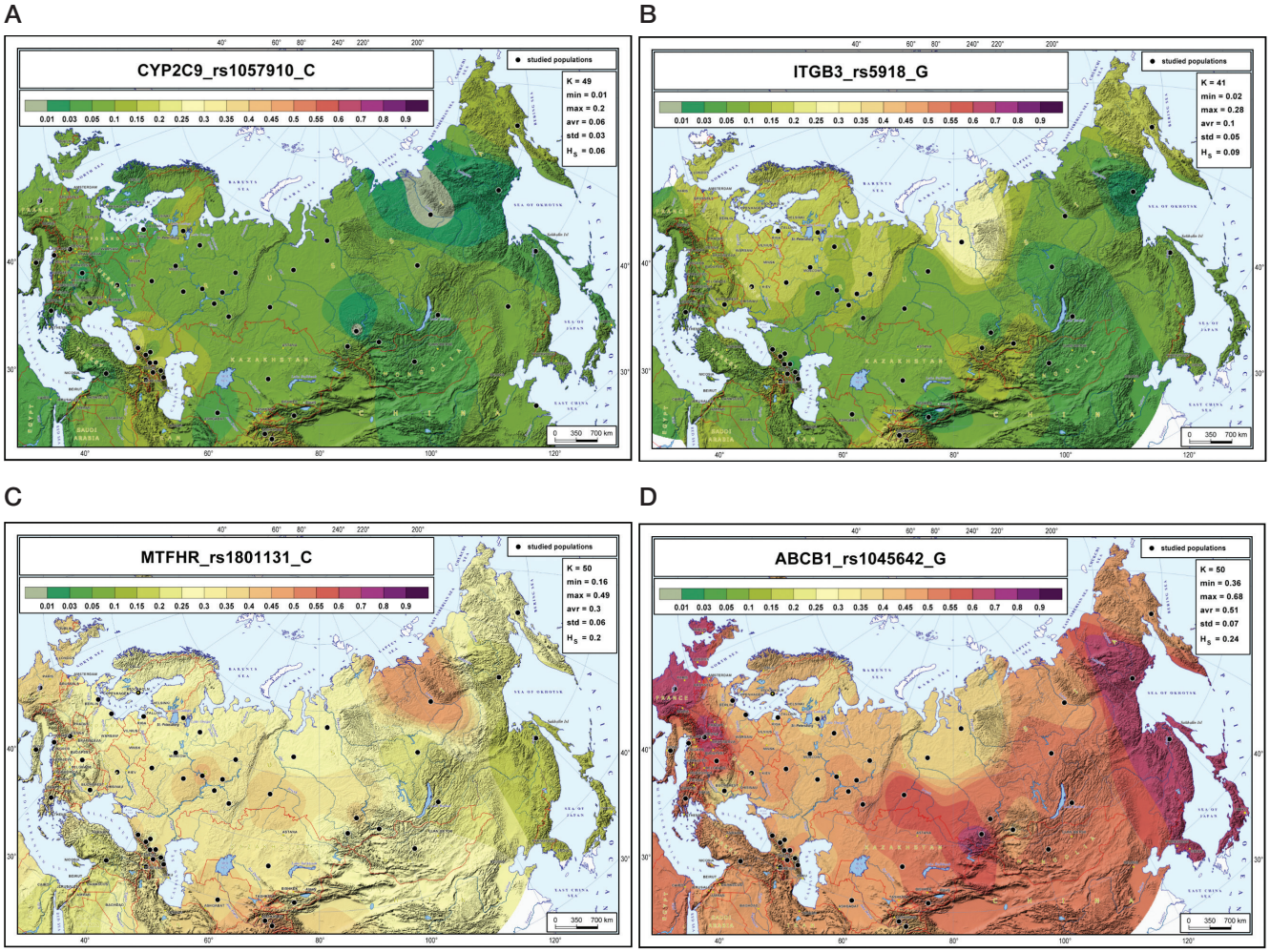


**Fig. 2.** Maps of frequency distribution for pharmacogenetic markers following the uniform distribution pattern: *CYP2C9* (rs1057910-C) (**A**); *ITGB3* (rs5918-G) (**B**) *MTFHR* (rs1801131-C) (**C**); *ABCB1* (rs1045642-G) (**D**)

pattern (see Fig. 1A): the domain of maximum frequencies ($0.55 \le q < 0.8$) is in the East of North Eurasia, but the domain of average frequencies ($0.40 \le q < 0.50$) is again in West Siberia. Minimum frequency values ($0.10 \le q < 0.25$) are observed in Western Europe and are lower than in the populations of Eastern Slavs.

For *IFNL3* (rs8099917-C) (Fig. 1D), the pattern of clinal variation is weakened by the narrow frequency range (0.25; see Table 2). However, similar to the previous map in Fig. 1B, the lowest frequencies of this polymorphism are concentrated in the East of North Eurasia, whereas its maximum frequencies ($0.24 \le q \le 0.28$) are found in Europe (from Karelia to Italy) and in the Caucasus, although they did not cover the entire European mainland. The narrow range of low frequencies ($0.02 \le q \le 0.28$) makes the pattern of clinal variation less distinct due the growing role of a sampling error.

### Uniform distribution

A feature with generally uniform spatial distribution will not necessarily occur at the same frequency at all points in space. Uniform distribution characterizes alleles that occur in almost every population under study but do not exhibit distinct clinal variation on the regional scale, although certain patterns might be identified in some parts of the studied region. Surges or dramatic falls in frequency in some parts of the region suggest a sampling error and the need to revise the number of the populations representing these areas and the sample size. But if these extreme behaviors are observed in more than one neighboring population, they are not evident of random frequency fluctuations but rather indicate a distinct frequency variation pattern in the studied group of populations.

The distribution of *CYP2C9* (rs1057910-C) across North Eurasia (Fig. 2A) is strikingly uniform. The average frequency ($q = 0.08$; Table 2) of the marker is low, slightly above 5%. But in most ethnic groups inhabiting North Eurasia, the marker occurs at nearly identical frequency which varies within a narrow range ($0.03 \le q \le 0.10$). The only identifiable pattern pertains to the increased frequency of this marker observed in almost all populations of the Caucasus and the Transcaucasian region. Single spikes in Karelia, Tadzhikistan and Chukotka and a steep decline below 1% (the gray zone of Yakutia) do not form distinct patterns.

Likewise, *ITGB3* (rs5918-G) (Fig. 2B) is characterized by uniform geographic distribution, its frequency varies within a very narrow range ($0.02 \le q \le 0.28$), and its average frequency is low ($q = 0.11$). However, deviations from the overall uniform distribution now form a pattern: the area of high frequencies stretches in an almost uninterrupted fashion from the Baltic region to the estuary of the Yenisey River. The second frequency peak occurs in the South of North Eurasia: in Tadzhikistan and South Siberia.

The map of *MTFHR* (rs1801131-C) frequency variation (Fig. 2C) shows that the marker is uniformly distributed across the vast territory, although its average frequency ($q = 0.31$; see Table 2) is 3 times higher than the average frequencies from the previous

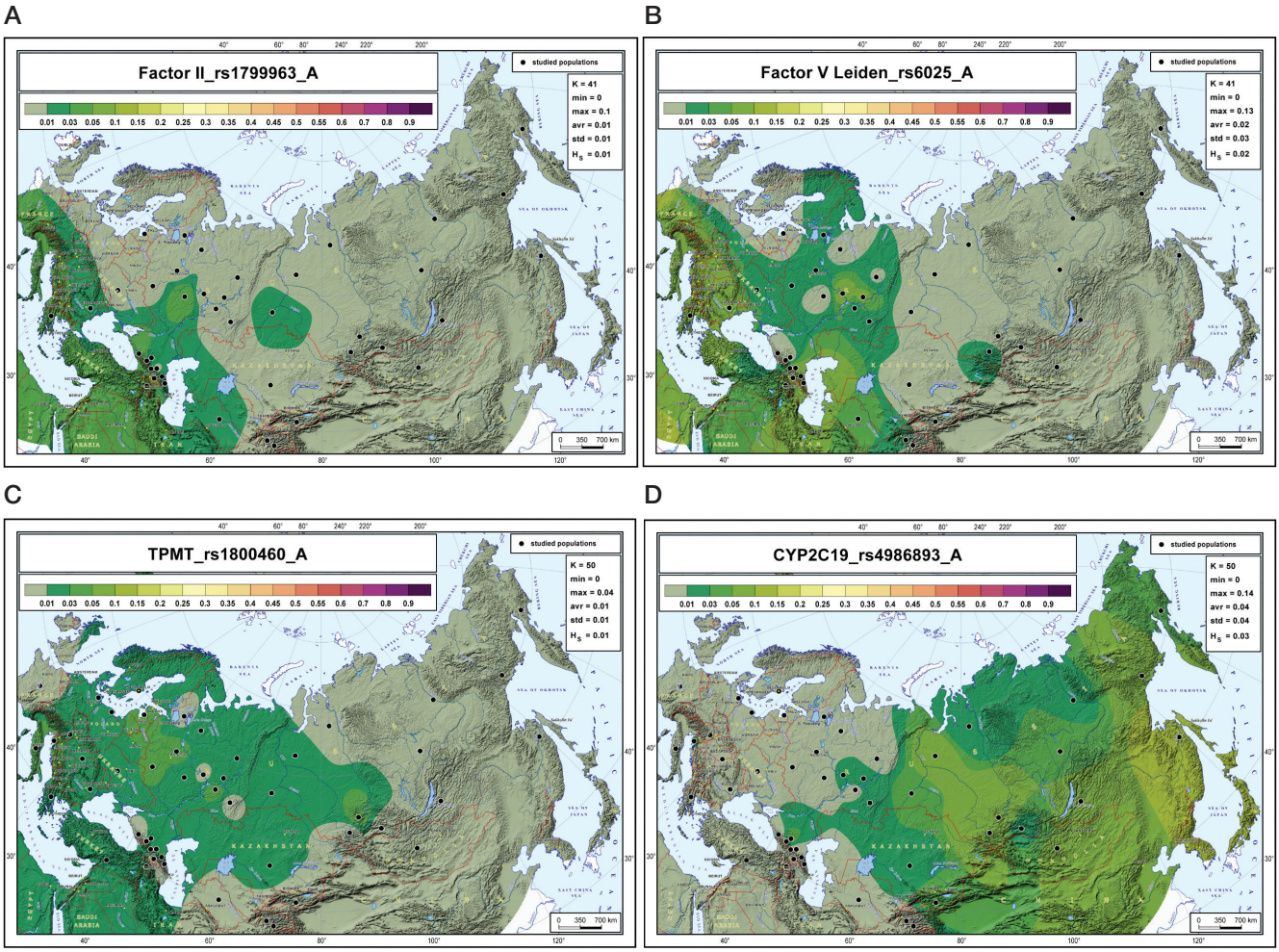


**Fig. 3.** Maps of frequency distribution for pharmacogenetic markers following the focal variation pattern: *Factor II* (rs1799963-A) (**A**); *Factor V Leiden* (rs6025-A) (**B**); *TPMT* (rs1800460-A) (**C**); *CYP2C19* (rs4986893-A) (**D**)

maps. The domain of average frequencies ($0.25 \le q < 0.35$) spreads throughout almost the entire North Eurasia, with two peaks in Yakutia and Tadzhikistan.

The average frequency of *ABCB1* (rs1045642-G) (Fig. 2D) is $q = 0.48$ (see Table 2); the scope of frequency variation is the same as on the previous map (0.32; see Table 2), slightly exceeding the scope of frequency variation in Fig. 2A (0.19) and Fig. 2B (0.26). Average frequencies ($0.40 \le q < 0.55$) cover almost the entire territory of North Eurasia and the neighbor states. Slightly lower frequencies are observed in the North of Middle Siberia in the cluster of Khanty, Mansi, Ket, Nenets and Selkup populations, as well as in two European populations (in Karelia and Moldova). Small areas of increased frequencies are scattered across North Eurasia: frequencies $q > 0.6$ occur in the South-West of Europe, in Ashkenazi Jews, Altaians, Evens and Amur region peoples. These local deviations do not disrupt the generally uniform distribution of the marker.

Similar to the previous set of maps, this set demonstrates that the variation pattern does not depend on the position of the frequency on the frequency spectrum: both high and low frequencies can follow a pattern of uniform distribution across vast territories.

## Focal variation

Uniform distribution and clinal variation are typical for all pharmacogenetic markers that occur almost everywhere in North Eurasia and the neighbor states. If a marker occurs in only one (large as it may be) part of the studied region and is not found in its other parts, the pattern of its distribution can be arbitrarily called “focal variation”. The “focal” frequencies are normally low. On the map, populations that either do not carry this marker in their genome or have it at < 1% frequency are highlighted in gray.

*Factor II* (rs1799963-A) (Fig. 3A) has a frequency range of $0 \le q \le 0.10$ (see Table 2). Its domain stretches uninterruptedly across the Southern regions of North Eurasia, from the Mediterranean to Turkmenia, covering all populations of the Caucasus, excluding the Ingush and Chechen peoples, and reaches its maximum frequency in the Transcaucasia region. Only two populations outside this area are carriers of the marker: Mordovians ($q = 0.05$) and Siberian Tatars ($q = 0.02$).

The frequency of *factor V Leiden* (rs6025-A) (Fig. 3B) varies within almost the same range ($0 \le q \le 0.13$; Table 2), but the area is concentrated in is much vaster, covering the territories on the previous map and the substantial share of the population in Eastern Europe and the Volga-Ural region. Peak frequencies are observed in the South of Europe, Caucasus, and the Transural region (Udmurt, Mari and Chuvash peoples). Outside this area, the polymorphism occurs only in the Altai region, where its frequency is below 1%.

Although the frequency range of *TPMT* (rs1800460-A) (Fig. 3C) is extremely narrow ($0 \le q \le 0.04$) and its peak frequency is below 5%, its domain stretches across the entire Europe (except for the far South-West, which might be explained by the small sample size representing Basque, Spanish, and

French populations; $n$ = 29), Ural, West Siberia, Kazakhstan, to Khakassia. Interestingly, this marker is not found in most populations of the Caucasus, except for the Central Caucasus (the Ingush, Ossetians, Chechens) and North Dagestan.

On its genogeographic map, *CYP2C19* (rs4986893-A) (Fig. 3D) occupies an enormous territory that spans the Asian part of the region, except for Tadzhikistan and Turkmenia, protruding into the European mainland, including Ciscaucasia (Nogais), the Caucasus (Ossetians), and the Volga region (the Mari, Udmurt, Tatar, and Chuvash). The frequency peaks in the Far East in the populations of the Amur region ($q$ = 0.14), Evens ($q$ = 0.10) and in Khakassia ($q$ = 0.10). For this marker, the scope of frequency variation is narrow ($0 \le q \le 0.14$; see Table 2), and the frequencies themselves are comparable to the markers in Fig. 3 that cover a much smaller area. Maps in Fig. 3 demonstrate that markers with low frequencies and low variation in frequency can cover from small to very extensive areas.

## DISCUSSION

There are two types of differences between populations that matter in pharmacogenetics: differences in the presence of pharmacogenetic markers and differences in their frequencies. The first type describes the alleles that are widespread in one population but almost non-existent in the other (some can even be region-specific). The second type pertains to different (sometimes contrasting) allele frequencies of the marker in different populations.

Of 3 patterns identified in our study, clinal variation and uniform distribution describe differences in allele frequencies between populations across the studied territory; importantly, the markers characterized by these two distribution patterns are found in almost every population of the studied territory. The focal variation pattern reflects differences in the presence of pharmacogenetic markers between different populations.

The clinal variation pattern (see Fig. 1) is characterized by the presence of 2 extremes on the frequency spectrum, with an area of intermediate values between them. The extremes are more common for the markers that exhibit great variation in frequency, although this may not always be the case: markers that have a very narrow frequency range often form a geographically distinct gradient pattern. In North Eurasia, the clinal variation pattern was observed for 6 of 45 markers (13% of the maps in the atlas; see Table 2).

With uniform distribution (see Fig. 2), differences in frequencies are either small or their spikes and falls occur sporadically across the entire studied territory. However, there are areas where allele frequencies follow certain patterns, which might become the object of future research. In North Eurasia, 12 of 45 markers followed the pattern of uniform distribution (27% of the maps; see Table 2).

A marker that follows the focal variation pattern is common for one population, but occurs at almost negligible frequency below 1% or is totally absent in another. The area where this marker occurs may vary in size (Fig. 3): from small compact foci to vast territories. Attempts to identify the pattern of marker variation in the "focal" area should account for the sampling error since small sample sizes and low marker frequencies can skew the picture. In our study, focal variation was the most common pattern observed for 27 of 45 markers (60% of the maps; see Table 2).

A cartographic atlas is a systematic collection of intertwined, mutually complementary maps that form a single entity [52]. Our pharmacogenetic atlas includes maps for 45 ADME markers in 21 genes, but the total number of markers with proved pharmacogenetic significance approaches a few hundred [2, 53]. This is one of the reasons why our collection of maps, which meets the formal requirements for a cartographic atlas [52], is referred to in this publication as the first draft version of the atlas. Our atlas covers only the key pharmacogenetic markers included in a genome-wide Illumina panel; other important markers are not included. The second reason is rooted in how comprehensive the study is. Our maps were based on a large number of populations ($K$ = 50), but the average sample size was relatively small ($n$ = 44); therefore, the maps reveal only general patterns of distribution for every studied marker, and the error in the frequency of a given marker in a given population might be substantial. This indicates the need for further data accumulation, which has already been initiated by a number of researchers [15].

Nevertheless, this first version of the pharmacogenetic atlas provides valuable information on the variation of each studied marker and allows for general conclusions to be drawn. For example, the atlas shows that the average frequency of the marker and its sporadic occurrence in individual populations should not be interpreted as an indication of its geographic distribution pattern. In order to identify the distribution pattern, other tools should be employed, of which a gene geographic map appears to be the most suitable. The analysis of all maps in aggregate can identify areas that display their own patterns of frequency distribution and therefore should be subjected to additional analysis. Among such areas are Western Europe, the Caucasus, the Far East, North Siberia, and some others. Other important patterns, not limited to the 3 patterns described in this paper are expected to be revealed across this vast territory as more populations are studied and more data is accumulated.

Our collection of maps provides a wealth of information that can be exploited in pharmacogenetic research: they show frequencies of each of 45 SNPs in different populations across the entire territory of Russia and the neighbor states. However, caution should be exercised when using the obtained figures, considering the following limitations: the sampling error; the fact that these frequencies represent indigenous populations; and the fact that they represent the entire population but not its single members.

The first limitation is an average sample size. In our study, it was 44 people (88 chromosomes). Consequently, if a marker has a frequency of 25%, the CI will include the margin of error of ± 6%. This CI is much narrower for the territory of Russia, where the samples are larger, and broader for its neighbor states (the samples of their populations available in the literature are quite small). To account for CI fluctuations, the maps for marker frequencies should be accompanied by reliability maps [55].

The second limitation is related to the fact that each region of the map is represented by the gene pool of its indigenous peoples. Therefore, the frequency of a pharmacogenetic marker in an ethnically Russian population of Siberia should be estimated from the maps of migration sources for this population, e.g. Central Russia, but not the maps of Siberia. Thus, knowing the genealogy of a patient, one can construct their map-based pharmacogenetic portrait in a situation when genotyping cannot be performed.

The third limitation is typical for all pharmacogenetic population studies: the genotype of an individual patient does not necessarily reproduce the average characteristics of the gene pool the patient represents; still, these average characteristics are the best possible approximation of an individual genotype. Therefore, it is possible to apply standard pharmacogenetic protocols to an individual patient, drawing on the average genotype based on the allele frequency

in a given population. This approach has obvious limitations, but there is an internationally held opinion that such protocols are the best solution in cases when an individual genotype of the patient is unknown or genotyping poses a financial burden. In our future studies, we will assess the significance of population frequencies for the economy by investigating the costs of inadequate drug dosing.

## CONCLUSION

Based on the data on 50 populations of Russia and its neighbor states, we created a cartographic atlas showing the geographic distribution of 45 pharmacogenetic markers. The gene geographic atlas is composed of 45 maps that demonstrate the frequency of the studied pharmacogenetic markers in a variety of populations across Russia. The maps are straightforward and easy to understand by specialists from different fields and with different qualifications.

The pharmacogenetic atlas of Russia and its neighbor states reveals 3 major spatial distribution patterns of ADME, drug target and hemostasis-controlling genes. Some of the studied markers follow the clinal variation pattern (a gradient change in frequency along the East–West axis), which is the main pattern of allele frequency variation in North Eurasian populations. Uniform distribution singles out a group of markers that occur at average frequency in most Russian regions. Focal variation is observed in the markers that are specific to a certain ethnicity/region and are absent on other populations.

The lack of information about the frequency of pharmacogenetic markers in Russia impedes the adoption of personalized treatment algorithms originally developed for West European populations. There are countless examples of insufficient efficacy of standardized dosing protocols in non-European populations. Adaptation of the existing protocols to Russian populations require information about the geographic distribution of clinically significant markers in Russian populations. This study has generated an extensive body of systematized data and thereby may contribute to research in this important clinical field that demands thorough planning and additional large-scale studies.

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# INTERRELATION BETWEEN miRNA AND mRNA EXPRESSION IN HT-29 LINE CELLS UNDER HYPOXIA

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Hypoxia accompanies various pathophysiological processes, including progression of tumors and metastasis. One of the mechanisms of molecular response of cells to hypoxia implies recruitment of specific miRNAs that regulate the expression of their target genes. This study aimed to evaluate the hypoxia-induced change in expression of miRNAs and their target genes in the HT-29 human colorectal adenocarcinoma cell line with the help of integrated miRNA and mRNA sequencing. To simulate hypoxia, the cells were treated with cobalt (II) chloride. We registered a significant change in expression of sixteen human miRNAs. Six of them (hsa-miR-18a-5p, hsa-miR-22-3p, hsa-miR-27a-5p, hsa-miR-182-5p, hsa-miR-215 -5p, hsa-miR-425-5p) had a significant proportion of target genes that had the expression changing in the opposite direction. Based on the bioinformatic analysis of interactions between differentially expressed transcription factors and miRNAs, we built a possible regulatory network with its main hubs being HIF-1α, p65, c-Myc, and Egr1 (encoded by the *HIF1A*, *RELA*, *MYC* and *EGR1* genes).

**Keywords:** hypoxia, miRNA, mRNA, transcriptome, sequencing, intestinal epithelium, HIF-1α, HT-29

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**Compliance with ethical standards:** the study was approved by the Ethics Committee of Amur State Medical Academy (Protocol № 10 dated November 20, 2019); the study conformed with the guidelines for the medical research involving human subjects. Voluntary informed consent was obtained from all the participants.

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# ВЗАИМОСВЯЗЬ ИЗМЕНЕНИЯ ЭКСПРЕССИИ миКРОРНК И мРНК В КЛЕТКАХ ЛИНИИ HT-29 В УСЛОВИЯХ ГИПОКСИИ

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Гипоксия возникает в различных патофизиологических процессах, включая прогрессирование опухолевых заболеваний и метастазирование. Один из механизмов молекулярного ответа клеток на гипоксию состоит в рекрутировании специфичных микроРНК, регулирующих экспрессию своих генов-мишеней. Целью работы было оценить изменения экспрессии микроРНК и их генов-мишеней в клеточной линии колоректальной аденокарциномы человека HT-29 в ответ на гипоксию с помощью интегрированного секвенирования микроРНК и мРНК. Для моделирования условий гипоксии клетки обрабатывали хлоридом кобальта (II). Было обнаружено достоверное изменение экспрессии 16 человеческих микроРНК, шесть из которых (hsa-miR-18a-5p, hsa-miR-22-3p, hsa-miR-27a-5p, hsa-miR-182-5p, hsa-miR- 215-5p, hsa-miR-425-5p) имели статистически значимую долю генов-мишеней с противоположным направлением изменения экспрессии. На основании биоинформатического анализа взаимодействий дифференциально экспрессированных факторов транскрипции и микроРНК была построена возможная регуляторная сеть, основными узлами которой оказались HIF-1α, p65, c-Myc и Egr1 (кодируемые генами *HIF1A*, *RELA*, *MYC* и *EGR1*).

**Ключевые слова:** гипоксия, микроРНК, мРНК, транкриптом, секвенирование, эпителий кишечника, HIF-1α, HT-29

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Hypoxia plays a role in the development and course of a number of pathological conditions, such as cardiovascular disorders [1] and tumor developments [2]. Hypoxia models staged *in vitro* allow studying the response to hypoxia at the molecular and cellular levels. One of the traditional models involves chemical agents that activate the hypoxia signaling pathways. $CoCl_2$ is one of the most common chemical hypoxia induction agents, since this substance causes direct and long-term stabilization of the hypoxia-induced factors 1 and 2 (HIF-1, HIF-2) [3].

MiRNAs are short non-coding RNAs; their main function is to suppress genes post-transcriptionally [4]. Usually, one miRNA has dozens of target genes, while 3'-UTR of a gene may have binding sites for hundreds of miRNAs [5]. It has been shown that interactions between miRNAs and their target genes play an important role in intercellular communication [6] and pathogenesis of many diseases, including various types of tumors [7, 8].

A number of studies aimed to investigate the role and functional activity of cellular miRNAs under the hypoxic stress conditions. It was found that some miRNAs, such as miR-210 or miR-27, are altered by hypoxia in many cells, and the differential expression of miRNAs and their targeting usually depend on the mechanism of hypoxia induction and cell type [9]. A relationship between the patterns of hypoxia-driven changes in miRNA expression and tumors has also been established: most of the miRNAs associated with the tumors can be affected by hypoxia [10].

This study aimed to investigate the effect of hypoxia on the miRNA profile and transcriptome in the HT-29 human colorectal adenocarcinoma line cells and to identify the potential key molecules involved in the response to hypoxia.

## METHODS

### Cell cultivation and processing

Human colorectal adenocarcinoma cells HT-29 (ATCC; USA) were cultured in McCoy's 5A medium (Thermo Fisher Scientific; USA) containing 10% fetal bovine serum (Thermo Fisher Scientific; USA). Penicillin (100 U/ml) and streptomycin (100 mg/ml) were added to the nutrient medium. The cells were seeded into 6-well plates in the amount of $4 \times 10^5$ cells per well, then cultured in a humidified atmosphere at +37 °C and 5% $CO_2$ for 48 h. To induce hypoxia, a water solution of cobalt chloride ($CoCl_2$) was prepared, added to the medium to the concentration of 300 μM, and incubated for 24 h. Three biological iterations were performed for both control and treatment group cells.

### RNA isolation

The cells were lysed in the Qiazol Lysis Reagent (Qiagen; Germany) for subsequent total RNA extraction with the help of the Qiagen miRNeasy Mini Kit (Qiagen, Hilden; Germany). The amount of isolated RNA was determined using a Nanodrop device (Thermo Fisher Scientific; USA). Agilent High Sensitivity DNA Kit (Agilent Technologies; USA) and a Bioanalyzer 2100 capillary electrophoresis instrument (Agilent Technologies; USA) enabled analysis of quality of the isolated RNA samples. The RIN (RNA integrity number) parameter value for all samples was above 9.0.

### Preparation of libraries and sequencing

The libraries for mRNA sequencing were obtained from the total RNA samples using the Illumina Stranded mRNA Library Prep Kit Illumina (Illumina; USA). Each sample was sequenced on an Illumina NextSeq 550 to obtain 75 nucleotide reading frames at one end.

The libraries for miRNA sequencing were prepared from the total RNA samples using the NEBNext Multiplex Small RNA Library Prep Kit for Illumina. Each sample was sequenced in the Illumina NextSeq 550 to obtain 50 unidirectional nucleotide reading frames.

We sequenced mRNA and miRNA for three biological iterations, with each of them having four technical iterations set up.

### Processing of the sequencing results

FASTQ file quality was assessed with the help of FastQC v0.11.9 (Babraham Bioinformatics; UK). One of the $CoCl_2$-treated replicates did not pass quality control at the microRNA sequencing stage. The adapters were cut using Cutadapt v2.10 [11]. The resulting mRNA fragment sequences were mapped to the human genome (GENCODE GRCh38.p13) using STAR v2.7.5b [12]. MiRDeep2 v2.0.1.2 package [13] enabled compilation of the miRNA expression matrix.

The sequencing library depths were normalized with the Trimmed Mean of M-values (TMM) algorithm available in the edgeR v3.30.3 package [14] with default background noise filtering. The same package was used to generate normalized mRNA and miRNA expression matrices in Reads Per Kilobase of transcript per Million mapped reads (RPKM) and Reads Per Million mapped reads (RPM) units, respectively. We took the logarithms of the values obtained with 2 as the base. For further processing, we only used the highly expressed transcripts and cut off the bottom 5% of genes and 50% of miRNAs based on their mean RPKM/RPM values.

### Evaluation of the differential expression and overrepresented signaling pathways

We used DESeq2 v1.28.1 [15] to analyze the differential expression and applied the Benjamini-Hochberg procedure to determine the false detection rate (FDR). The differences with FDR below the threshold value of 0.05 were considered significant. DAVID v6.8 online service [16] enabled the analysis of overrepresented signal pathways.

### Prediction of the miRNA targets

At the first stage of miRNA target prediction we exported a list of miRNA-gene interactions from TargetScan v7.2 [17]. Then, we selected the negative expression correlation miRNA-gene pairs from The Cancer Genome Atlas Colon Adenocarcinoma (TCGA-COAD) cohort [18]. The initial miRNA/mRNA expression matrices for tumor samples were obtained from the GDC Data Portal (https://portal.gdc.cancer.gov/) and converted into the RPKM/RPM tables using the procedure described above. Next, we calculated the Spearman correlation for each miRNA and the predicted target gene. The thresholds of 0.05 and –0.1 were applied to the FDR and correlation values, respectively.

### Building the regulatory network of interactions between transcription factors and miRNA

We took the information on the regulatory interactions of transcription factors and miRNAs from the curated TransmiR v2.0 database [19]. The resulting interaction network was built and visualized in the yED Graph Editor (yWorks GmbH; Germany).

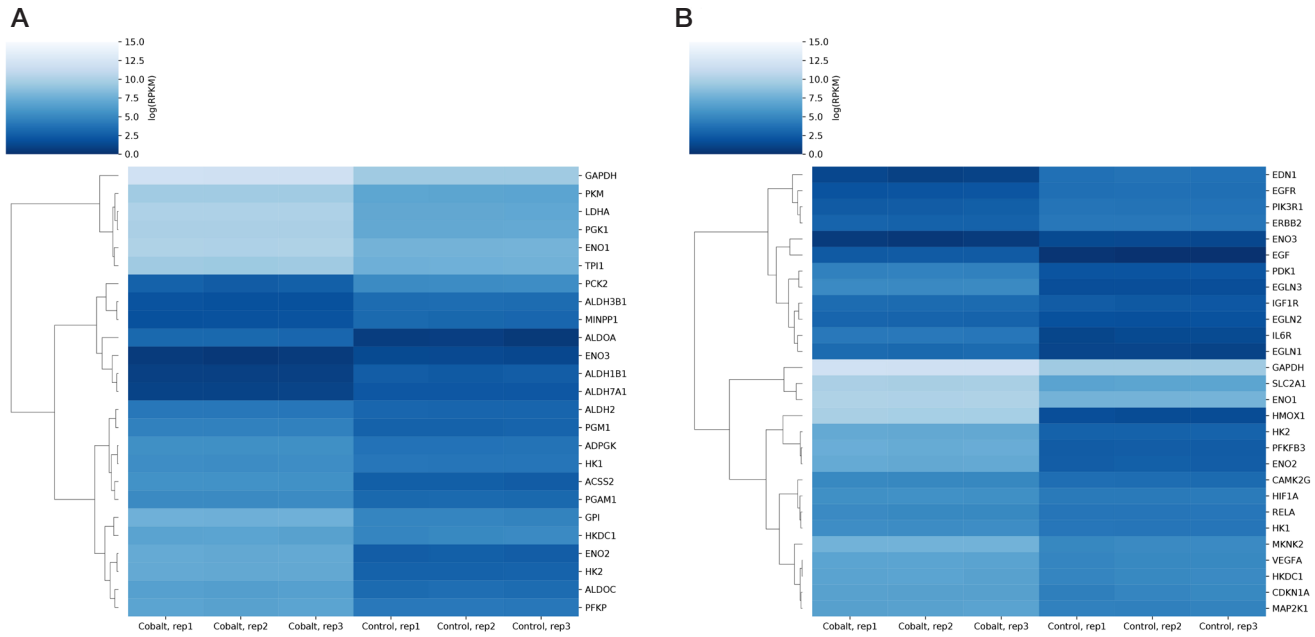


**Fig. 1.** Differential expression of genes associated with hypoxia. **A**. Glycolysis/gluconeogenesis. **B**. HIF-1 signaling pathway

RESULTS

### Effect of Cobalt (II) Chloride on gene expression in HT-29 cells

To chemically induce hypoxia, HT-29 cells were treated with cobalt (II) chloride for 24 h. Analysis of sequencing of the RNA isolated from HT-29 control cells and treated with cobalt chloride showed a statistically significant change in the expression of 2511 genes that encode proteins, this change being 2-fold and greater in response to hypoxia. The search for overrepresented metabolic pathways identified 25 genes associated with the switch of aerobic metabolism to anaerobic glycolysis (KEGG pathway hsa00010 Glycolysis/Gluconeogenesis, FDR = $2.04 \times 10^{-4}$; Fig. 1A), and also demonstrated activation of the HIF-1 signaling pathway (KEGG pathway hsa04066 HIF-1 signaling pathway, FDR = $4.45 \times 10^{-3}$) that allows cobalt chloride to simulate hypoxia (Fig. 1B). In addition to the HIF-1 pathway, several other signaling cascades involved in the response to hypoxia were activated, including NF-κB [20] and AMPK [21] (attachment 1).

The most overrepresented category corresponded to the genes encoding proteins of proteasome complexes (KEGG pathway hsa03050 Proteasome, FDR = $2,02 \times 10^{-16}$). In particular, 33 genes were significantly activated in response to hypoxia, including 6 out of 6 ATPases, 11 out of 12 subunits of the 26S proteasome lacking ATPase activity, 7 out of 8 α-subunits and 7 out of 11 β-subunits of the 20S proteasome, as well as the proteasome maturation protein *POMP* and proteasome activator subunit *PSME4* (attachment 2). We have registered an increase in the expression of *UBB*, *UBC*, *UBA52* and *RPS27A* genes, the increase being 3.1, 8.7, 2.0 and 1.6-fold, respectively. These genes encode ubiquitin, which is needed for proteasome-dependent protein degradation.

**Table 1.** Differentially expressed miRNAs

| MiRNA | Mean expression level in control (RPM) | Expression change, times* | FDR |
|---|---|---|---|
| hsa-miR-210-3p | 372.38 | 2.40 | $4.01 \times 10^{-20}$ |
| hsa-miR-4521 | 452.54 | –2.48 | $2.31 \times 10^{-18}$ |
| hsa-miR-615-3p | 739.12 | –1.90 | $5.30 \times 10^{-10}$ |
| hsa-miR-22-3p | 1032.99 | 1.65 | $5.21 \times 10^{-6}$ |
| hsa-miR-425-5p | 751.22 | –1.44 | $8.52 \times 10^{-4}$ |
| hsa-let-7a-3p | 631.34 | –1.44 | $2.87 \times 10^{-3}$ |
| hsa-miR-32-5p | 594.48 | –1.43 | $3.18 \times 10^{-3}$ |
| hsa-miR-215-5p | 2604.64 | 1.49 | $6.61 \times 10^{-3}$ |
| hsa-miR-224-5p | 4385.37 | 1.41 | 0.0123 |
| hsa-miR-182-5p | 3935.22 | 1.49 | 0.0144 |
| hsa-miR-1260b | 550.76 | –1.35 | 0.0187 |
| hsa-miR-1260a | 531.53 | –1.34 | 0.0241 |
| hsa-miR-27a-5p | 158.10 | 1.51 | 0.0317 |
| hsa-miR-30b-5p | 1509.37 | 1.31 | 0.0317 |
| hsa-miR-10a-3p | 2102.20 | –1.29 | 0.0417 |
| hsa-miR-18a-5p | 225.40 | –1.39 | 0.0444 |

**Note:** * — positive and negative values indicate the cellular miRNA level drop or growth in response to hypoxia.

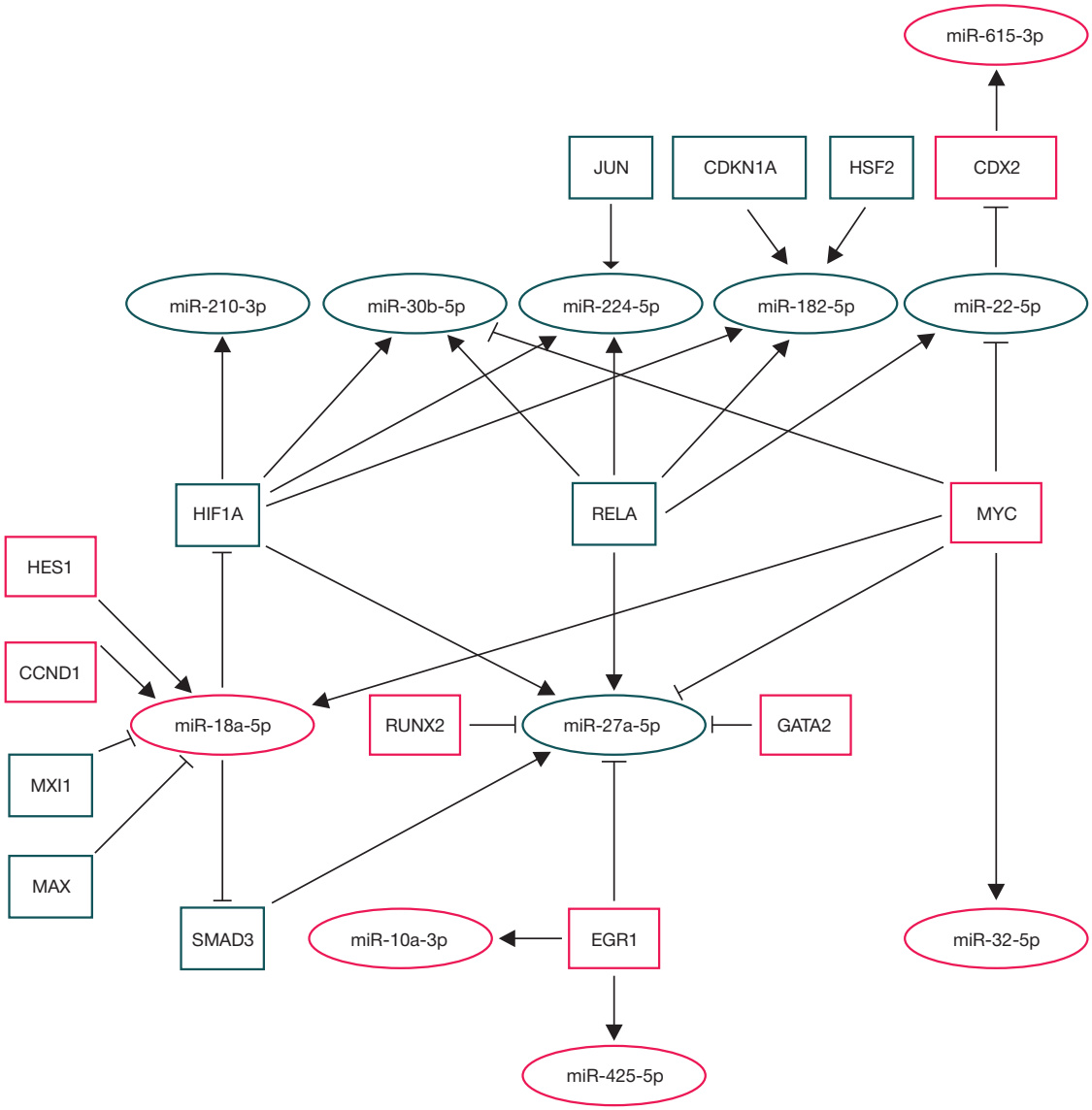


**Fig. 2.** Regulatory network of interactions between transcription factors (TF) and miRNA, induced by hypoxia. *Rectangles* are TFs, *ellipses* — miRNAs. Expression growths and drops are shown in *green* and *red*, respectively. *Arrows* indicate activation of the expression, *T-shaped lines* — its suppression

We have also detected a change in the expression of genes that encode proteins involved in focal adhesion (integrins and laminins). Accordingly, the expression levels of laminin subunits α3, β3, Υ1, and Υ2 were increased 5.5, 4.6, 3.1 and 4.5-fold, respectively. Three of them (α3, β3, Υ2) can form a heterotrimer and thus generate laminin 332, also known as laminin-5 [22]. The expression of integrin subunits changed in a different direction: subunits αE, αV and β1 showed the growth of 1.8, 2.2, 1.8 times, while for subunits α1, α2, β3 and β8 the registered expression level decrease was 2.3, 2.1, 1.7 and 2.7 times.

### Effect of hypoxia on the expression of miRNAs and their target genes

We have registered 16 miRNAs (Table 1) showing a significant change in the expression as a response to the treatment of cells with cobalt chloride. One of them was hsa-miR-210-3p, the only miRNA that is cited in all papers published as increasing expression in response to hypoxia [23]. The level of several confirmed hsa-miR-210-3p mRNA targets involved in mitochondrial metabolism and apoptosis induction decreased in response to hypoxia: *GPD1L* by 2.3 times, *CASP8AP2* by 1.7 times, and *AIFM3* by 8 times.

The following analysis was performed to assess the overall functional effects of the cellular miRNA content fluctuations induced by hypoxia. The TargetScan resource enabled compilation of the list of potential miRNA targets. Since some interactions between miRNA and target mRNA inhibit translation without affecting the level of mRNA expression, analyzing the samples from 441 colon adenocarcinoma patients (taken from the TCGA-COAD database, Table S3) we searched for miRNA–target mRNA pairs showing a significant negative correlation. Next, we crossed the resulting list with the list of genes that had the expression changed at least two-fold in the direction opposite that peculiar to the corresponding miRNA. The result were six miRNAs with a statistically significant number of deregulated target genes (hypergeometric test; $p < 0.05$): hsa-miR-18a-5p, hsa-miR-22-3p, hsa-miR-27a-5p, hsa-miR -182-5p, hsa-miR-215-5p, hsa-miR-425-5p (attachment 4).

### Building the network of regulatory interactions between transcription factors and miRNA

To better understand the mechanisms underlying the aberrant expression of microRNAs, we analyzed the possibility of regulation of microRNAs with transcription factors (TF). In particular, we

**Table 2.** MiRNAs showing differential expression patterns specific to the particular miRNA arms

| MiRNA | Mean expression level in control (RPM) | Expression change, times* | FDR |
|---|---|---|---|
| hsa-let-7a-5p | 21537.87 | 1.07 | 0.899 |
| hsa-let-7a-3p | 631.34 | –1.44 | $2.87 \times 10^{-3}$ |
| hsa-miR-10a-5p | 100119.91 | 1.09 | 0.872 |
| hsa-miR-10a-3p | 2102.20 | –1.29 | 0.0417 |
| hsa-miR-27a-5p | 158.10 | 1.51 | 0.0317 |
| hsa-miR-27a-3p | 6321.04 | 1.04 | 0.929 |

**Note:** * — positive and negative values indicate the cellular miRNA level drop or growth in response to hypoxia.

considered TFs that had their mRNA representation significantly changing (two-fold or greater) in response to hypoxia and searched for the miRNAs they regulate in the TransmiR database of regulatory TF-microRNA interactions. As a result, we identified 30 TF–miRNA interactions between 15 TFs and 11 miRNAs. We have also considered the reciprocal miRNA-induced TF silence to build a complete regulatory network at these nodes (Fig. 2). As is shown, the four TFs encoded by the *EGR1*, *HIF1A*, *MYC*, and *RELA* genes simultaneously regulate several miRNAs, while most miRNAs are regulated by the TF ensembles.

## DISCUSSION

This study investigated changes in the transcriptome landscape of HT-29 cells in response to hypoxia induced by cobalt (II) chloride. The investigation relied on the mRNA and miRNA integrative sequencing technique. In addition to the activation of HIF-1, the canonical signaling pathway, we have shown that the expression of integrins and laminins changes, too. These proteins play a critical role in cell adhesion and interactions with the extracellular matrix. The latter is of particular importance, since recent data indicate a close relationship between the microenvironment formed by hypoxia and the metastatic progression of tumors, including colon adenocarcinoma [24]. One of the possible mechanisms for the metastatic spread of tumors is associated with laminin 332. By interacting with various receptors on the cell surface (including integrins α6β4 and α3β1, epidermal growth factor receptor and syndecan 1), as well as some other components of the basal membrane, laminin 332 regulates the process of oncogenesis, promotes invasion and survival of tumor cells [25]. The increased expression of the ϒ1-arm of laminins (encoded by the *LAMC1* gene) may also play a role in the progression of tumor diseases, as has been shown in uterine carcinoma [26].

The analysis of profile of the small noncoding RNAs revealed several miRNAs expressed differentially in response to hypoxia. Some of these miRNAs were already reported as having their expression changed under hypoxia, including hsa-miR-210-3p [23], hsa-miR-27a-5p [27], hsa-miR-182-5p [28]. Four miRNAs (hsa-miR-30b-5p, hsa-miR-32-5p, hsa-miR-425-5p, hsa-miR-1260a, and hsa-miR-1260b) were not previously covered in the published papers in connection with the cellular response to hypoxia. This can be explained both by the cellular specificity of the response and by the cobalt chloride's stream effects.

Particular attention should be paid to the miRNAs that show differential patterns of expression specific to a particular miRNA arm (arm-specific differential expression patterns). Namely, this applies to the miRNAs that had only the passenger arms hsa-let-7a, hsa-mir-10a and hsa-mir-27a regulated, while the expression of their guide arms did not change (Table 2). We have recently reported a similar observation for miR-21-3p (passenger arm) in mouse lungs; it showed an eight-fold increase in expression in response to the SARS-CoV infection, while the guide arm of the same miRNA increased only threefold [29]. One of the most promising theories that could explain this phenomenon is the regulation of miRNA arms by RNA-binding proteins [30].

The analysis of regulatory interactions between TF and miRNA showed that HIF-1, p65, c-Myc, and EGR1 (encoded by the *HIF1A*, *RELA*, *MYC* and *EGR1* genes) are the key factors regulating transcription of differentially expressed miRNAs (see Fig. 2). Three of the considered miRNAs demonstrated reciprocal capabilities and suppressed some of the TFs. In particular, *HIF1A* is a confirmed target for hsa-miR-18a-5p that is suppressed by multiple TFs.

## CONCLUSION

The performed integrative sequencing of miRNA/mRNA allowed revealing significant changes in the transcriptome and miRNA profile in HT-29 cells in response to the $CoCl_2$-induced hypoxia. We have shown that differential expression of several of the miRNAs can cause significant changes in the expression of their target mRNAs. The analysis of the regulatory interactions between transcription factors and miRNAs revealed possible mechanisms underlying the observed response to hypoxia.

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# IMPACT OF P53 MODULATION ON INTERACTIONS BETWEEN P53 FAMILY MEMBERS DURING HACAT KERATINOCYTES DIFFERENTIATION

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HaCaT cell line is a widely used model for studying normal human keratinocytes. However, mutations of TP53 gene are typical for this cell line, which have a substantial impact on functions of the encoded protein. The features of this regulatory circuit should be considered when using HaCaT cells for assessment of human skin physiology and pathology *in vitro*. The study was aimed to assess the features of differentiation realization in HaCaT cells with modulated activity of p53 protein. The expression of p53 was reduced by knockdown of *TP53* gene by shRNA (by 2.2 times, $p < 0.05$), and the elevated concentration of the p53 active forms was achieved via exposure of cells to Nutlin-3a, the MDM2 inhibitor and the major negative regulator of p53. It has been found that regulation of at least three differentiation markers, *CASP14*, *IVL* (expression increase by 3.9 and 3.7 times respectively in the p53-knockdown cells, $p < 0.05$) and *TGM1* (twofold expression decrease in the p53-knockdown cells, and 1.7-fold expression increase under exposure to Nutlin-3a, $p < 0.05$) in HaCaT cells is p53-mediated. The positive correlation has been revealed for expression of *TGM1* and p53 that might be realized indirectly via ΔNp63 expression alteration. At the same time, modulation of p53 does not result in significant alterations in expression of cytokeratins.

**Keywords:** HaCaT, keratinocytes differentiation, p53, p63, ΔNp63, TAp63, Nutlin-3a, shRNA, knockdown

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**Compliance with ethical standards:** the study was carried out in accordance with the World Medical Association Declaration of Helsinki.

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# ВЛИЯНИЕ МОДУЛЯЦИИ АКТИВНОСТИ P53 НА ВЗАИМОДЕЙСТВИЕ ЧЛЕНОВ СЕМЕЙСТВА P53 В ПРОЦЕССЕ ДИФФЕРЕНЦИРОВКИ КЕРАТИНОЦИТОВ ЛИНИИ НАСАТ

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Клетки линии HaCaT являются распространенной моделью для исследования нормальных кератиноцитов человека. Однако для клеток этой линии характерны мутации в гене *TP53*, что существенно сказывается на функциях кодируемого белка. Особенности данного регуляторного контура необходимо учитывать при использовании клеток HaCaT в качестве объекта исследований физиологии и патологии кожи человека *in vitro*. Целью исследования было изучить особенности реализации программы дифференцировки в клетках линии HaCaT в условиях модуляции активности белка p53. Снижения уровня экспрессии p53 добивались нокдауном гена *TP53* с помощью shRNA (в 2,2 раза, $p < 0,05$), и, напротив, увеличения концентрации активных форм данного белка в клетках достигали за счет воздействия на клетки Nutlin-3a — ингибитора MDM2, основного негативного регулятора p53. Установлено, что регуляция как минимум трех маркеров дифференцировки, *CASP14*, *IVL* (увеличение экспрессии в 3,9 и 3,7 раза соответственно при нокдауне p53, $p < 0,05$) и *TGM1* (уменьшение экспрессии вдвое при нокдауне *TP53* и увеличение в 1,7 раза при воздействии на клетки Nutlin-3a, $p < 0,05$), в клетках HaCaT зависит от активности p53. При этом в отношении экспрессии *TGM1* и p53 отмечена прямая зависимость, которая, возможно, реализуется опосредованно, через изменение экспрессии ΔNp63. В то же время модуляция активности p53 не приводит к значительным изменениям экспрессии цитокератинов.

**Ключевые слова:** HaCaT, дифференцировка кератиноцитов, p53, p63, ΔNp63, TAp63, Nutlin-3a, shRNA, нокдаун

**Финансирование:** работа, включающая нокдаун гена p53, ИФА и ПЦР-исследования, выполнена в рамках Программы фундаментальных научных исследований государственных академий наук на 2013–2020 годы; эксперименты с применением Nutlin-3a были проведены на базе ООО НПО «Перспектива» при поддержке РФФИ, научно-исследовательский проект 18-44-540031/19.

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Terminal differentiation of keratinocytes is one of the possible programmed cell death pathways. This process ensures normal stratification of epidermal cells required for the normal epidermal barrier formation. Various impairments of terminal differentiation underlie clinical manifestations of many chronic skin disorders. Recently, the role of p53 proteins in regulation of keratinocyte differentiation has been actively studied [1–4].

It is known that p53 protein accumulates and induces apoptosis in epidermal cells in response to sunburn [5] and a number of other cytotoxic effects. Furthermore, active forms of the proteins are most often detected in proliferating keratinocytes, but not in differentiated cells [6]. In one view, p53 may play a dual role in survival of epidermal cells supporting "healthy" proliferative cells and inducing apoptosis in severely damaged cells [7].

According to some authors, p53 promotes proliferation and slows down differentiation of normal human keratinocytes by inactivation of signaling pathway mediated by MYC proto-oncogene [7]. Yet in p53-knockdown cells the significantly increased expression of such cell differentiation markers as involucrin, keratins KRT1 and KRT10, and filaggrin was observed. Generally, cells with inactivated p53 were characterized by higher rate of stratification and detachment [7].

However, the specific role of p53 family proteins and their interactions at different stages of the human keratinocytes' differentiation in healthy and diseased organism is poorly understood.

The role of p53 family proteins in regulation of proliferation and differentiation of HaCaT line keratinocytes, the spontaneously immortalized non-carcinogenic human keratinocyte cell line, is even less studied [8]. This cell line is widely used as a model for studying the normal human keratinocyte functions [9, 10]. However, HaCaT keratinocytes are characterized by abnormal stratification and aberrant differentiation marker expression [11].

It is known that genome of HaCaT keratinocytes contains two alleles of *TP53* gene (H179Y and R282Q) with two gain-of-function (GOV) mutations acquired as a result of spontaneous immortalization (mutp53) [12]. In HaCaT cell line, mutp53 promotes proliferation and cell growth; it contains over 7,000 sites for DNA biding. The protein functions related to apoptosis induction are preserved [4].

Moreover, unlike normal keratinocytes, HaCaT cells predominantly express ΔNp63α isoform of p63, and TA isoform is almost undetectable [13].

Understanding HaCaT cells' physiology and underlying functions is necessary to assess the limitations when using such cells as a model. Furthermore, studying the p53 family proteins' interactions in the presence of mutp53 in cell genome will provide new data relevant for the study of carcinogenesis [14, 15].

The study was aimed to assess the role of p53 family proteins in regulation of proliferation and differentiation of the HaCaT cells. Modulation of p53 protein was provided during the study: activation was achieved via exposure to Nutlin-3a (inhibitor of MDM2, the major negative regulator of p53), and expression of p53 was suppressed by anti-TP53 shRNA.

## METHODS

### Cell lines and culture conditions

HaCaT cell line was obtained from the cell culture collection of German Cancer Research Center (DKFZ, Heidelberg; Germany). The cells were cultured at 37 oC with 5% $CO_2$ in DMEM/F12 culture medium (1:1, Gibco; USA) containing 1% GlutaMAX supplement (Thermo Fisher Scientific; USA), penicillin/streptomycin solution at a concentration of 100 U/ml and 100 μg/mL respectively (Gibco; USA), as well as 10% fetal bovine serum (Dia-M; Russia), i.e. in the complete culture medium. The cells were grown in culture flasks with a surface area of 25 $cm^2$ or in Petri dishes with a diameter of 60 mm (Corning; USA). The medium was replaced with the fresh one every 48 hours of cell growth.

### *TP53* gene knockdown using shRNA

TP53 gene was knocked down with lentiviral vector, which encoded anti-*TP53* shRNA. In order to construct the lentiviral vector, the HEK2937T cell line was transfected withpLKO-p53-shRNA vector (Addgene, #25637) in accordance with the standard polyethyleneimine (PEI) transfection protocol [16]. Conditioned medium containing the lentiviral particles was prepared 48–72 hours of incubation after transfection. In order to enhance the transduction efficiency, protamine sulfate (50 μg/mL) was added to the culture medium containing the lentiviral particles. For transduction, HaCaT keratinocytes were grown to 40–50% confluence, and the culture medium was replaced by the lentiviral particles containing medium. Then the cells were centrifuged at 800g for 1.5 hours. After centrifugation the conditioned medium was replaced by the complete culture medium. The wild-type control (WT HaCaT) transduced with the lego-ig2 vector (Addgene #27341) containing no puromycin resistance gene was used for selection with puromycin. The transduced cells of both cell lines (control and treated) were cultured at 37 °C with 5% $CO_2$ for 4 days. After that culture medium was replaced by puromycin containing medium (1 μg/mL), and the cells were cultured up to 100% control cells death. During this period the non-transduced cells of the treated cell line also died.

### Assessment of gene expression level

The cells were grown in the 60 mm Petri dishes with the complete culture medium at 37 °C with 5% $CO_2$. Upon reaching 60% confluence, the culture medium was replaced by the Nutlin–3a–containing medium (Merck; Germany). The cells were incubated for 24 hours and used for further experiments.

RNA was isolated using the RNeasy Kit (QIAGEN; USA) in accordance with the manufacturer's protocol. The isolated RNA was quantified using the NanoDrop 2000c system (Thermo Scientific; USA). Reverse transcription reaction was performed using MMLV RT kit (Evrogen; Russia) in accordance with the standard protocol by adding 1 μg of RNA at a time. R eal-time PCR (qPCR) was carried out using the qPCRmix-HS SYBR+LowROX reaction mixture (Evrogen; Russia). For each group three biological samples were used, and reactions were carried out in three iterations for each gene and each sample. GAPDH was used as a reference gene. The primers are listed in Table 1.

### Metabolic activity assessment

Cells' metabolic activity was defined using the MTT assay [17]. The cells were plated in the 96-well plate (Corning; USA) at $3.0 \times 10^3$ cells per well 48 hours before exposure, 6 wells for each concentration of Nutlin-3a. After two days of growth the culture medium was replaced by the fresh one containing Nutlin-3a at a concentration of 0.2–50 μM, then the cells were incubated for 24 hours. After cultivation the medium was replaced by the fresh one containing MTT (Dia-M; Russia) at

**Table 1.** Primers

| Gene | Primer nucleotide sequence |
|---|---|
| *GAPDH* | Forward 5`-TCGACAGTCAGCCGCATCTTCTTT-3`<br>Probe R6G–5`-AGCCGAGCCACATCGCTCAGACACCAT-3`-Q<br>Reverse 5`-ACCAAATCCGTTGACTCCGACCTT-3` |
| *TP53* | Forward 5`-CTCACCATCATCACACTGGAA-3`<br>Probe FAM–5`-TACTGGGACGGAACAGCTTTGAGG-3`-Q<br>Reverse 5`-CCAGGACAGGCACAAACA-3` |
| *ΔNp63* | Forward 5`-AGAAGAAAGGACAGCAGCATTGAT-3`<br>Probe FAM–5`-TCCTGAACAGCATGGACCAGCAGA-3`-Q<br>Reverse 5`-GGACGAGGAGCCGTTCTGA-3` |
| *TAp63* | Forward 5`-CCAGAGCACACAGACAAATG-3`<br>Probe FAM–5`-ACAGCCTATATGTTCAGTTCAGCCCA-3`-Q<br>Reverse 5`-TGATGGTTCATCCACAAAGTTC-3` |
| *TP63* | Forward 5`-CGTACAGGCAACAGCAACAG-3`<br>Probe FAM–5`-CAGCAGCACCAGCACTTACTTCAGA-3`-Q<br>Reverse 5`-CACAGAAGGCAGCTTGTTCA-3` |
| *TGM1* | Forward 5`-TGCTGGATGCCTGCTTAT-3`<br>Probe FAM–5`-TGGTGAACTCCCTGGATGACAATGG-3`-Q<br>Reverse 5`-ACCAGACCAGTTCCCAATC-3` |
| *IVL* | Forward 5`-CCAAAGCCTCTGCCTCAG-3`<br>Probe FAM–5`-AGATGTCCCAGCAACACACACTGC-3`-Q<br>Reverse 5`-GTATTGACTGGAGGAGGAACAG-3` |
| *KRT14* | Forward 5`-CTGAAGAAGAACCACGAGGA-3`<br>Probe FAM–5`-AGGTGGGTGGAGATGTCAATGTGG-3`-Q<br>Reverse 5`-TCTCTGCCATCTTCTCATACTG-3` |
| *KRT10* | Forward 5`-AGCATGGCAACTCACATCA-3`<br>Probe FAM–5`-ATTTGCTGTAGTCACGAGGCTCCC-3`-Q<br>Reverse 5`-GTCGATCTGAAGCAGGATGTT-3` |
| *CASP14* | Forward 5`-CCTGTCGAGGAGAACAAAGG-3`<br>Probe FAM-5`-AAAGACAGCCCACAAACCATCCCA-3`-Q<br>Reverse 5`-TGCAAGGCATCTGTGTATGT-3` |

a concentration of 1 mg/mL. The cells were incubated for 2 hours, and formazan formed granules were dissolved in DMSO (Helicon; Russia). Then the optical density was measured at a wavelength of 490 nm. The experiment was carried out in three biological replicates.

### Enzyme-linked immunosorbent assay (ELISA)

Semiquantitative analysis of p53 was performed using the ab205713 kit (Abcam; UK) in accordance with the manufacturer's instructions. The cells were plated in the 96-well plate in three replicates at $1 \times 10^4$ cells per well. The signal intensity was measured using the iMark spectrophotometer (Bio-Rad; USA) at a wavelength of 450 nm.

### Immunofluorescence staining

For immunofluorescence studies, the cells were cultured on coverslips in 6-well plates. The cells were fixed in 4% formalin, permeabilized by 0.1% Triton X-100, and stained with primary antibodies to ΔNp63 (#619002, Biolegend; USA), TAp63 (#618902, Biolegend; USA), KRT5 (ab52635, Abcam; UK), KRT10 (ab9025, Abcam; UK) and secondary Alexa Fluor 488-conjugated (ab150105, Abcam; UK) or Texas Red-conjugated (ab6793, Abcam; UK) antibodies. All preparations were stained simultaneously using the same reagent kit (dilutions of antibodies and buffers). The experiment was carried out in three biological replicates.

The preparations were sequentially visualized with the LSM 710 confocal laser scanning microscope (Carl Zeiss; Germany) using the same settings. At least 5 fields of view were photographed for each preparation. The images were processed with CellProfiler 3.1.9 software [18]. At least 100 cells were analyzed for each sample, and mean fluorescence intensity (cell fluorescence intensity divided by cell area) was calculated. The mean fluorescence intensity values were normalized to mean fluorescence intensity of the control.

### Data analysis

The data of three biological replicates were used for analysis. The results were processed using the R programming language [19]. The differences in the values of the studied parameters between groups were determined using the Student's *t*-test with Benjamini-Hochberg adjustment for multiple comparisons. The differences were considered significant when $p < 0.05$. The data are presented as $M \pm m$.

## RESULTS

The knockdown of *TP53* in the HaCaT cell line was confirmed by enzyme-linked immunosorbent assay (ELIZA). The anti-*TP53* shRNA transduction resulted in significantly decreased (by 2.2 times, $p < 0.05$) intracellular p53 concentration, by not to complete lack of intracellular p53 (ELIZA) (Fig. 1A).

Metabolic activity of wild-type cells and TP53-knockdown cells when exposed to inhibitor of MDM2, Nutlin-3a, was assessed using the MTT assay (Fig. 1B). The 24-hour exposure to Nutlin-3a resulted in the dose-dependent decrease in amount of formazan produced by wild-type HaCaT cells. The IC10 and IC50 values were 0.18 ± 0.10 μM and 129.11 ± 167.78 μM respectively.

Similar treatment of *TP53*-knockdown cells did not affect the production of formazan. Significant differences in the amount of formazan produced by these cells compared to wild-type cells were observed when exposed to Nutlin-3a concentrations of

10 μM and more. Therefore, Nutlin-3a concentration of 10 μM was used in subsequent experiments.

The identified features of the Nutlin-3a effect of *TP53*-knockdown cells may be associated with significantly reduced level of active p53 forms in these cells. Nevertheless, blocking MDM2-mediated degradation of p53 by Nutlin-3a does not appear to result in an increase in its concentration sufficient for realization of p53 major effects (cell cycle arrest, apoptosis).

Expression levels of a number of proteins' genes which reflected the activity of keratinocyte differentiation processes in intact wild-type cells and TP53-knockdown cells were different (Fig. 2A). Thus, higher expression of genes encoding involucrin and caspase-14, and lower expression of *TGM1* gene was observed in the knockdown cells compared to wild-type cells. No significant differences in the levels of cytokeratin expression (PCR, fluorescence microscopy) were detected (Fig. 2 A, B, C). Furthermore, the knockdown cells demonstrated decreased expression of genes encoding p63, ΔNp63 and TAp63 isoforms. Reduced level of the listed proteins in cytoplasm and nucleus was also detected (microscopy) (Fig. 3 A, B).

Exposure of wild-type cells to Nutlin-3a resulted in twofold increase of *P21* gene activity, which indicated the accumulation of p53 active forms in the cells. We also detected the increased expression of *TGM1* gene responsible for synthesis of p63 isoform (*ΔNp63*), and the slightly increased expression of *IVL*. However, the level of the latter remained significantly lower compared to knockdown cells. At the same time, no significant alterations in the levels of *CASP14* and cytokeratins 14 and 10 expression were detected (PCR). Cytokeratin 10 expression level was confirmed by microscopy. We also detected an increase in the level of cytokeratin 5 by 1.54 times (microscopy). It should be noted that along with the elevated expression of ΔNp63, a decrease in the level of this protein was observed both in nucleus and in cytoplasm of the cells. The level of TAp63 in nucleus and cytoplasm increased.

## DISCUSSION

The study was aimed to investigate the effect of p53 family proteins on HaCaT keratinocyte differentiation. We assessed the differentiation markers' expression alterations in relation to activity of p53: the reduced expression of p53 was obtained by knockdown of *TP53*, and activity of the protein was increased by Nutlin-3a added to the culture medium.

Proteins, the expression of which in normal keratinocytes increases as epidermal cells move from stratum basale to stratum corneum, have been selected as keratinocytes' differentiation markers (IVL, TGM1 and CASP14). Thus, involucrin (IVL) is the structural protein of keratinocytes responsible for mechanical strength of the epidermal barrier. Furthermore, complexes of involucrin with lipids (omega-hydroxyceramides) are involved in the corneocyte lipid envelope formation [20]. Involucrin is one of the major substrates of transglutaminase 1 (TGM1), which catalyzes the formation of cross-links between lysine residues in structural proteins of epidermis [21]. TGM1 is critical for epidermal barrier formation [21]. Unlike other proteins of the discussed family, caspase-14 (CASP14) is specifically expressed in epidermis, which plays particularly no role in realization of apoptosis, and is responsible for regulation of keratinocyte differentiation [22].

To assess the degree of cell differentiation, we studied the expression of keratins, KRT10, KRT5, KRT14 (components of the keratinocyte cytoskeletal intermediate filament proteins). It is known that high levels of KRT1 and KRT10 are most typical for differentiating keratinocytes [23], in contrast to keratins KRT5 and KRT14 actively expressed in cells of stratum basale [24].

The expression of other p53 family members, ΔNp63 and TAp63, was assessed using the same experimental conditions, which made it possible to evaluate its relationship to p53 activity.

Earlier the effect of reduced p53 expression (resulting from the *TP53* knockdown) on the normal human keratinocytes differentiation markers' expression had been studied [7]. Thus, the comparison of experimental results with literature data may enable us to obtain information about similarities and differences in the effects of p53 proteins on cell differentiation in the normal keratinocytes and HaCaT cell line.

It should be noted that during our study we failed to achieve the complete suppression of the p53 expression in the HaCaT cells. This could be due to the cell line resistance to reagents for transduction/transfection (compared to epithelial cells of internal organs) [25]. However, the p53 expression reduction was significant (2.2-fold).

Among the studied markers of keratinocyte differentiation, *IVL*, *CASP14* and *TGM1* genes were to a greater extent

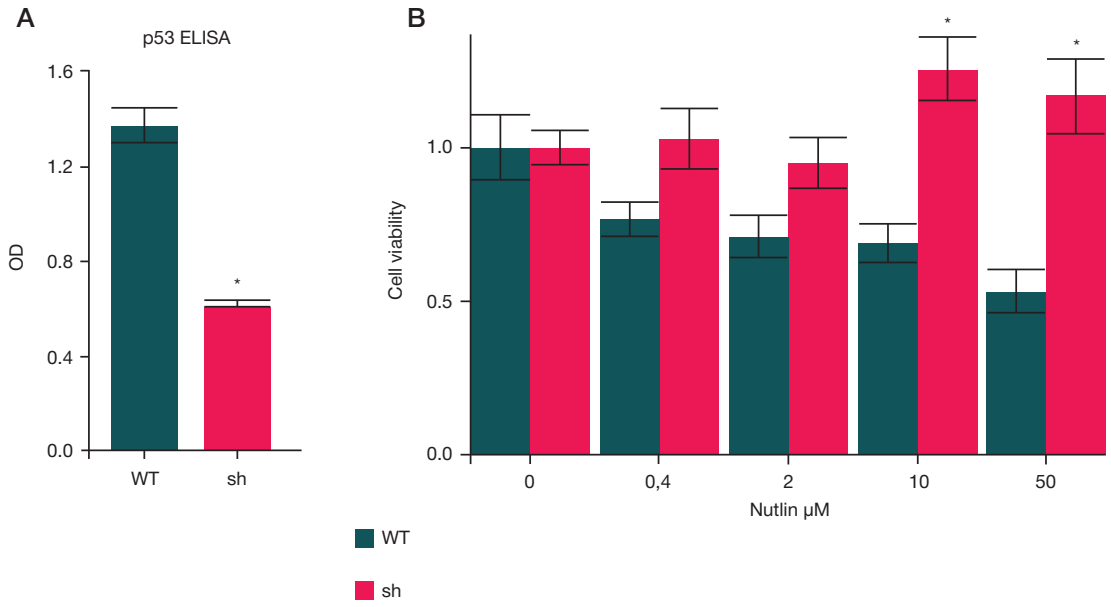


**Fig. 1.** Effects of TP53 modulation by knockdown and exposure to Nutlin-3a. **A**. Semiquantitative analysis of p53 in wild-type cells (WT) and *TP53*-knockdown cells (sh). **B**. Metabolic activity of cells (MTT assay) after exposure to Nutlin-3a. * — significant differences between groups WT and sh ($p < 0.05$)

associated with alterations in expression of p53. The expression of *TGM1* gene was the most p53-dependent: the expression level significantly decreased in *TP53*-knockdown cells and increased under exposure to Nutlin-3a.

It should be noted that the expression levels of *TGM1* and involucrin, the protein being a substrate for *TGM1*, as well as the dynamics of these parameters' values in the studied experimental situations did not match up. The expression of involucrin significantly increased in the TP53-knockdown cells. However, it also slightly increased under exposure to Nutlin-3a. Despite the obvious functional relationship between TGM1 and involucrin, the expression of those in HaCaT cells is regulated by different mechanisms.

Unlike normal keratinocytes, unresponsive to alterations in p63 expression in response to p53 knockdown [7], in our study the modulation of p53 activity in HaCaT cells resulted in altered expression of p63 isoforms. The decreased expression of TP63 in HaCaT cells with p53 knockdown had been reported before [4].

It is known that p63 isoforms, especially ΔNp63, inhibit activity of p53 [1]. It cannot be excluded that a high baseline expression of ΔNp63 in HaCaT cells is required for inactivation of mutp53 effects. In this context the detected alteration of p53 and p63 isoforms (ΔNp63, TAp63) ratio in TP53-knockdown cells compared to intact cells seems to be natural: in the absence of protein to be inhibited (p53) the reduced expression of p63 isoforms is observed. The detected discrepancy in dynamics of ΔNp63 expression when exposed to Nutlin-3a (elevated expression, according to PCR data, along with reduced level of protein in the cytoplasm and nucleus) may result from ΔNp63 protein involvement in p53 inactivation, for example, due to formation of ΔNp63/p53 heterodimers [26].

At the same time, the increase in TAp63 protein amount in the cells with no significant alterations in the expression of the appropriate gene in the same experimental situation may be associated with the MDM2 inhibition. According to literary sources [27] and our results, MDM2 may be a negative regulator of both p53 and TAp63.

It is known that the activity of p53 increases as keratinocytes move from stratum basale to higher layers, partly because of gradual decrease in ΔNp63 inhibitory effect. As a result, p53 implements its functions required to complete keratinization. In particular, it induces expression of TGM1, the substrate of which (involucrin) is produced in lower epidermal layers and is regulated by other mechanisms.

It cannot be excluded that the major regulator of TGM1 expression in HaCaT cells is ΔNp63 protein, but not p53 itself. As can be seen, the dynamics of the discussed protein gene expression is ipsidirectional to the expression of gene, which encodes TGM1. Such ΔNp63 expression alteration is evidently due to altered intracellular p53 activity.

In HaCaT cells, the impact of p53 activity on the expression of the other keratinocyte differentiation marker, caspase-14, is obvious. In the cells with knockdown TP53 the expression of gene encoding CASP14 is significantly increased. Presumably, in HaCaT cells p53 directly or indirectly inhibits the expression of this protein. However, the activity of such an impact is rather high: the

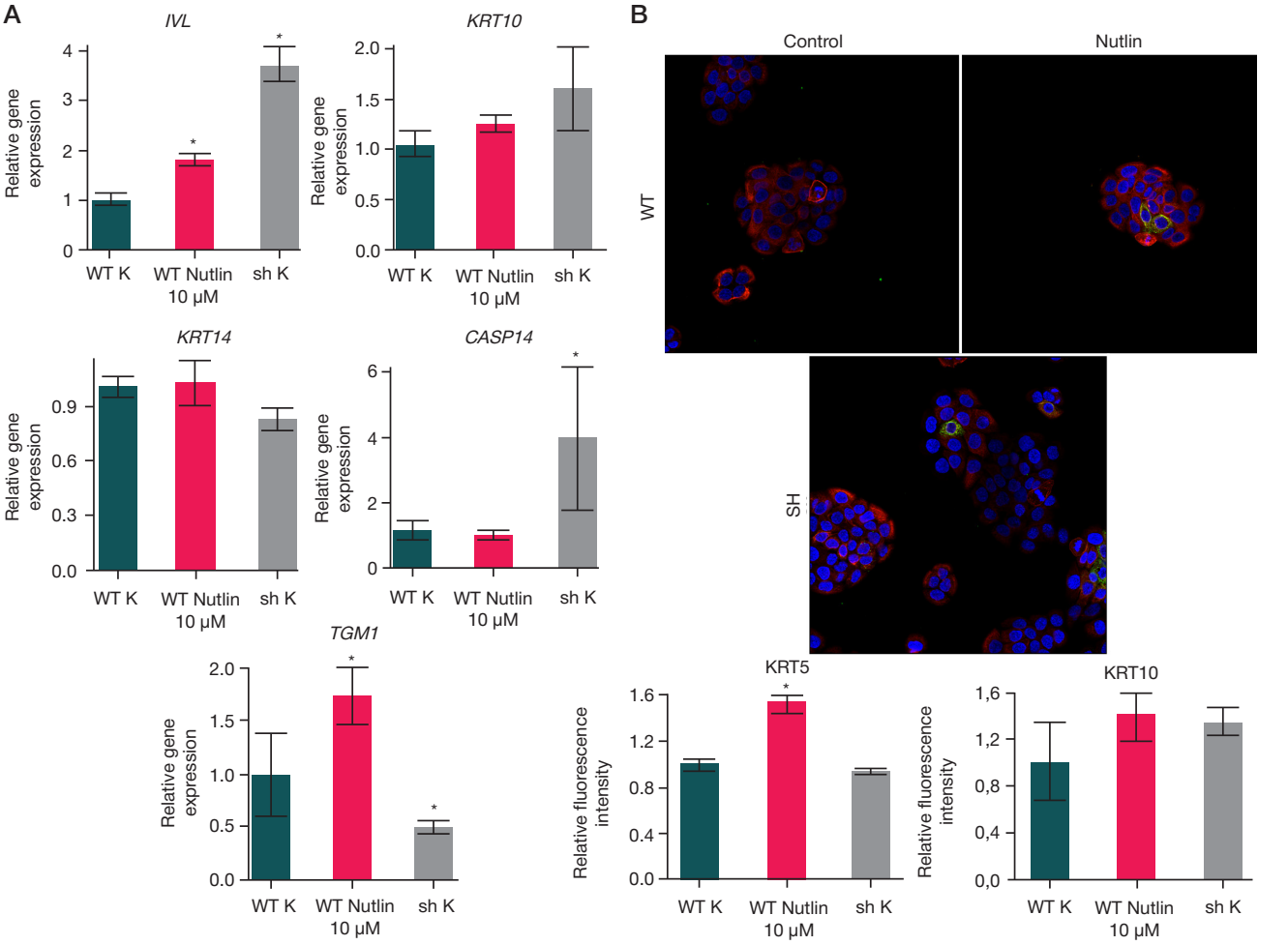


**Fig. 2.** Expression of differentiation markers in HaCaT cells. **A.** Expression of *IVL*, *KRT10*, *KRT14*, *CASP14*, *TGM1*. **B.** Immunofluorescence microscopy: KRT5 (*red*), KRT10 (*green*), DAPI (*blue*, nuclei), x400 magnification. **C.** Generalized diagram of the cells' relative fluorescence intensities. * significant differences between treated cells (sh, Nutlin-3a) and intact cells (WT K), $p < 0.05$

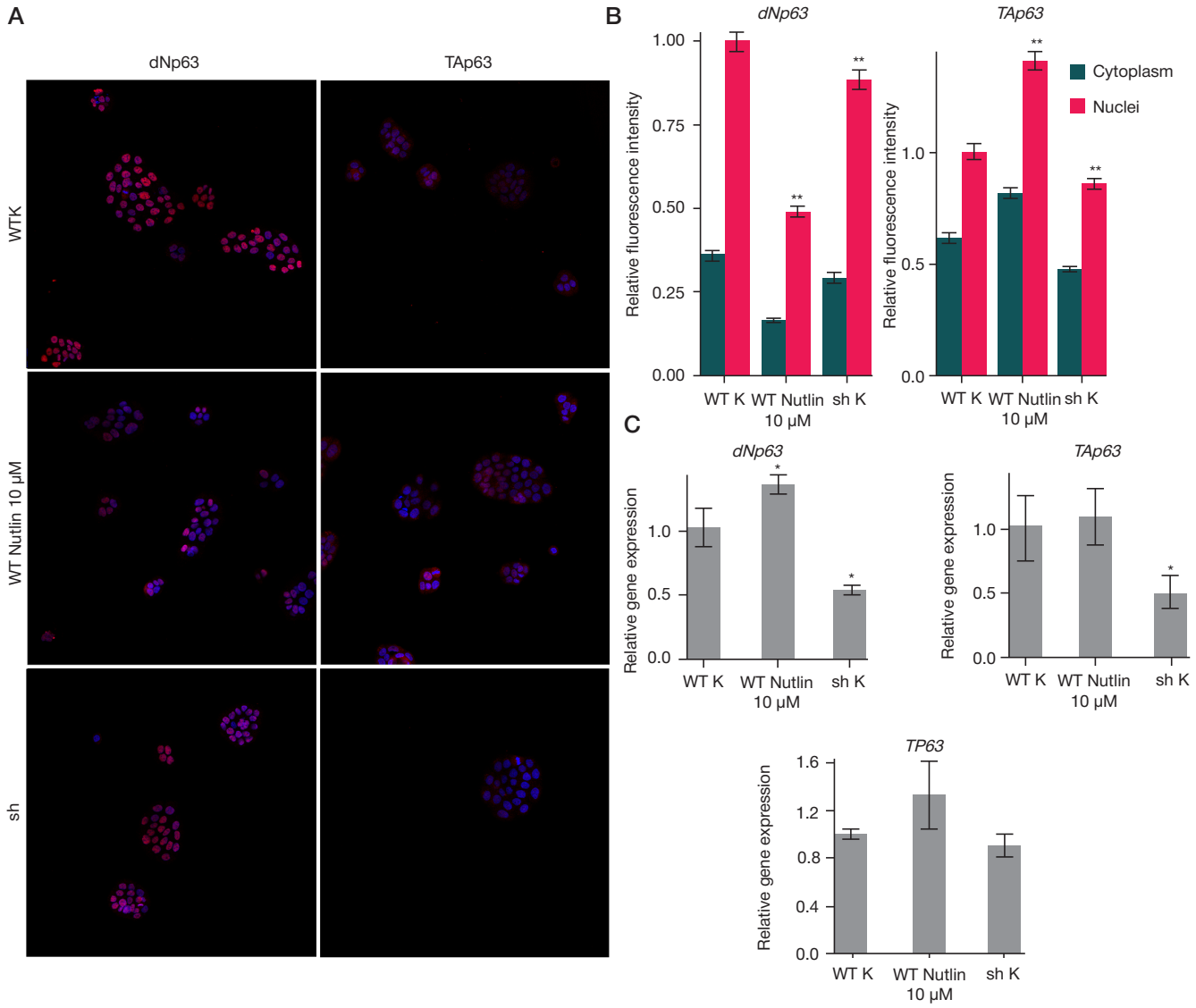


**Fig. 3.** Alterations in p63 isoforms' expression. **A.** Immunofluorescence microscopy: dNp63, TAp63 (*red*), DAPI (*blue*), x400 magnification. **B.** Generalized diagram of relative fluorescence intensities of cytoplasm and nucleus. **C.** Expression of TP63 isoforms (dNp63, TAp63). * — significant differences between treated cells (sh, Nutlin-3a) and intact cells (WT K), $p < 0.05$

increased activity of p53 resulting from exposure of cells to Nutlin-3a does not lead to significant decrease in expression of *CASP14*.

It should be noted that with modulation of p53 activity in HaCaT cells no significant alterations in expression of cytokeratins were observed. An exception was KRT5, the marker of the lower epidermal layer cells. The expression of KRT5 slightly increased under exposure to Nutlin-3a, perhaps, due to activation of mutp53 effects contributing to cell proliferation [4]. Expression of KRT10 typical for differentiating keratinocytes [23] in the cells with p53 knockdown did not change. Yet the suppressed function of p53 in normal keratinocytes is associated with elevated expression of the discussed cytokeratins [7]. In HaCaT cells, mutp53 inhibits the expression of these proteins, even under conditions of incomplete knockdown of TP53.

## CONCLUSION

Under conditions of p53 activity modulation in HaCaT cells, the expression of certain cell differentiation markers is altered, particularly, the expression of caspase-14, involucrin and transglutaminase 1, but not the expression of KRT10. Unlike normal human keratinocytes, HaCaT cells respond to reduced activity of p53 by reduced expression of p63 isoforms. The study results may be taken into account when studying the processes associated with keratinocyte differentiation, as well as when using HaCaT cell line as a cell culture model of skin. The data obtained may be used for in-depth assessment of the role of p53 family proteins expressed by HaCaT cell line in various physiological processes.

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# ALTERED NEUROMETABOLIC POTENTIAL OF GUT MICROBIOME IN HEALTHY CHILDREN OF DIFFERENT AGE

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Recently much attention is paid to investigation of the gut microbiome impact on children's mental health. The study was aimed to detect alterations in the taxonomic composition and content of bacterial genes encoding key enzymes involved in the metabolism of neuroactive compounds in the metagenomes of healthy young children and adolescents. The whole metagenome sequencing was used to obtain the metagenomic data of the faecal specimens. The bioinformatics algorithm developed and the catalogue of homologs created were used to identify the changes in abundance of bacterial genes and metagenomic signatures in the studied metagenomes. The core neurometabolic signature of the healthy children gut microbiota included the *Bacteroides uniformis*, *Faecalibacterium prausnitzii* and *Lachnospiraceae bacterium* species, as well as genes involved in production of acetic, propionic and butyric acids, glutamate and enzymes possessing antioxidant activity. Comparison of metagenomes in children of different age groups revealed significant ($p < 0.1$) changes in the average abundance for 3 bacterial genera and 18 species. The higher alpha diversity of the adolescents' microbiota was observed both at the genus and species level. Furthermore, in the adolescents' microbiota metagenomes the increased average relative abundances for the genes encoding enzymes involved in production of SCFAs, glutamate, tryptophan and compounds with antioxidant properties, histidine degradation and linoleic acid conjugation were observed ($p < 0.1$). The study results support the evidence that healthy gut microbial communities become more diverse and functional as their human hosts become older.

**Keywords:** gut microbiota, gut-brain axis, metagenomic signatures, neurodevelopment, neuroactive compounds

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# СТРУКТУРА И НЕЙРОМЕТАБОЛИЧЕСКИЙ ПОТЕНЦИАЛ МИКРОБИОТЫ КИШЕЧНИКА У ЗДОРОВЫХ ДЕТЕЙ РАЗНОГО ВОЗРАСТА

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В последние годы большое внимание уделяется изучению влияния кишечной микробиоты на здоровье детей, в том числе психическое. Целью данной работы было определить изменения в таксономическом составе и содержании бактериальных генов, кодирующих ферменты, участвующие в метаболизме нейроактивных соединений, в метагеноме микробиоты кишечника детей младшего и подросткового возраста. Данные для анализа были получены при помощи секвенирования полного метагенома. Для определения изменения представленности бактериальных генов и метагеномных сигнатур использовали разработанный биоинформатический алгоритм и каталог гомологов генов. В результате построена коровая нейрометаболическая сигнатура кишечной микробиоты здоровых детей младшего возраста, включающая в себя виды *Bacteroides uniformis*, *Faecalibacterium prausnitzii* и *Lachnospiraceae bacterium* и гены, участвующие в образовании уксусной, пропионовой и масляной кислот, глутамата и ферментов с антиоксидантной активностью. Сравнение метагеномов детей разных возрастных групп показало статистически значимое (P-value < 0,1) изменение представленности для 3 родов бактерий и 18 видов. Альфа-разнообразие микробиоты подростков выше как на родовом, так и на видовом уровнях. Кроме того, в микробиоте подростков повышена (P-value < 0,1) представленность генов, кодирующих ферменты, участвующие в образовании короткоцепочечных жирных кислот, глутамата, триптофана и ферментов с антиоксидантной активностью и деградации гистидина, конъюгации линолевой кислоты. Полученные результаты подтверждают имеющиеся данные об увеличении биоразнообразия и развитии функциональных свойств кишечного микробного сообщества со взрослением человека.

**Ключевые слова:** микробиота кишечника, ось кишечник-мозг, метагеномные сигнатуры, развитие нервной системы, нейроактивные соединения

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Today, human gut microbiota is considered an important organ, which plays a vital part in preserving human health. Gut microbiota is the microbial population colonizing the gastrointestinal tract. The healthy gut microbial communities, which contribute to preserving the metabolic homeostasis, live inside the host maintaining the immunological tolerance. Due to symbiotic relationship, human gut microbiota carries out various tasks contributing to the host's physiology [1]. The host co-evolves with the microbiota; the composition of gastrointestinal tract microbial community changes in response to various internal and external stimuli. Bacterial species colonizing the gastrointestinal tract in infancy affect the host's health later in life [2]. The gut microbiota's bacterial composition becomes stable after the first three years of life getting closer to the adult gut microbiota profile [3].

Clinical and experimental data demonstrate the significant impact of human gut microbiota on the broad range of behaviors, including the social behavior, mood, emotions, anxiety and nuitrition [4]. Gut bacteria affect various human neurological conditions via the microbiota-gut-brain axis [4]. The gut microbiota composition may affect the neural network formation during the early nervous system development [5]. Bacteria influence the central nervous system (CNS) and enteric nervous system (ENS) in a variety of ways via metabolites and hormones of immune system and afferent nerves. Bacteria produce hundreds of compounds, which may affect the host's physiology. The gut microbiota composition alterations may result in major changes in metabolite production. Since the host is constantly exposed to such molecules, those may facilitate the development of various neuropsychiatric disorders, including depression [6].

Adolescence and puberty are critical periods for the developing nervous system with numerous structural, neurochemical and molecular changes occurring in response to genetic and environmental signals. At this age microbiota is also subject to significant shifts in composition and functioning. Steroid hormones cause the sex-specific differences in the gut microbial composition. Maturation of human gut microbiota runs alongside with the dynamic brain development; both processes have similar critical periods of development [7].

The use of next-generation sequencing (NGS) provides a better understanding of gut microbiota composition and allows one to explore its structural changes throughout the lifespan [8]. During our work, we used the shotgun metagenomic sequencing to study the human gut microbiota. The method is based on sequencing of the complete genomic material of the microbiota sample, which makes it possible both to obtain full data on bacterial composition, and to assess the overall microbiota metabolic functions and the functional capabilities of all bacteria. Moreover, the method may be used for strain-level microbiota analysis. The study was aimed to detect alterations in the taxonomic composition and content of bacterial genes encoding key enzymes involved in the metabolism of neuroactive compounds and biomarker metabolites in the metagenomes of healthy children of different age groups: 3–5 years old (children's metagenomes (ChM) and 15 years old (adolescent metagenomes (AM).

## METHODS

### Cohorts and metagenome sequencing

The study included the previously sequenced gut microbiota metagenomes isolated from 23 healthy neurotypical children aged 3–5 (ChM group) [9] and 7 adolescents aged 15 (AM group) [10] living in Moscow Region. Inclusion criteria: age; no gastrointestinal disorder prior to sampling; geographic region of origin — Moscow and Moscow Region; no exposure to antibiotics, probiotics and prebiotics within 2 months before sampling; no mental disorder (depression, schizophrenia, bipolar disorder, etc.); no diarrhea. Faecal specimens obtained from each volunteer were stored in sterile plastic containers before analysis at a temperature of –80 °C.

Metagenomic DNA isolation, library construction and sequencing using the Illumina HiSeq system (Illumina; USA) were carried out in accordance with the previously reported algorithm [9]. Metagenomic reads were deposited in the Sequence Read Archive (SRA) NCBI (the ChM BioProject ID was PRJNA516054, and the AM ID was PRJNA380118). The raw sequence data quality control was performed using the FastQC tool, and the Trimmomatic tool was used for trimming [11, 12]. The bases with quality score Q < 20 and sequences shorter than 50 bp were removed. In order to remove human DNA, all reads were mapped to the human genome (hg19 assembly) using the bowtie2 tool [13]. The metagenomic reads were assembled into contigs using the metaSPADes software [14].

Parameters of the sequenced samples and the resulting assemblies are presented in Table 1.

### Catalogue creation

The catalogue of gene homologs involved in synthesis and metabolism of various neuroactive compounds, which was introduced before [9, 15], was updated and expanded. The genes involved in synthesis and metabolism of various compounds and metabolites reported as biomarkers of depression were added to the catalogue [16]. The amino acid sequences of these genes' homologs were selected in accordance with the previously reported algorithm [9] (Table 2).

### Taxonomic and statistical analysis

The taxonomic composition was defined using the Kraken2 [17] and TAGMA [18] software. The analyses were carried out separately for the taxonomic levels of phylum, genus and species. The alpha diversity (Shannon's diversity index) was assessed using the R programming language.

The significant differences in the taxonomic composition at the genus and species level were defined using the Wilcoxon signed-rank test and the multiple testing correction based on permutation test (1000 permutations), the significance threshold was set to P-value < 0.1. This value was chosen due to the relatively small number of samples in the AM group.

### Signature identification in metagenomic data

Metagenomic signature is a combination of genes found in the metagenome and bacteria containing such genes [9]. In order to define the signatures, the metagenomic assemblies were analyzed using the previously reported algorithm [15]. The search for open reading frames (ORF) was performed using the MetaGeneMark software (USA) [19]. The ORFs were annotated using the catalogue created and BLASTp with the following parameters: homology ≥ 60%; relative alignment score ≥ 80%. Bacterial origin of ORFs was defined at the taxonomic level of species using the Kraken2 software. All unclassified sequences were designated as "Unclassified". Thus, a set of pairs was obtained for each species (species; gene). In order to assess the relative abundance of the pair, the reads were mapped to the appropriate ORFs using the Burrows-Wheeler Alignment

**Table 1**. Characteristics of studied metagenomic samples

| № | Reads | | | Assemblies | | |
|---|---|---|---|---|---|---|
| | Group | Sample name | Size, billion base pairs | Size, Mb | Number of contigs | N50, bp |
| 1 | ChM | HC_1 | 2.99 | 160.03 | 197683 | 2827 |
| 2 | ChM | HC_2 | 1.91 | 129.61 | 194544 | 3020 |
| 3 | ChM | HC_3 | 2.63 | 166.00 | 210193 | 3795 |
| 4 | ChM | HC_4 | 3.46 | 189.25 | 209685 | 7667 |
| 5 | ChM | HC_5 | 1.85 | 154.83 | 238146 | 2148 |
| 6 | ChM | HC_6 | 4.71 | 182.04 | 174019 | 4487 |
| 7 | ChM | HC_7 | 5.16 | 178.69 | 194821 | 2532 |
| 8 | ChM | HC_8 | 7.15 | 154.62 | 142565 | 3426 |
| 9 | ChM | HC_9 | 5.86 | 256.49 | 259614 | 2096 |
| 10 | ChM | HC_10 | 6.77 | 149.53 | 156608 | 2114 |
| 11 | ChM | HC_11 | 5.06 | 192.06 | 153535 | 14058 |
| 12 | ChM | HC_12 | 6.26 | 201.55 | 175868 | 9029 |
| 13 | ChM | HC_13 | 6.09 | 168.69 | 141685 | 9188 |
| 14 | ChM | HC_14 | 6.16 | 168.31 | 121753 | 6826 |
| 15 | ChM | HC_15 | 7.2 | 226.17 | 236984 | 2053 |
| 16 | ChM | HC_16 | 6.18 | 178.16 | 153050 | 4611 |
| 17 | ChM | HC_17 | 5.83 | 280.50 | 311115 | 1871 |
| 18 | ChM | HC_18 | 5.23 | 214.81 | 205380 | 3613 |
| 19 | ChM | HC_19 | 5.26 | 140.45 | 116040 | 13178 |
| 20 | ChM | HC_20 | 4.73 | 172.67 | 125060 | 15088 |
| 21 | ChM | HC_21 | 8.31 | 248.61 | 205319 | 6630 |
| 22 | ChM | HC_22 | 9.86 | 277.61 | 238778 | 7011 |
| 23 | ChM | HC_23 | 8.51 | 172.41 | 163607 | 3065 |
| 24 | AM | D3F | 10.1 | 108.11 | 61735 | 9284 |
| 25 | AM | D4F | 9 | 241.5 | 152814 | 5677 |
| 26 | AM | D5F | 9.9 | 142.83 | 53487 | 21016 |
| 27 | AM | D6F | 8.6 | 206.81 | 202650 | 3438 |
| 28 | AM | D11F | 7.7 | 38.29 | 31806 | 13412 |
| 29 | AM | D12F | 7.5 | 76.07 | 130779 | 760 |
| 30 | AM | D13F | 10.6 | 180.43 | 335100 | 617 |

(BWA) tool [20]. The counts were normalized by the Trimmed Mean of M-values (TMM) method using the edgeR library [21]. The comparison of relative abundance values for the ChM and AM groups was carried out using the Wilcoxon signed-rank test and the multiple testing correction based on permutation test (1000 permutations; P-value < 0.1).

## RESULTS

### Expanded gene catalogue

The previously constructed reference catalogue of gene homologs involved in the various neuromodulators metabolism has been expanded by adding genes, which encode enzymes involved in the new compounds production and the various neuroactive metabolites destruction [9, 15]. The resulting catalogue comprises 742 amino acid sequences for gene homologs encoding the 68 bacterial enzymes. The new enzymes involved in γ-aminobutyric acid (GABA), nitric oxide, γ-hydroxybutyric acid and p-cresol decomposition, isovaleric acid, inositol and glutamate synthesis and decomposition, as well as antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) have been added to the catalogue. The full list of genes included in the updated catalogue is presented in Table 2.

### Core neurometabolic signature of the healthy children gut microbiota

In the first phase, the search for bacterial genes encoding the key enzymes involved in the synthesis of neuroactive compounds and biomarkers of depression, which may affect the development and functioning of the child's nervous system during his/her early life was carried out in the metagenomes of the ChM group (Fig. 1). Only those genes found in more than 50% of samples were taken into account. The most abundant genes were those encoding methylmalonyl-CoA decarboxylase (propionic acid production), phosphotransacetylase (acetic acid production), glutamate decarboxylase (GABA synthesis), gamma-aminobutyrate antiporter (GABA transport) and histidine ammonia-lyase (histidine destruction). Gene homologs involved in metabolic pathways of GABA, serotonin, melatonin, butyric acid, conjugated linoleic acid, spermidine, isovaleric acid, inositol, γ-hydroxybutyric acid, glutamate, creatinine, indole, tryptophan, superoxide dismutase, catalase and glutathione peroxidase were also detected.

**Table 2.** Updated catalogue of homologs

| № | Enzyme | Function | Number of homologs |
|---|---|---|---|
| 1 | DOPA decarboxylase | Synthesis of serotonin, dopamine and norepinephrine | 10 |
| 2 | Glutamate decarboxylase | GABA synthesis | 28 |
| 3 | Gamma-aminobutyrate antiporter | GABA transport | 20 |
| 4 | 4-Aminobutyrate aminotransferase (gabT, puuE), glycine amidinotransferase | GABA decomposition | 17 |
| 5 | Histidine decarboxylase | Histamine synthesis | 13 |
| 6 | Serotonin N-acetyltransferase | Decomposition of serotonin for synthesis of melatonin | 24 |
| 7 | Acetylserotonin O-methyltransferase | Synthesis of melatonin | 8 |
| 8 | Nitric oxide synthase | Nitric oxide formation | 6 |
| 9 | Nitric oxide dioxygenase, nitric oxide reductase (norB, norC) | Nitric oxide decomposition | 13 |
| 10 | Aromatic amino acid hydroxylases | Catecholamine synthesis | 7 |
| 11 | Monoamine oxidase | Decomposition of serotonin, dopamine and norepinephrine | 5 |
| 12 | Phosphotransacetylase | Acetic acid formation | 43 |
| 13 | Butyrate kinase | Butyrate synthesis | 16 |
| 14 | Butyryl-CoA dehydrogenase | Butyric acid synthesis | 32 |
| 15 | Lactoyl-CoA dehydratase, propionaldehyde dehydrogenase, methylmalonyl-CoA decarboxylase | Propionic acid formation | 55 |
| 16 | Linoleic acid isomerase | Linoleic acid conjugation | 23 |
| 17 | Spermidine synthase | Spermidine synthesis | 26 |
| 18 | Tyrosine decarboxylase | Synthesis of tyramine and dopamine | 11 |
| 19 | 2-Oxoisovalerate dehydrogenase (alpha, beta), dihydrolipoyl dehydrogenase | Isovaleric acid synthesis (KADH pathway) | 24 |
| 20 | Aldehyde dehydrogenase, pyruvate decarboxylase | Isovaleric acid synthesis (KADC pathway) | 11 |
| 21 | Myo-inositol-1 (or -4) -monophosphatase, myo-inositol-1-phosphate synthase | Inositol synthesis | 11 |
| 22 | Myo-inositol 2-dehydrogenase | Inositol decomposition | 13 |
| 23 | 4-Hydroxybutyrate dehydrogenase | Decomposition of γ-hydroxybutyric acid | 13 |
| 24 | Glutamate synthase (gltB, gltD) | Glutamate II synthesis | 22 |
| 25 | Glutamate mutase (glmS, glmE), methylaspartate ammonia-lyase | Glutamate II decomposition | 24 |
| 26 | 4-Hydroxyphenylacetate decarboxylase | P-cresol synthesis | 8 |
| 27 | "4-Cresol dehydrogenase, Protocatechuate 3,4-dioxygenase (pcaG, pcaH)" | P-cresol decomposition | 15 |
| 28 | Creatinin amidohydrolase | Creatinin synthesis | 5 |
| 29 | D-lactate dehydrogenase | D-lactic acid formation | 13 |
| 30 | Glutathione synthetase (gshAB, gshB) | Glutathione synthesis | 12 |
| 31 | Glutathione S-transferase, glutathione reductase, gamma-glutamyl transpeptidase | Glutathione decomposition | 35 |
| 32 | Histidine ammonia-lyase | Histidine decomposition | 20 |
| 33 | Vinylphenol reductase | Synthesis of 4-ethylphenol | 7 |
| 34 | Tryptophanase | Metabolization of tryptophan into indole | 7 |
| 35 | Chorismate mutase | Prephenate synthesis | 8 |
| 36 | Prephenate dehydrogenase | Synthesis of 4-hydroxyphenylpyruvate | 10 |
| 37 | Tyrosine-specific transport protein | Tyrosine transport | 6 |
| 38 | Tyrosine aminotransferase | Tyrosine synthesis | 6 |
| 39 | Phenylalanine aminotransferase | Phenylalanine synthesis | 3 |
| 40 | Phenylalanine-specific permease | Phenylalanine transport | 6 |
| 41 | Tryptophan synthase (alpha and beta) | Tryptophan synthesis | 26 |
| 42 | Tryptophan-specific transport protein, tryptophan permease | Tryptophan transport | 7 |
| 43 | Superoxide dismutase ([Mn], [Fe], [Cu-Zn]), catalase, glutathione perxidase | Antioxidant | 73 |

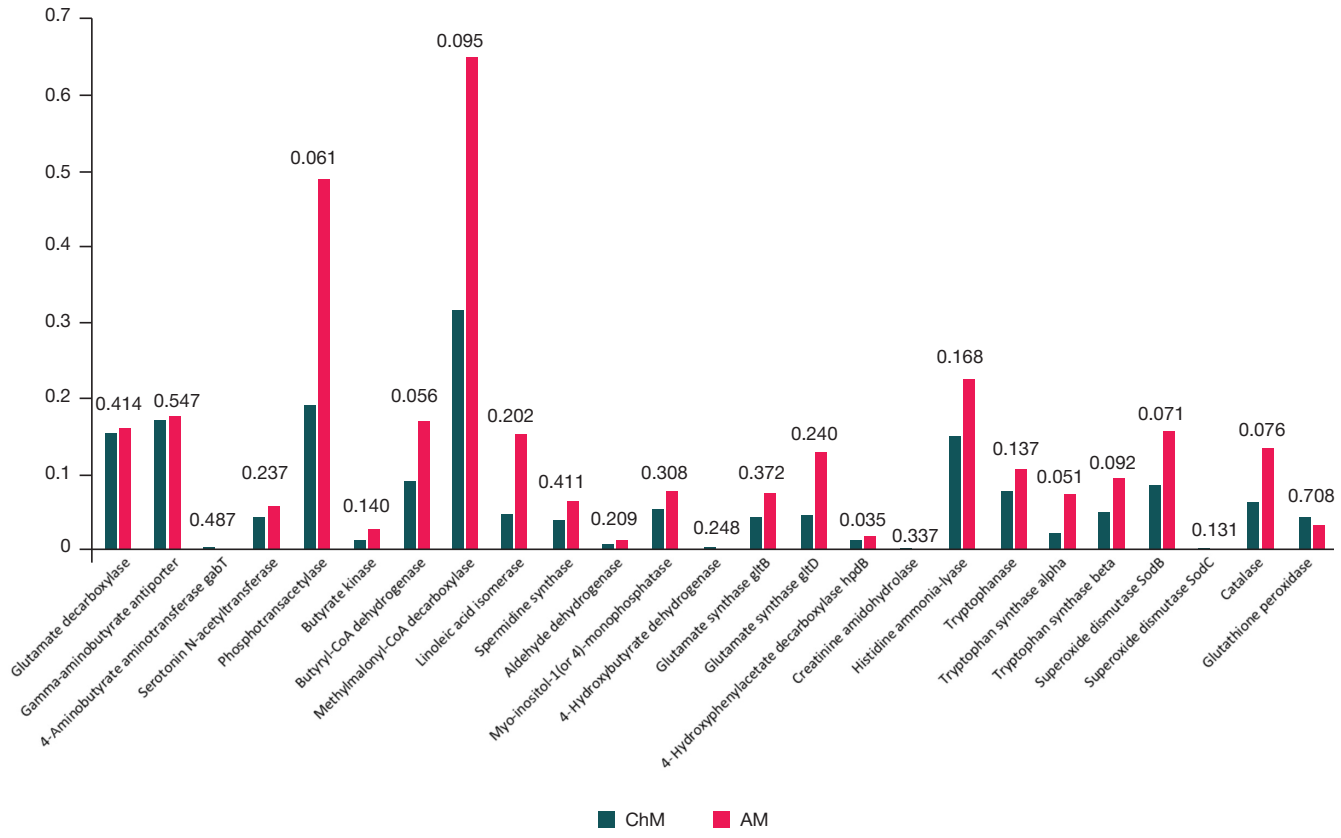


**Fig. 1.** Relative abundance of genes found in the ChM and AM groups. The figure shows the median relative abundance values for each gene found in more than 50% of samples. The values for the ChM group are *green* the values for the AM group are *red* The values in the head are P-values obtained using the Wilcoxon signed-rank test and the multiple testing correction based on permutation test, 1000 permutations

Then, the bacterial origin of genes at the species level was defined, and the signature pairs were constructed (Fig. 2A). The pairs defined in the vast majority of samples (over 70%) comprised the core neurometabolic signature of the ChM (Fig. 2B). The ChM core neurometabolic signature included four species (*Bacteroides uniformis*, *Faecalibacterium prausnitzii*, *Lachnospiraceae bacterium и Parabacteroides distasonis*) and genes encoding 15 enzymes (glutamate decarboxylase, gamma-aminobutyrate antiporter, serotonin-N-acetyltransferase, phosphotransacetylase, butyrate kinase, butyryl-CoA dehydrogenase, methylmalonyl-CoA decarboxylase, linoleic acid isomerase, spermidine synthase, two subunits of glutamate synthase, histidine ammonia-lyase, tryptophanase, beta-subunit of tryptophan synthase and superoxide dismutase).

### Age-related alterations in the neurometabolic signature of the human gut microbiota

Metagenomic samples for AM were analyzed using the same algorithm as the ChA samples. First, the search for gene homologs from the catalogue was carried out (see Fig. 1). On average, the increased relative abundance was noted for all genes found in more than 50% of the AM group samples compared to the ChM group. No homologs of genes encoding 4-aminobutyrate aminotransferase, creatinine amidohydrolase and superoxide dismutase (gene *sodC*) were found in the AM, however, this could be due to small sample size. Thus, the statistical power of the tests was low. The significant (adjusted P-value < 0.1) abundance increase was detected in AM for genes encoding phosphotransacetylase, butyryl-CoA dehydrogenase, methylmalonyl-CoA decarboxylase, 4-hydroxyphenylacetate decarboxylase, alpha- and beta-subunits of tryptophan synthase, superoxide dismutase (gene *sodB*) and catalase.

Then the metagenomic signatures for AM were constructed, and the abundance of pairs (species; gene) was compared with ChM (Fig. 2B). The significant abundance increase was detected for the following pairs: (*Alistipes onderdonkii*; catalase), (*A. onderdonkii*; glutamate decarboxylase), (A. onderdonkii; histidine ammonia-lyase), (*A. onderdonkii*; 4-hydroxyphenylacetate decarboxylase), (*Bacteroides vulgatus*; gamma-aminobutyrate antiporter), (*Bacteroides thetaiotaomicron*; methylmalonyl-CoA decarboxylase) and (*Barnesiella viscericola*; methylmalonyl-CoA decarboxylase). However, the observed alterations in the abundance of pairs comprising the core signature were not significant.

### Comparative taxonomic analysis of gut microbiota in children of different age groups

All metagenomes were analyzed using the Kraken2 software. The alpha diversity comparison for ChM and AM is presented in Fig. 3. The average value of the Shannon's diversity index for AM was higher both at the genus (Fig. 3A) and species level (Fig. 3B).

The taxonomic composition of the ChM and AM was defined for the taxonomic levels of phylum, genus and species. At the phylum level, the AM were characterized by significant increase in abundance of Proteobacteria (8.99% vs. 3.37% in ChM and AM respectively, P-value = 0.001) (Fig. 3C). The differences were also revealed for phyla Actinobacteria (4.85% vs. 2.77%; P-value = 0.735), Bacteroidetes (60.55% vs. 66.94%; P-value = 0.421), Firmicutes (21.08% vs. 24.42%; P-value = 0.758) and Verrucomicrobia (0.40% vs. 1.36%; P-value = 0.298), however, the differences were not significant.

The comparison of abundance values at the genus (Table 3) and species (Table 4) level included only taxa identified in

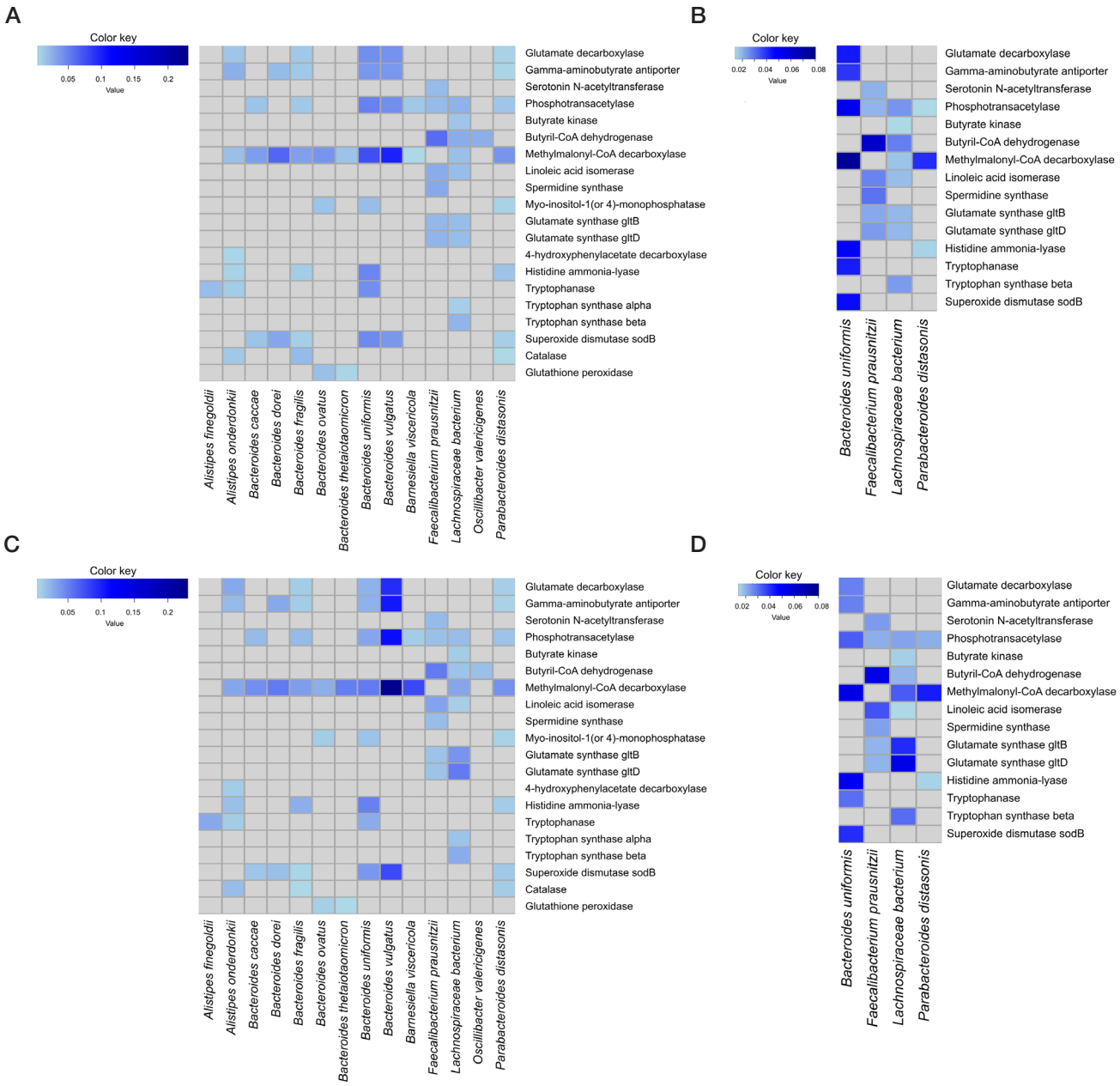


**Fig. 2.** Metagenomic signature and core metagenomic signature of human gut microbiota constructed for ChM group (**A** and **B** respectively), alterations of signature pairs abundance for AM group (**C** and **D** respectively). Color gradient shows the average relative abundance of the pairs (species; gene). Fig. (**A**) and (**B**) show only pairs found in more than 50% of samples, Fig. (**C**) and (**D**) show pairs found in more than 70% of samples

more than 50% of samples. As a result, the significant abundance increase (P-value < 0.1) was detected for genera *Butyrivibrio*, *Gordonibacter* and *Prevotella*. At the species level, there was a significant (P-value < 0.1) increase in abundance of *Alistipes communis*, *Alistipes megaguti*, *Alistipes sp*. dk3624, *Butyrivibrio fibrisolvens*, *Butyrivibrio proteoclasticus*, *Eggerthella sp*. YY7918, *Lactobacillus reuteri*, *Lactobacillus ruminis*, *Prevotella dentalis*, *Prevotella denticola*, *Prevotella enoeca*, *Prevotella jejuni*, *Prevotella oris* and *Prevotella ruminicola*, and the decrease in abundance of *Bacteroides sp*. A1C1, *Gordonibacter pamelaeae*, *Enterococcus faecalis* and *Streptococcus thermophiles*.

In addition, the analysis of strain diversity for the studied samples was carried out using the TAGMA software (Russia) [18] (Table 5). In AM, the increased median number of strains compared to ChM was observed for the *Clostridium botulinum*, *Clostridium perfringens*, *Escherichia coli* and *Streptococcus pneumonia* species. The larger number of strains per sample was also detected for the *Enterococcus faecium* species. However, the maximum number of strains was higher in the ChM group. Lower strain diversity in the AM group was observed for the *Bacteroides fragilis* species. Furthermore, in *Klebsiella pneumonia*, the median numbers of strains per sample were identical in both groups. However, the maximum number of strains was significantly higher in AM.

## DISCUSSION

In order to study the possible mechanisms of the human gut microbiota impact on the early childhood neurostructural and neurocognitive development in healthy children, we focused on the group of bacterial genes encoding the enzymes involved in metabolism of neuroactive compounds, which correlate with dysregulations resulting in neurometabolic disorders and depression. The use of the constructed catalogue of homologs to genes of the selected group made it possible to

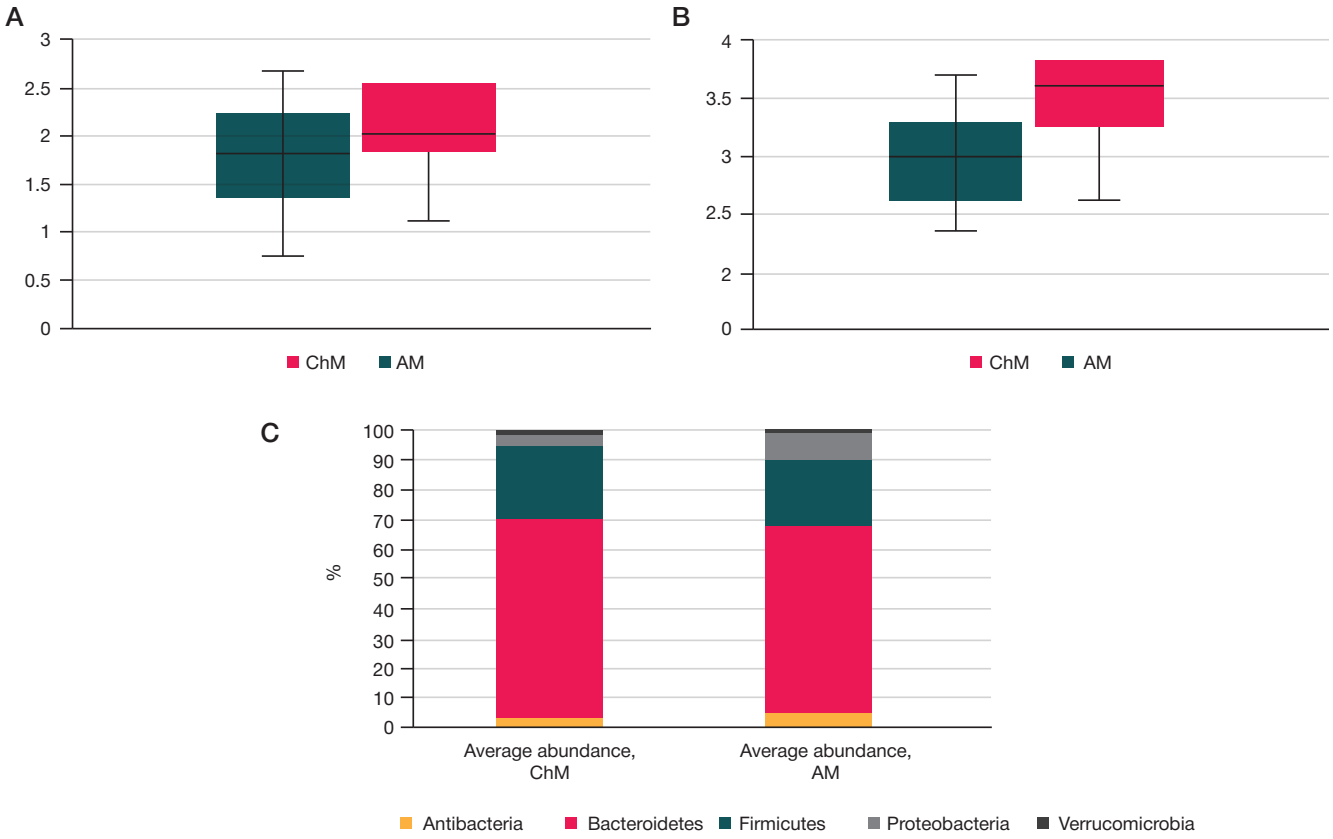


**Fig. 3.** Differences in taxonomic composition of gut microbiota for the ChM and AM groups. Alpha diversity for both groups was defined using the Shannon's diversity index at the genus (**A**) and species (**B**) level. The taxonomic composition alterations at the phylum level (**C**) are displayed as percentage values. The vertical error bars show the standard deviation

define the neurometabolic signature of the ChM. The signature approach was used to reveal the bacterial species comprising the largest number of genes (more than seven genes) responsible for production of various neuroactive compounds and thus having greater potential to affect the child's brain development and functioning. The revealed species were *B. uniformis*, *F. prausnitzii*, *L. bacterium* and *P. distasonis* being the gut commensals in healthy young children [22]. These bacteria contain genes encoding the proteins involved in production of acetic, propionic and butyric acids, GABA,

**Table 3.** Relative abundance of bacterial genera found in the ChM and AM groups

| Species | Abundance in ChM group, % | Abundance in AM group, % | Ratio ChM/AM | Adjusted P-value | Proportion of identified samples (out of 30), % |
|---|---|---|---|---|---|
| *Akkermansia* | 1.36 ± 3.33 | 0.38 ± 0.75 | 0.28 | 0.182 | 90 |
| *Alistipes* | 8.98 ± 7.60 | 7.22 ± 5.25 | 0.8 | 0.54 | 100 |
| *Bacteroides* | 49.16 ± 20.00 | 39.73 ± 18.84 | 0.81 | 0.261 | 100 |
| *Bifidobacterium* | 1.64 ± 2.93 | 3.08 ± 7.50 | 1.88 | 0.763 | 97 |
| *Blautia* | 1.05 ± 1.63 | 0.31 ± 0.16 | 0.3 | 0.237 | 100 |
| *Butyricimonas* | 0.33 ± 0.39 | 0.60 ± 0.84 | 1.84 | 0.232 | 100 |
| *Cupriavidus* | 0.02 ± 0.04 | 0.51 ± 1.30 | 22.3 | 0.178 | 80 |
| *Faecalibacterium* | 5.15 ± 4.85 | 3.42 ± 1.91 | 0.66 | 0.595 | 100 |
| *Flavonifractor* | 1.00 ± 1.57 | 0.24 ± 0.18 | 0.24 | 0.015 | 100 |
| *Lachnospira* | 1.22 ± 2.76 | 0.29 ± 0.29 | 0.24 | 0.18 | 100 |
| *Odoribacter* | 0.75 ± 0.79 | 1.28 ± 0.97 | 1.7 | 0.109 | 100 |
| *Parabacteroides* | 3.15 ± 4.03 | 2.63 ± 0.95 | 0.83 | 0.529 | 100 |
| *Paraprevotella* | 0.50 ± 0.89 | 0.88 ± 0.78 | 1.76 | 0.129 | 100 |
| *Phascolarctobacterium* | 1.05 ± 2.16 | 0.93 ± 1.26 | 0.89 | 0.652 | 57 |
| *Prevotella* | 0.22 ± 0.35 | 2.06 ± 3.31 | 9.24 | 0.033 | 100 |
| *Pseudomonas* | 0.17 ± 0.11 | 0.57 ± 0.40 | 3.43 | 1 | 100 |
| *Roseburia* | 1.40 ± 1.40 | 1.21 ± 1.23 | 0.87 | 0.485 | 100 |
| *Ruminococcus* | 1.59 ± 3.04 | 0.61 ± 0.82 | 0.39 | 0.457 | 97 |
| *Xanthomonas* | 0.07 ± 0.08 | 0.66 ± 0.59 | 10 | 1 | 100 |

**Note:** the table presents only genera found in more than 50% of samples with abundance of at least 0.5%.

**Table 4.** Relative abundance of bacterial species found in the ChM and AM groups

| Species | Abundance in ChM group, % | Abundance in AM group, % | Ratio ChM/AM | Adjusted P-value | Proportion of identified samples (out of 30), % |
|---|---|---|---|---|---|
| *Akkermansia muciniphila* | 1.35 ± 3.33 | 0.37 ± 0.75 | 0.28 | 0.127 | 90 |
| *Alistipes communis* | 0.94 ± 1.57 | 1.20 ± 0.91 | 1.27 | 0.075 | 100 |
| *Alistipes dispar* | 0.65 ± 1.39 | 0.29 ± 0.26 | 0.44 | 0.299 | 100 |
| *Alistipes finegoldii* | 2.55 ± 4.74 | 1.53 ± 2.25 | 0.6 | 0.662 | 100 |
| *Alistipes onderdonkii* | 2.65 ± 3.10 | 1.40 ± 2.10 | 0.53 | 0.3 | 100 |
| *Alistipes shahii* | 1.41 ± 2.37 | 1.82 ± 2.19 | 1.29 | 0.322 | 100 |
| *Bacteroides caccae* | 2.03 ± 3.24 | 2.21 ± 1.58 | 1.09 | 0.174 | 100 |
| *Bacteroides cellulosilyticus* | 1.69 ± 4.02 | 3.52 ± 6.67 | 2.09 | 0.111 | 100 |
| *Bacteroides dorei* | 7.92 ± 7.74 | 4.15 ± 3.17 | 0.52 | 0.358 | 100 |
| *Bacteroides fragilis* | 3.52 ± 3.84 | 1.62 ± 0.88 | 0.46 | 0.101 | 100 |
| *Bacteroides ovatus* | 4.87 ± 5.50 | 1.74 ± 0.97 | 0.36 | 0.218 | 100 |
| *Bacteroides sp. A1C1* | 1.77 ± 1.19 | 1.03 ± 0.67 | 0.58 | 0.05 | 97 |
| *Bacteroides sp. CBA7301* | 0.28 ± 0.34 | 0.75 ± 1.35 | 2.65 | 0.252 | 100 |
| *Bacteroides thetaiotaomicron* | 2.39 ± 2.29 | 1.38 ± 0.81 | 0.58 | 0.515 | 100 |
| *Bacteroides uniformis* | 5.87 ± 3.97 | 3.44 ± 2.51 | 0.59 | 0.109 | 100 |
| *Bacteroides vulgatus* | 7.78 ± 8.96 | 9.41 ± 10.14 | 1.21 | 0.54 | 100 |
| *Bacteroides xylanisolvens* | 2.17 ± 3.21 | 0.94 ± 0.83 | 0.43 | 0.629 | 100 |
| *Bifidobacterium adolescentis* | 0.41 ± 1.08 | 2.55 ± 6.60 | 6.17 | 0.227 | 93 |
| *Bifidobacterium longum* | 0.76 ± 2.14 | 0.19 ± 0.36 | 0.25 | 0.568 | 97 |
| *Blautia sp. SC05B48* | 0.87 ± 1.56 | 0.18 ± 0.10 | 0.21 | 0.315 | 100 |
| *Butyricimonas faecalis* | 0.33 ± 0.39 | 0.60 ± 0.84 | 1.84 | 0.208 | 100 |
| *Faecalibacterium prausnitzii* | 5.15 ± 4.85 | 3.42 ± 1.91 | 0.66 | 0.571 | 100 |
| *Flavonifractor plautii* | 1.00 ± 1.57 | 0.24 ± 0.18 | 0.24 | 0.012 | 100 |
| *Lachnospira eligens* | 1.22 ± 2.76 | 0.29 ± 0.29 | 0.24 | 0.151 | 100 |
| *Odoribacter splanchnicus* | 0.75 ± 0.79 | 1.28 ± 0.97 | 1.7 | 0.134 | 100 |
| *Parabacteroides distasonis* | 1.63 ± 2.03 | 1.35 ± 0.38 | 0.83 | 0.261 | 100 |
| *Paraprevotella xylaniphila* | 0.50 ± 0.89 | 0.88 ± 0.78 | 1.76 | 0.16 | 100 |
| *Roseburia intestinalis* | 0.99 ± 1.35 | 0.91 ± 1.08 | 0.92 | 0.878 | 100 |
| *Ruminococcus bicirculans* | 1.45 ± 2.99 | 0.41 ± 0.82 | 0.29 | 0.306 | 97 |
| *Xanthomonas euvesicatoria* | 0.05 ± 0.08 | 0.64 ± 0.58 | 11.73 | 1 | 100 |

**Note:** the table presents only species found in more than 50% of samples with abundance of at least 0.5%.

as well as antioxidant enzymes having a positive impact on the people's mental health. *B. uniformis*, *F. prausnitzii* and *L. bacterium* comprise the core neurometabolic signature of the healthy children gut microbiota, which may be used as an early childhood biomarker of normal microbiota.

Here we report the pilot study. This is the early stage of exploring the alterations in metabolic potential of gut microbiota in healthy children from early childhood to adolescence. So far, the small number of adolescents' samples was used for comparison. We had to determine whether the alterations in microbiota occurred during the child's development from infancy to adulthood. For this purpose, we compared taxonomic profiles and the content of bacterial genes encoding the key enzymes involved in the metabolism of neuroactive compounds.

We revealed differences in the quantitative content of bacterial genes responsible for production and destruction of neuroactive compounds in the compared metagenomes of children of different age groups, which was considered the most important result of the study. In AM, the two-fold increase in abundance of genes encoding the enzymes involved in the propionic, acetic and butyric acids, glutamate, and tryptophan production, histidine degradation, as well as in production of conjugated linoleic acid and antioxidant proteins was detected. As is known, all the listed above compounds have a positive impact on the gut and brain functioning, and contribute to homeostasis maintenance. The impact of the short-chain fatty acid levels on the energy homeostasis of the host has been reported [23]. Tryptophan is a substrate for the synthesis of neurotransmitter, serotonin [24]. Neurotransmitters serotonin and glutamate play a critical part in depression [25]. Conjugated linoleic acid and antioxidant proteins are the important factors of defense against oxidative stress. The content of other detected genes was negligible and demonstrated small differences. These data are likely to represent the varying contribution of the studied bacterial genes to maintaining the normal nervous system development in healthy children. The further transcriptome and metabolome analyses will be carried out to validate the results of the metagenomes bioinformatics analysis.

Earlier studies (based on the analysis of the 16S rRNA genes) aimed at the comparison of gut microbiota in children of different age groups revealed significant differences in the taxonomic composition [26]. In our study we used the data obtained by the shotgun metagenomic sequencing for the comparative metagenomic analysis. Although a small sample

**Table 5.** Strain diversity of bacterial species for the ChM and AM groups defined using the TAGMA software

| Species | ChM | | AM | |
|---|---|---|---|---|
| | Number of samples; proportion of samples (out of 23) | Average strains per sample [min; max] | Number of samples; proportion of samples (out of 7) | Average strains per sample [min; max] |
| *Anaerostipes hadrus* | 20; 0.87 | 2 [1; 2] | 7; 1.00 | 2 [1; 2] |
| *Anaerotruncus colihominis* | 23; 1.00 | 1 [1; 2] | 7; 1.00 | 1 [1; 1] |
| *Bacteroides cellulosilyticus* | 22; 0.96 | 2 [1; 3] | 7; 1.00 | 2 [1; 3] |
| *Bacteroides clarus* | 22; 0.96 | 1 [1; 2] | 6; 0.86 | 1 [1; 1] |
| *Bacteroides dorei* | 14; 0.61 | 3 [3; 3] | 5; 0.71 | 3 [1; 3] |
| *Bacteroides faecis* | 18; 0.78 | 1 [1; 2] | 4; 0.57 | 2 [1; 2] |
| *Bacteroides finegoldii* | 21; 0.91 | 2 [1; 2] | 7; 1.00 | 2 [2; 3] |
| *Bacteroides fragilis* | 20; 0.87 | 7 [2; 11] | 7; 1.00 | 2 [1; 11] |
| *Bacteroides intestinalis* | 22; 0.96 | 2 [1; 2] | 7; 1.00 | 2 [1; 2] |
| *Bacteroides ovatus* | 22; 0.96 | 5 [1; 5] | 7; 1.00 | 5 [1; 5] |
| *Bacteroides vulgatus* | 21; 0.91 | 3 [1; 4] | 7; 1.00 | 3 [2; 4] |
| *Bacteroides xylanisolvens* | 21; 0.91 | 3 [1; 4] | 7; 1.00 | 3 [2; 4] |
| *Bifidobacterium adolescentis* | 14; 0.61 | 2 [1; 3] | 6; 0.86 | 2 [1; 3] |
| *Bifidobacterium longum* | 20; 0.87 | 4 [1; 6] | 5; 0.71 | 4 [3; 5] |
| *Blautia obeum* | 23; 1.00 | 3 [3–3] | 7; 1.00 | 3 [3; 3] |
| *Butyrivibrio crossotus* | 22; 0.96 | 2 [1; 2] | 7; 1.00 | 2 [1; 2] |
| *Catenibacterium mitsuokai* | 14; 0.61 | 1 [1; 1] | 5; 0.71 | 1 [1; 1] |
| *Clostridium asparagiforme* | 21; 0.91 | 1 [1; 1] | 3; 0.43 | 1 [1; 1] |
| *Clostridium botulinum* | 23; 1.00 | 2 [1; 6] | 7; 1.00 | 3 [1; 5] |
| *Clostridium pasteurianum* | 22; 0.96 | 1 [1; 2] | 7; 1.00 | 1 [1; 2] |
| *Clostridium perfringens* | 23; 1.00 | 2 [1; 5] | 7; 1.00 | 4 [2; 5] |
| *Clostridium sporogenes* | 17; 0.74 | 1 [1; 2] | 5; 0.71 | 2 [1; 2] |
| *Coprococcus catus* | 13; 0.57 | 1 [1; 1] | 6; 0.86 | 1 [1; 1] |
| *Coprococcus comes* | 19; 0.83 | 1 [1; 2] | 6; 0.86 | 1 [1; 2] |
| *Dialister invisus* | 14; 0.61 | 2 [1; 2] | 4; 0.57 | 2 [1; 2] |
| *Dorea formicigenerans* | 23; 1.00 | 2 [1; 3] | 7; 1.00 | 2 [1; 3] |
| *Eggerthella lenta* | 16; 0.70 | 2 [1; 2] | 2; 0.29 | 2 [1; 2] |
| *Enterococcus faecium* | 23; 1.00 | 1 [1; 8] | 6; 0.86 | 2 [1; 2] |
| *Escherichia coli* | 20; 0.87 | 10 [2; 54] | 7; 1.00 | 25 [4; 40] |
| *Eubacterium ramulus* | 22; 0.96 | 1 [1; 1] | 7; 1.00 | 1 [1; 1] |
| *Eubacterium rectale* | 23; 1.00 | 2 [1; 2] | 7; 1.00 | 2 [1; 2] |
| *Eubacterium ventriosum* | 13; 0.57 | 1 [1; 1] | 5; 0.71 | 1 [1; 1] |
| *Faecalibacterium prausnitzii* | 23; 1.00 | 5 [5; 5] | 7; 1.00 | 5 [5; 5] |
| *Klebsiella pneumoniae* | 17; 0.74 | 2 [1; 29] | 6; 0.86 | 2 [1; 5] |
| *Parabacteroides merdae* | 15; 0.65 | 3 [2; 3] | 5; 0.71 | 2 [2; 3] |
| *Roseburia intestinalis* | 23; 1.00 | 4 [4; 4] | 7; 1.00 | 4 [4; 4] |
| *Roseburia inulinivorans* | 23; 1.00 | 2 [1; 2] | 7; 1.00 | 2 [2; 2] |
| *Ruminococcus bromii* | 23; 1.00 | 1 [1; 2] | 7; 1.00 | 1 [1; 2] |
| *Ruminococcus gnavus* | 23; 1.00 | 2 [1; 2] | 7; 1.00 | 2 [1; 2] |
| *Ruminococcus lactaris* | 23; 1.00 | 1 [1; 1] | 7; 1.00 | 1 [1; 1] |
| *Ruminococcus torques* | 23; 1.00 | 2 [1; 2] | 7; 1.00 | 1 [1; 2] |
| *Streptococcus pneumoniae* | 14; 0.61 | 1 [1; 4] | 3; 0.43 | 5 [1; 10] |
| *Streptococcus suis* | 12; 0.52 | 1 [1; 1] | 4; 0.57 | 1 [1; 1] |
| *Veillonella parvula* | 12; 0.52 | 1 [1; 3] | 3; 0.43 | 1 [1; 3] |

of AM was used for comparison, the results obtained also demonstrated typical differences in the taxonomic composition of gut microbiota in children of different age groups. The significant increase in bacteria of the phylum *Proteobacteria* and no significant differences for phyla *Actinobacteria*, *Bacteroidetes* and *Firmicutes* were revealed in AM. The alpha diversity of AM was higher both at the genus and species level, which was consistent with the published data on the more diverse microbiota of adolescents compared to young children [26]. High biodiversity often correlates with the higher content of probiotic bacteria. Our study revealed higher content of bifidobacteria (*B. adolescentis*) and lactobacilli in AM. It is

known that bifidobacteria and lactobacilli exhibit probiotic properties. Recently those were proposed as psychobiotics due to their ability to produce neuromodulators and affect the brain–gut interactions [27]. The significant relative content increase for the *Prevotella* genus representatives was detected in AM, as well as the decreased content of *A. muciniphila*, which demonstrated the negative correlation with obesity and inflammation [28]. Perhaps, the microbiota composition alterations observed in children as they mature are due to the impact of the diet and hormones. In turn, alterations in the composition if microbiota may affect the development of different brain areas [29].

Our findings revealed the strain diversity in both groups of metagenomes. The median increase of the bacterial strains in the AM for pathogenic bacteria *C. botulinum*, *C. perfringens*, *E. coli* and *S. pneumonia* was detected. Perhaps, that could be due to increased exposure to antibiotics during maturation. It is interesting that in the AM group there were more strains per sample (on average) for the *E. faecium* species, and less strains per sample for *B. fragilis*. Alterations in the strain-level microbiota composition may change its metabolic activity due to strain-specific production of various active compounds by bacteria. Combining the shotgun sequencing with the metagenome signature approach and the bioinformatics tools allowing one to perform the strain-level taxonomic analysis could put us closer to constructing the strain-level metagenomic signatures. This, in turn, would help to reveal the potential for specific production of neuroactive compounds in the new strains. This information could then be used to develop the methods for diagnosis of such neuropsychiatric disorders as depression, as well as to develop the targeted therapy for these disorders based on the use of pharmaceutical compounds, probiotics, prebiotics and/or psychobiotics [30].

## CONCLUSION

The study results confirm and expand the knowledge that the gut microbial communities become more diverse and functional as their human hosts become older. The gut microbial communities significantly enrich themselves with genes involved in metabolism of neuroactive compounds and compounds possessing anti-inflammatory and antioxidant activity necessary for the nervous system function. These alterations occur in response to external and internal factors, such as diet, antibiotics, hormones, stress, etc. The detected neurometabolic signature of the healthy children gut microbiota may be used as a marker of the normal human gut microbiota condition. Future research should be focused on identification of the gut microbiota metagenomic signature in healthy children of different age groups from different backgrounds.

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# CONTEMPORARY APPROACH TO DIAGNOSIS OF ISCHEMIC STROKE PATHOGENETIC VARIANTS IN PATIENTS WITH ATHEROSCLEROSIS AND ARTERIAL HYPERTENSION

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The angio- and neurovisualization methods vigorously developing in recent decades determine the relevance of improvement of etiopathogenetic ischemic stroke classification used for the treatment tactics selection and for secondary prevention of the disorder. The study was aimed to clarify the capabilities of clinical diagnosis for pathogenetic variants of ischemic stroke. For that, in 125 postmortem cases, the macro and microscopic examination of brain and cardiovascular system was carried out in order to verify the stroke pathogenesis established as a result of the previous patients' examination. The study demonstrates the great potential of the major pathogenetic stroke subtypes (large-artery atherosclerosis, cardioembolism, small-artery occlusion) diagnosis using the complex of contemporary clinical and instrumental methods and the main morphological criteria of these subtypes in accordance with the TOAST classification. Moreover, the clinical and pathomorphological assessment allowed us to differentiate stroke resulting from various alterations of single cerebral artery, the atherothrombotic occlusion (44% of cases for the subtype), arterio-arterial embolism (13%) and critical stenosis (10%), as well as stroke resulting from cerebrovascular insufficiency (33%), within the "large-artery atherosclerosis" subtype. Thus, the high informativity of the existing examination methods allows for a more differentiated understanding of the cause of ischemic stroke, which is fully in line with modern personalized medicine.

**Keywords:** ischemic stroke, cerebral infarction, pathogenesis, atherosclerosis, arterial hypertension

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**Compliance with ethical standards:** the study was approved by the Ethics Committee of the Research Center of Neurology (protocol № 11/14 dated November 19, 2014)

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# СОВРЕМЕННЫЙ ПОДХОД К ДИАГНОСТИКЕ ПАТОГЕНЕТИЧЕСКИХ ВАРИАНТОВ ИШЕМИЧЕСКОГО ИНСУЛЬТА ПРИ АТЕРОСКЛЕРОЗЕ И АРТЕРИАЛЬНОЙ ГИПЕРТОНИИ

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Активное развитие в последние десятилетия методов ангио- и нейровизуализации определяет актуальность совершенствования этиопатогенетической классификации ишемического инсульта, используемой для выбора целенаправленной тактики лечения и вторичной профилактики этого заболевания. С целью уточнить возможности клинической диагностики патогенетических вариантов ишемического инсульта в 125 случаях выполнено посмертное макро- и микроскопическое исследование мозга и сердечно-сосудистой системы для верификации патогенеза инсульта, установленного в результате предшествующего обследования пациентов. Показаны широкие возможности диагностики ведущих патогенетических подтипов инсульта (атеросклероз крупной артерии, кардиоаортальная эмболия, окклюзия мелкой артерии) при использовании комплекса современных клинико-инструментальных методов и основных морфологических критериев этих подтипов, отмеченных в общепризнанной классификации TOAST. Вместе с тем, клиническое и патоморфологическое исследование позволило выделить в подтипе «атеросклероз крупной артерии» инсульты, обусловленные различными изменениями одной церебральной артерии — атеротромботической окклюзией (44% инсультов этого подтипа), артерио-артериальной эмболией (13%) и критическим атеростенозом (10%), а также инсульты, возникающие по механизму сосудистой мозговой недостаточности (33%). Таким образом, высокая информативность существующих методов исследования позволяет более дифференцированно подходить к установлению причины ишемического инсульта, что в полной мере отвечает персонализированной направленности современной медицины.

**Ключевые слова:** ишемический инсульт, инфаркт головного мозга, патогенез, атеросклероз, артериальная гипертония

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Over the past decades ischemic stroke remains one of the most significant medical and social problems due to high morbidity and mortality [1, 2]. The solution to the problem is somewhat limited by the complexity of pathogenesis studies resulting from the polymorphism of the changes in cardiovascular system and brain associated with atherosclerosis and arterial hypertension (AH), which contribute to the vast majority of ischemic stroke cases. During the second half of the last century the new

classification systems based on the concept of cerebrovascular pathology heterogeneity have been developed. These were adopted for treatment tactics selection and secondary prevention of the disorder, as well as for clinical trials and epidemiological studies' standardization. The TOAST (Trial of Org 10172 in Acute Stroke Treatment) classification system has been internationally recognized, inter alia in Russian Federation. [3–5]. However, the significant rate of stroke of undetermined etiology (up to 30–40%) indicates the lack of certainty in the diagnostic criteria for ischemic stroke variants defined by this classification [6, 7].

The strong growth of clinical and diagnosis capabilities in recent decades, particularly introducing the advanced modifications of magnetic resonance imaging of the brain, was followed by the TOAST classification system refining. This made it possible to reduce the rate of stroke of undetermined etiology by several times [8, 9]. The refined version of the discussed classification system, the Stop Stroke Study (SSS), defines the criteria of obvious, probable and possible variants of the following major pathogenetic subtypes of ischemic stroke: large-artery atherosclerosis, cardioembolism, and small-artery occlusion.

Introduction of a number of other classification systems is due to urgent need for improvement of etiopathogenetic stroke classification in accordance with contemporary diagnosis and treatment options [10–15]. The most significant criteria of major stroke subtypes defined by the TOAST classification continue to be the focus of discussion, however, the new classification systems propose the additional features of these subtypes. The most disputed issues are as follows: minimum severity of monofocal and multifocal intracranial atherosclerotic stenosis (atherostenosis), resulting in cerebrovascular disease; size, localization and other morphological features of infarctions in different stroke subtypes; significance of various diffuse and focal brain lesions in diagnosis of lacunar stroke resulting from hypertension, which is classified as small-artery occlusion subtype. Some classification systems propose classification of stroke with different mechanisms both as existing pathogenetic subtypes and distinct subtype based on the certain clinical and morphological features.

Pathomorphological studies make it possible to obtain extensive and most reliable data on the ischemic cerebral circulatory disorders, provided the detailed assessment of brain and cardiovascular system changes. These studies assume particular importance while being compared with the data of previous patients' examinations allowing one to clarify the criteria of clinical and instrumental diagnosis of pathogenetic stroke subtypes. The importance of pathomorphological studies, as well as the clinical and pathomorphological comparisons involving the diversity of brain ischemia pathogenesis in patients with atherosclerosis, AH and the frequent combinations of those, is emphasized by scarcity of such studies and the controversial nature of the data obtained.

The study was aimed to clarify the capabilities of clinical diagnosis for pathogenetic variants of ischemic stroke associated with atherosclerosis and AH.

## METHODS

The clinical and pathomorphological comparison of 125 postmortem ischemic stroke cases observed at the Research Center of Neurology from 1990 to 2015 was carried out. Inclusion criteria: deceased men and women aged 45–74 with cerebral infarction. Exclusion criteria: no vascular disease in the form of aortic and cerebral arterial atherosclerosis, AH, coronary heart disease (CHD). Male patients predominated (78%), and the average patients' age was 62 years. Aortic and cerebral arterial atherosclerosis of various degrees together with AH was identified in all patients, and different CHD types were revealed in 64% of patients. In most cases death was caused by cerebral edema in the presence of infarctions or hemorrhages, and in the absence of such the death was due to pulmonary embolism, heart failure associated with CHD, as well as renal or multiple organ dysfunction upon severe infectious disease.

Pathogenetic stroke subtype was defined in accordance with the SSS-TOAST classification based on the appropriate subtype criteria [8]. For this purpose we assessed data obtained by computed tomography (56% of cases) or 0.5T and 1.5T magnetic resonance imaging (44%), as well as the changes in aorta and cerebral arteries revealed by color duplex sonography of the major arteries of the head, inter alia combined with transcranial Doppler embolodetection (16% of cases), computed tomography angiography (28%) or magnetic resonance angiography (34%). The history of pre-stroke episodes of cardiovascular and blood pressure (BP) instability was taken into account, as well as data of ophthalmological and endocrinological examination, electrocardiography, including Holter monitoring (24 hours and longer), and transesophageal echocardiography (22%).

During the pathomorphological examination, the cerebral infarction characteristics were taken into account, such as localization, size, appearance ("white" or hemorrhagic), and organization phase; arterial alterations from aortic arch to small vessels on the surface of the brain — emboli, complicated atherosclerotic lesions (intraplaque hemorrhage, plaque ulceration, thrombosis), atherosthenosis degree (in accordance with the clinical and instrumental examination method); liquor system condition; microhemorrhages and enlarged perivascular spaces (criblurs). The atherosclerosis and AH-related cardiac structure alterations, as well as cardiogenic emboli in the branch arteries of the aortic arch were detected.

During microscopic examination we clarified the complicated atherosclerotic lesions, the nature of large and small superficial arteries' occlusion, and the nature of focal brain lesion; the condition periventricular white matter was assessed. In each case, 5–10 brain tissue blocks sized 2.5 x 3 cm and 0.5 cm thick were cut out. The large arteries were examined using blocks of the same thickness, which were cut out perpendicular to the longitudinal axis of the blood vessel. Prior to microscopic examination the paraffin embedded 5–6 μm-thick brain slices were stained with Carazzi's hematoxylin and eosin using the Weigert Van Gieson method (assessment of arterial alterations) and the Kluver–Barrera method (assessment of white matter condition). Analysis of the resulting data made it possible to define the cause of each infarction.

The differences were revealed using the Mann–Whitney *U* test; the *p* values of less than 0.05 were regarded as statistically significant. The results were processed with the Statistica software (StatSoft Inc.; США), version 13.3.

## RESULTS

In accordance with SSS-TOAST, 76 stroke cases (of 125) were classified as large-artery atherosclerosis based on the arterial alterations in the form of occlusion or atherostenosis, both severe (≥ 50%) and moderate (30–49%), ipsilateral to the infarction in the absence of cardiac pathology and aorta with high risk of embolism. Clinical and instrumental examination followed by pathomorphological assessment revealed

four reasons of stroke of the discussed subtype: critical atherostenosis (70–90%) of single artery — 10%; multifocal atherostenosis — 33%; atherosclerotic lesion disruption with superimposed thrombosis (atherothrombosis) — 44%; arterio-arterial embolism (13%) when the atherosclerotic plaque with parietal thrombus was found in the vessels located proximal to the occlusion area (Fig. 1).

In cases with stenosis of single artery, stroke resulted from rapid and significant increase in the volume of atherosclerotic plaque due to extensive hemorrhage or parietal thrombosis. Occlusive thrombosis resulted from atherosclerotic plaque rupture due to thinned and disrupted cap in the foci of atheromatosis and calcification. In cases of multifocal stenosis the degree of the vessel lumen narrowing ranged from 30 to 70%, moreover, in 10 observations (of 25) the maximum vessel lumen narrowing degree did not exceed 50%. We determined the diversity of the infarction size and localization with a slight predominance (by 1.5 times, $p < 0.05$) of large infarctions (Fig. 2).

It has been noted that occlusion or stenosis of single artery determine the infarctions occurring strictly in the altered blood vessel system, whereas the multifocal stenosis results in focal lesions in the areas of adjacent cerebral blood supply and lacunar infarctions. According to microscopy data, there are arteries with "recalibrated" (narrowed) lumen due to tunica intima sclerosis or tunica intima components' proliferation with formation of extra elastic membrane in the areas of infarctions occurring in the areas of adjacent blood supply and lacunar infarctions. Such patients had no diabetes mellitus, occlusions, severe atherostenoses and severe hypertensive alterations in extra- and intracerebral arteries with a diameter of 200–500 μm in the examined foci area, which are considered the major causes of focal brain ischemia [3, 14, 16, 17].

The 32 stroke cases were classified as cardioembolism subtype based on the lack of cerebral artery stenosis ≥ 50% ipsilateral to the infarction and the presence of cardiac pathology and aorta with high risk of embolism. An almost twofold prevalence of atrial fibrillation ($p < 0.05$) was detected; atrial fibrillation is considered the most common cause of cardioembolic stroke [18, 19] (Fig. 3).

The postmortem study confirmed the presence of embologenic plaque in the aortic arch, as well as the listed forms of CHD. Focal atrial fibrosis was the morphological marker of atrial fibrillation. In the arteries located proximal to the occlusion area there were plaques, which narrowed the lumen not by more than 30%, with no alterations determining the risk of embolism.

In cases of this stroke subtype the infarctions could involve most of the internal carotid or middle cerebral artery system, but more often the infarctions occurred in the system of branches 1–2 of the middle or anterior cerebral artery (62% of cases). Systemic embolic events and infarctions with a hemorrhagic component were detected in 40% and 47% of cases respectively, and the cardiogenic emboli in the branches of the abdominal aorta with infarctions in kidney and spleen identified during the pathomorphological examination were asymptomatic due to small size of foci (1–2 cm).

SSS-TOAST classification system admits the possibility of the cardioembolism subtype establishment in case of signs of embolism from the heart or aorta, however, this can also raise some difficulties. Thus, echocardiography and postmortem examination revealed intracardiac thrombi only in 12 cases (of 31), and the cerebral artery occlusion was detected in only half of the cases, mostly during localization of emboli in the internal carotid or middle cerebral artery. It was impossible to detect the occlusion in patients admitted to hospital 4 or more days after the stroke onset due to the well known vanishing occlusion phenomenon resulting from the spontaneous emboli fragmentation and distal migration of fragments. This phenomenon was observed in a number of postmortem cases, in which the embolism of the middle or anterior cerebral artery branches was verified only by microscopy with the detection of emboli in the small arteries of pia mater near the infarction.

A small number (17) of stroke cases were classified as the small-artery occlusion subtype based in the detection of infarction with a diameter of ≤ 20 mm in the artery systems penetrating hemispheres and brainstem in the absence of the extra- and intracranial arteries alterations in the form of occlusion or stenosis (including the moderate stenosis) ipsilateral to the focal lesion. In 11 stroke cases the detected foci sized 1–2 cm were located in the knee and posterior hip area of the internal capsule, and in six cases the foci were located in thalamus and deep inside the basilar part of the pons. The foci were considered hypertensive lacunar infarctions, which was confirmed by episodes of high BP (180/110 mmHg and higher) in the medical history or upon admission to hospital, as well as by the presence of hypertensive retinopathy and left ventricular hypertrophy. According to the pathomorphological examination, the size of hypertensive lacunar infarctions along with the infarctions resulting from atherosclerosis was smaller compared to the size measured during neurovisualization (0.5–1.5 cm), which was due to their organization. Pathogenetic relationship between such infarctions and AH was confirmed,

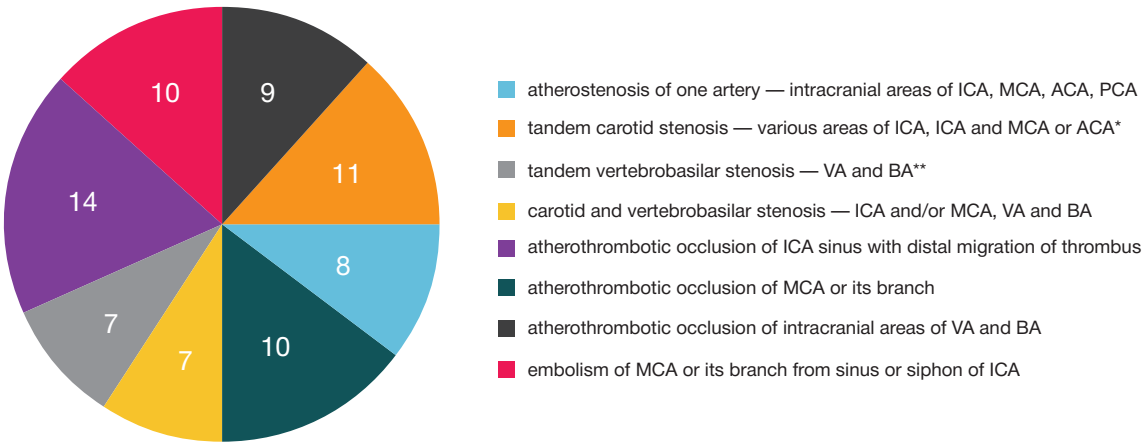


**Fig. 1.** Causes of stroke of the large-artery atherosclerosis subtype. ICA — internal carotid artery, MCA — middle cerebral artery, ACA — anterior cerebral artery, PCA — posterior cerebral artery, VA — vertebral arteries, BA — basilar artery; * — in combination with contralateral stenosis of ICA or MCA, ** — in combination with contralateral stenosis of VA

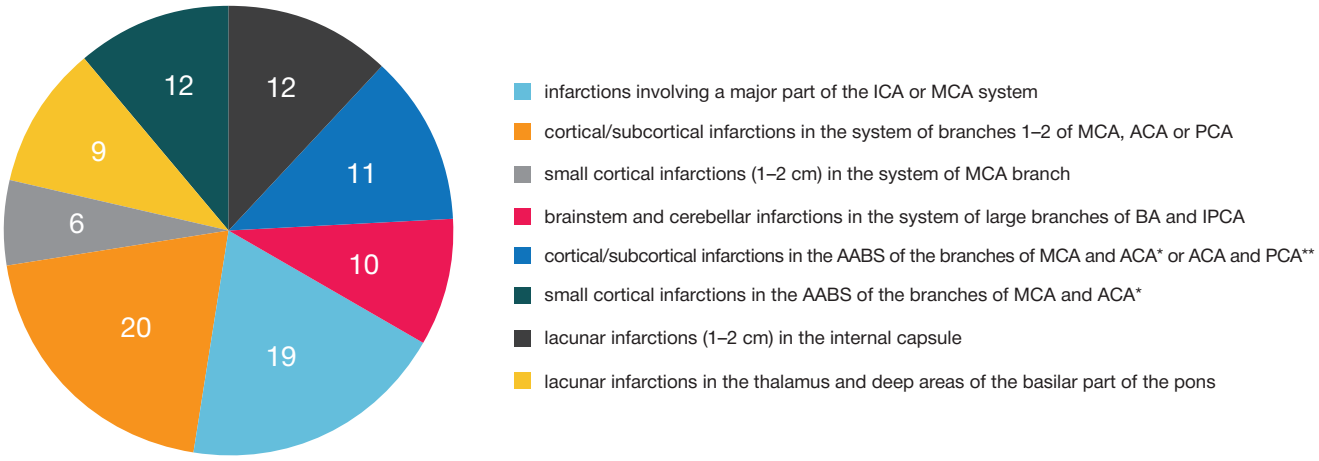


**Fig. 2.** Infarctions in patients with stroke of the large-artery atherosclerosis subtype. ICA — internal carotid artery, MCA — middle cerebral artery, ACA — anterior cerebral artery, PCA — posterior cerebral artery, BA — basilar artery, IPCA — inferior posterior cerebellar artery, AABS — area of adjacent blood supply; * — in the area of superior frontal sulcus and on the border between the superior and middle thirds of the central gyri, ** — in the area of intraparietal sulcus and in the inferior temporal gyrus

since the arteries with specific alterations (hyalinosis or plasmorrhagia with foci of fibrinoid necrosis associated with sharp narrowing of the lumen until the closure is complete) were detected in the close proximity to the foci.

Mention should be made of multiple infarctions, including the silent ones, within the same arterial system, detected in 49% stroke cases or the large-artery atherosclerosis subtype. The majority of silent ischemic foci were represented by single and multiple lacunar infarctions (54 of 78), the relationship of which with AH was excluded by microscopy. The other asymptomatic infarctions were small cortical infarctions, which resulted from critical atherostenoses and occlusions in the small arteries of the brain surface, and, much like lacunar infarctions, most often resulted from severe stenoses of larger arteries.

## DISCUSSION

The study had shown the diversity of ischemic brain lesions and cardiovascular system alterations in cases of cerebral artery atherosclerosis combinations with CHD and AH. Clinical diagnosis or these alterations requires the use of a set of methods, which, according to our study, should be applied during the first 1–3 days after the stroke onset. Contemporary research methods make it possible to obtain considerable information on the ischemic stroke morphology. Thus, color duplex sonography of the major arteries of the head allows not only to define the degree of stenosis with accuracy of 95%, but also to reveal ulceration, hemorrhages and other structural features of atherosclerotic plaques associated with high risk of stroke [20]. Active use of echocardiography and Holter monitoring, as well as implementation of prolonged ambulatory ECG monitoring (during 30 days and more) and cardiac magnetic resonance imaging possesses great potential for verification of cardioembolism [21–23]. Neurovisualization methods provide a comprehensive source of information on acute focal brain lesions, diffuse white matter changes and cerebral perfusion, and also allow one to visualize the complicated atherosclerotic lesions [24–26]. Magnetic resonance angiography has become the method of choice for the diagnosis of intracranial artery alterations, including the occlusions and stenoses of perforating arteries in the areas of their origin, which are considered one of the main causes of focal brain lesion [27].

This study is one of the few Russian studies showing the great potential of the use of contemporary clinical and instrumental methods, as well as the stroke subtypes defined by the SSS-TOAST classification system, for diagnosis of major stroke pathogenetic subtypes (large-artery atherosclerosis, cardioembolism, small-artery occlusion) based on the pathomorphological verification. Furthermore, the study highlights inconsistency of the discussed in the literature additional features of cardioembolism (systemic embolic events and infarctions with a hemorrhagic component) and large-artery atherosclerosis (multiple infarctions, including the silent ones, occurring within the same arterial system) subtypes [8, 28]. Small number of lacunar stroke cases resulting from hypertension made it impossible to evaluate the significance of other features reported as additional markers of this type of stroke (diffuse loss of white matter and criblures caused by persistent edema, dilated ventricular system and subarachnoid space in the region of hemisphere sulci, microhemorrhages, combination of clinically significant lacunar infarctions with similar silent infarctions located in the other arterial system) [13]. All features were detected in no more than two cases both by neurovisualization and pathomorphological examination.

In our opinion, high informativity of currently available clinical and instrumental methods allows for a more in-depth diagnosis of pathogenetic stroke variants. The in-depth differential diagnosis is particularly important for the large-artery atherosclerosis subtype, which, according to the clinical, instrumental and pathomorphological data, includes stroke types of different etiology, i.e. resulting from critical atherostenosis or arterio-arterial embolism of intracranial artery, atherothrombotic occlusion of an extra- or intracranial artery, as well as from maximum degree (≥ 50%) of multifocal atherostenosis. Lower degree of maximum stenosis in patients with multiple atherosclerotic lesions compared to monofocal stenosis may be associated with local obstacles to collateral circulation through the circle of Willis anastomotic system and the arterial network of the brain surface. It seems that we should accept the opinion that in case of stroke resulting from multifocal stenosis the total cerebral arteries plaque burden should be assessed rather than maximum stenosis degree [15].

In cases of multifocal atherostenoses infarctions evolved in accordance with the cerebrovascular insufficiency mechanism, the doctrine of which was developed in the 50s by D. Denny-Brown and E. Corday. According to the doctrine, the development of infarctions depends not only on local obstacles to blood supply, but also on hemodynamics. According to the well known Schneider–Zulch's "last meadow" concept, in cases of general hemodynamic disorders ischemic lesions occur in the periphery of vascular territory, i.e. in the areas of adjacent blood supply to the branches of the internal carotid and/or

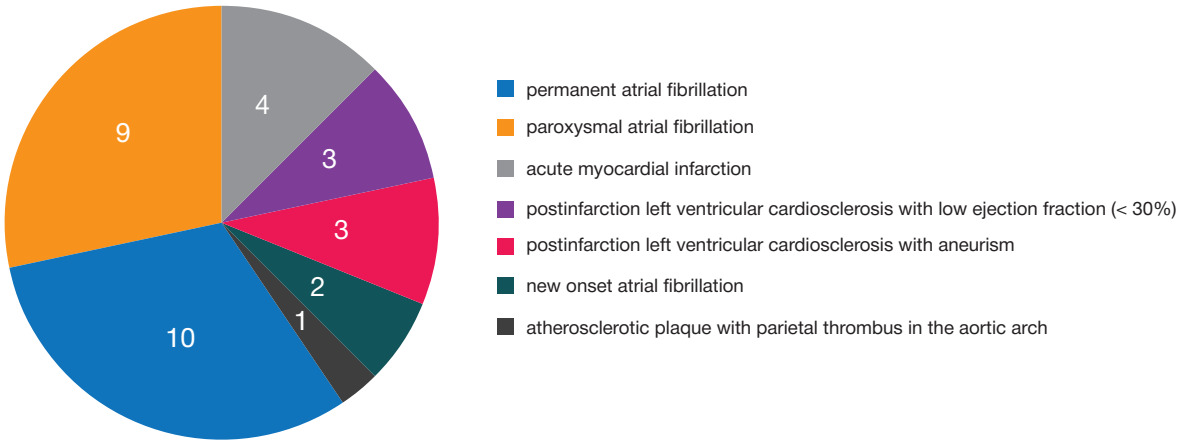


**Fig. 3.** Pathology with high risk of cerebral artery embolism

vertebral arteries, which remain intact with a sufficient level of systemic BP and total blood flow. The areas furthest away from arteries of the brain surface are the regions located deep in the cerebral hemispheres and pons, which determines the possibility of lacunar infarctions in the listed regions. In cases with multifocal stenoses the following signs of infarctions in the areas of adjacent blood supply or lacunar infarctions resulting from cerebrovascular insufficiency were observed: pre-stroke episodes of fluctuations in BP with a tendency to hypotension resulting from angina attack, decompensated heart failure in patients with postinfarction cardiosclerosis, and the taking the excessive dose of antihypertensive drug. Stenotic arteries with sclerotic wall changes were the pathomorphological feature of the detected infarctions, which may be considered the adaptive response to reduced blood flow resulting from atherostenosis of proximal large arteries. Pathogenesis features and clear signs of stroke resulting from cerebrovascular insufficiency made it possible to classify these infarctions as the distinct hemodynamic subtype in accordance with classification system developed in the Research Center of Neurology [11, 28].

The importance of in-depth differential diagnosis of pathogenetic stroke variants is reflected in other classifications of stroke. In one of those there is a distinct group within the large-artery atherosclerosis subtype which includes stroke resulting not only from cerebrovascular insufficiency, but also from atherostenosis or atherothrombosis of the brain surface artery with occlusion of penetrating artery in the area of its origin, and the aorto- and arterio-arterial embolism [14]. Such distinct pathogenetic variants of stroke have been assessed in some recent clinical trials aimed at further development of targeted treatment methods and prevention of brain ischemia, which is quite reasonable in terms of modern personalized medicine [29, 30].

## CONCLUSION

The study has shown the diversity of ischemic brain lesion pathogenesis in cases of cerebral artery atherosclerosis combined with coronary heart disease and arterial hypertension. It has also shown the possibility of effective clinical diagnosis of the major ischemic stroke pathogenetic subtypes based on the criteria defined in the latest version of the TOAST classification. In our opinion, high informativity of the existing examination methods allows for a more differentiated understanding of the cause of ischemic stroke, which is fully in line with modern personalized medicine. Such approach should be used for the large-artery atherosclerosis subtype, which includes stroke resulting from various alterations of single cerebral artery (critical atherostenosis, atherothrombotic occlusion and arterio-arterial embolism) and stroke resulting from cerebrovascular insufficiency. The latter deserve special attention, since their pathogenetic features are defined by the following triad: cortical and cortical/subcortical infarctions in the areas of adjacent blood supply or lacunar infarctions in the regions located deep in the cerebral hemispheres and pons; multifocal atherostenoses (incuding the contralateral ones) the maximum degree of which may not exceed 50%; pre-stroke extracerebral factors of cerebral blood flow reduction.

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# BRAIN ATROPHY PATTERNS IN PATIENTS WITH FRONTOTEMPORAL DEMENTIA: VOXEL-BASED MORPHOMETRY

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Frontotemporal dementia (FTD) is a neurodegenerative disorder characterized by language and behaviour deficits, which is considered the second most common cause of early-onset dementia. Detection of brain atrophy patterns is important for FTD diagnosis. However, the visual assessment of magnetic resonance imaging data may not be sensitive enough requiring the use of objective gray matter (GM) volume determination method. The study was aimed to assess the GM atrophy pattern in patients with FTD compared to control group patients using voxel-based morphometry (VBM). The study included 16 patients with FTD (12 patients with nonfluent agrammatic variant primary progressive aphasia (nfvPPA), three patients with behavioral variant of FTD, and one patient with logopenic variant PPA) and 10 healthy volunteers. VBM of patients with FTD and healthy controls revealed three significant (pFWE-corr < 0.05) atrophy areas in the left inferior frontal, left fusiform, and left supramarginal gyri. Taking into account the predominance of patients with nfvPPA in the group of FTD patients, the additional VBM of this group and control group was carried out, which revealed a distinct atrophy pattern: the reduced GM volume was detected in the left inferior frontal and left middle frontal gyri (pFWE-corr < 0.05). The results obtained indicate that regardless of the clinical variant, there is a certain atrophy pattern characteristic of FTD, which involves both frontotemporal areas and parietal lobe. The example of nfvPPA shows that each variant of the disease is associated with distinct localization of atrophy.

**Keywords:** frontotemporal dementia, voxel-based morphometry, primary progressive aphasia, behavioral variant frontotemporal dementia

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**Compliance with ethical standards:** the study was approved by the Ethics Committee of the Research Center of Neurology (protocol № 12-7/19 dated December 25, 2019).

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# ПАТТЕРНЫ АТРОФИИ ГОЛОВНОГО МОЗГА ПРИ ЛОБНО-ВИСОЧНОЙ ДЕМЕНЦИИ: ДАННЫЕ ВОКСЕЛЬ-ОРИЕНТИРОВАННОЙ МОРФОМЕТРИИ

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Лобно-височная деменция (ЛВД) — нейродегенеративное заболевание, вторая по частоте деменция с ранним началом, проявляющаяся речевыми и поведенческими нарушениями. Выявление паттернов атрофии важно для диагностики данной патологии. Однако визуальная оценка данных магнитно-резонансной томографии может быть недостаточно чувствительной, что требует использования объективного метода определения объема серого вещества (СВ). Целью исследования было оценить паттерн атрофии СВ у пациентов с ЛВД в сравнении с контрольной группой при помощи воксель-ориентированной морфометрии (ВОМ). В исследование включены 16 пациентов с ЛВД (12 — с аграмматическим вариантом первичной прогрессирующей афазии (авПФА), три — с поведенческим вариантом ЛВД, один — с логопеническим вариантом ППА) и 10 здоровых добровольцев. При проведении ВОМ в группе ЛВД и контрольной группе выявлено три статистически значимые (pFWE-corr < 0,05) зоны атрофии — в левой нижней лобной извилине, левой фузиформной и левой надкраевой извилинах. В связи с преобладанием в группе ЛВД пациентов с авПФА дополнительно проводили ВОМ в этой группе и группе контроля, при которой был выявлен иной паттерн атрофии: уменьшение объема СВ обнаружено в левой нижней лобной и левой средней лобной извилинах (pFWE-corr < 0,05). Полученные результаты показывают, что для ЛВД независимо от клинического варианта характерен свой определенный паттетрн атрофии, захватывающий как лобно-височные отделы, так и теменную долю. На примере авПФА было показано, что у каждого из вариантов заболевания локализация атрофического процесса имеет отличный от других характер.

**Ключевые слова:** лобно-височная деменция, воскель-ориентированная морфометрия, первичная прогрессирующая афазия, поведенческий вариант ЛВД

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Frontotemporal dementia (FTD) is a neurodegenerative disorder characterized by behaviour and/or language deficit progression. FTD usually affects people aged 45–65. It is considered to be the second most common cause of early-onset dementia (before age 65), second only to Alzheimer disease (AD) [1].

The following three clinical subtypes of the disorder are distinguished based on the most prevalent deficit: behavioral variant of FTD (bvFTD), semantic variant primary progressive aphasia (svPPA), and nonfluent variant primary progressive aphasia (nfvPPA). The logopenic variant PPA (lvPPA) is typically classified as atypical AD, however, up to 24% lvPPA cases may be attributed to as FTD based on the pathomorphological investigation results [2]. BvFTD is characterized by progressive deficits in executive function, apathy, behavioural disinhibition, eating disorder, altered motor activity and euphoria. The patients demonstrate risky impulsive behaviour and indifference to people around them. In some of the patients, irritability and sleep disturbances are observed [3]. In patients with primary progressive aphasia (PPA), language impairment is one of the most prominent and disabling manifestations. In patients with svPPA, the single word comprehension deficits and anomia are observed. The early manifestation is poor comprehension of low frequency words. As symptoms progress, the patients also lose knowledge about more familiar words and objects. The main symptoms of nfvPPA are agrammatisms involoving verbal and subsequently written language, and speech apraxia. As time progresses, difficulties arise with comprehension of syntactically complex sentences, the speech becomes limited, often consisting of single short sentences and subsequently of short phrases. Most patients with lvPPA experience word-finding difficulties and are unable to repeat long sentences, since the deficit affects the phonological working memory. The above FTD variants manifestations may overlap with motor impairment, such as motor neuron disease (MND) or parkinsonian syndromes (corticobasal syndrome or progressive supranuclear palsy syndrome) [4].

In addition to the clinical manifestations diversity, FTD is characterized by genetic and morphological heterogeneity. The proportion of familial cases is as large as 40%. To date, over 20 genes have been identified, the mutant variants of which are involved in FTD. Yet, the vast majority of genetic cases of the disease are associated with mutations of three genes: *C9orf72*, *GRN*, *MAPT* [5]. Histological analysis of FTD specimen has revealed pathological accumulation of tau protein, TDP-43 or FET family proteins making it possible to classify FTD into three molecular subtypes [6].

Given the prominent FTD heterogeneity, the study and diagnosis of the disease are a major challenge. The existing diagnostic criteria are based on clinical manifestations and neuroimaging data, especially the visual assessment of brain atrophy on magnetic resonance imaging (MRI) and/or computed tomography (CT), or hypoperfusion/hypermetabolism on positron emission tomography (PET) and/or single-photon emission computed tomography (SPECT). The affected areas characteristic of each variant have been distinguished: bilateral frontal and anterior temporal lobe atrophy for bvFTD, frontal and insular lobe atrophy with predominant left hemisphere involvement for nfvPPA, anterior temporal lobe atrophy for svPPA, and left parietal lobe atrophy with predominant left posterior perisylvian atrophy for lvPPA [7, 8].

However, in recent years it has been shown that visual assessment of MRI data may be insuffucient to identify a characteristic atrophy pattern. According to a number of reports, the accuracy of MRI in diagnosis of bvFTD varies between 59– 70% [9, 10]. One of the methods for the MRI objectivity improvement is voxel-based morphometry (VBM), the voxel-wise comparison of brain volume between two studied groups in order to detect the significant gray matter (GM) atrophy.

The use of VBM to study FTD has shown that the pathogenetic process is not limited to frontal and temporal lobes. In patients with distinct variants of the disorder, the parietal and occipital areas, cerebellum, insular lobes and subcortical structures may be also affected [11–15]. However, with the new knowlege it becomes clear, that the findings of studies vary significantly. Thus, meta-analysis of publications on the use of VBM in patients with bvFTD revealed the significant frontal and insular lobes atrophy, as well as the bilateral striatum atrophy, but showed no significant temporal lobes lesions (one of the bvFTD diagnosis criteria) reported by a number of authors [16].

Moreover, some reports show that clinical manifestations of PPA may vary depending on the patient's native language, therefore, the FTD-associated atrophy patterns may vary depending on the studied population [17, 18]. However, no studies of characteristic FTD-associated atrophy features in Russian population have been performed.

The study was aimed to reveal the characteristic patterns of brain atrophy common to distinct FTD variants in the Russian population.

## METHODS

The study was carried out at the Research Center of Neurology. Inclusion criteria: patients who fulfilled the diagnostic criteria of FTD; age over 18 years. Exclusion criteria: contraindications for MRI; serious health condition requiring the advanced life support; structural focal brain lesions (tumors, effects of cerebrovascular accident or traumatic brain injury, etc.). A cohort of 16 FTD patients (6 men and 10 women; average age 61.2 ± 9.4 years), and 10 healthy volunteers (4 men and 6 women; average age 55.6 ± 11.3 years) were included in the study. The groups were matched for age and gender.

Twelve (68.75%) patients of index group were diagnosed with nfvPPA, three patients (18.75%) were diagnosed with bvFTD, and one patient (6.25%) had lvPPA. The patient with lvPPA underwent lumbar puncture with amyloid-β level assessment. The normal amyloid-β level value made it possible to exclude AD. One of the patients with nfvPPA had signs of MND (FTD-MND phenotype). The average age of nfvPPA patients was 60.6 ± 7.5 years, the four of them were males.

At the time of the study, the disease duration ranged from 12 to 84 months, and the average duration of the disease was 47.6 ± 21.3 months. The disease severity was assessed using the Frontotemporal Dementia Rating Scale (FTD-FRS) [19]: 3 patients were rated as having very mild FTD, 4 patients had mild FTD, and 7 patients were diagnosed with moderate FTD; the group also included one patient with severe and one patient with extremely severe FTD. The total Frontal Assessment Battery (FAB) score ranged from 3 to 15, the average value was 9.3 ± 3.9. It was difficult to use the Montreal Cognitive Assessment (MoCA) test in 8 patients due to severe language and/or behaviour deficits (apathy, restlessness, refusal to perform tests). The average score of patients tested was 22.25 ± 6.04. In addition, all patients were tested for literal and semantic verbal fluency. The significant literal and semantic verbal fluency decline was revealed (an average of 3 and 7 words per minute, respectively).

MRI of the brain in the 3D-T1 MPR (multiplanar reconstruction) mode was performed in all patients using the Magnetom Verio 3T system (Siemens; Germany). The MRI data

post-processing and statistical analysis were carried out using the SPM12 software (Statistical Parametric Mapping; Institute of Neurology, UK) written using Matlab R2019b (Mathworks; USA).

Post-processing included spatial normalization of images to MNI (Montreal Neurological Institute) reference space, MR images segmenting into GM, white matter and cerebrospinal fluid using the DARTEL (Diffeomorphic Anatomical Registration Through Exponentiated Lie Algebra) algorithm, as well as spatial smoothing using the isotropic Gaussian filter kernel with full width at half maximum (FWHM) size 8 mm in order to align the individual structural characteristics.

The Easy Volumes utility (Institute of Neurology; UK) was used to calculate total GM volume, as well as bilateral GM volume in frontal, temporal, parietal, occipital, insular lobes and basal ganglia.

VBM data vizualiation, statistical analysis, data withdrawal and coordinates localization were performed using the xjView software [20].

The assessment of VBM results included cluster analysis using the two-sample t-test with the whole brain voxel-wise comparison of GM volume between the studied groups. The threshold for the individual voxels of the cluster was set to $p < 0.0001$. The analysis included the GM clusters of cerebral hemispheres with minimum area volume of ≥ 100 voxels and significance level of $p < 0.05$ adjusted for multiple comparisons to control the family-wise error (FWE) rate. The analysis of the results was performed at the cluster and peak levels.

Statistical data processing was performed using the IBM SPSS Statistics 23.0 software (IBM; USA). The differences in the brain GM volume between two groups were assessed using the Mann–Whitney *U* test (Bonferroni adjusted). The relationship between clinical data and brain GM atrophy was defined by correlation analysis using the Spearman's rank correlation coefficient.

## RESULTS

VBM revealed a significant decline in GM volume in the left inferior frontal, supramarginal and fusiform gyri in patients with FTD compared to controls (Table 1, Fig.). The greatest degree of atrophy was observed in the left inferior frontal gyrus.

Due to the significant prevalence of nfvPPA among patients with FTD, the additional analysis of this group was carried out. VBM revealed significant degree of atrophy in the small areas of left middle and inferior frontal gyri in patients with nfvPPA compared to controls (see Table 1).

At the next stage the calculation of total GM volume and GM volume in frontal, temporal, parietal, occipital and insular lobes, as well in basal ganglia of the right and left hemispheres, was carried out in both groups. The differences between two groups were significant ($p < 0.05$) in all listed areas (Table 2).

Correlation analysis revealed a significant negative correlation between the left temporal lobe GM volume and the duration of the disease (Spearman's rank correlation coefficient –0.53; $p = 0.035$). No correlations with other clinical manifestations and neuropsychological findings (total MoCA and FAB score, semantic and literal verbal fluency, disease severity) were detected.

## DISCUSSION

The study showed that in the group of patients with FTD the significant GM volume decline was observed in the following areas: left inferior frontal, supramarginal and fusiform gyri.

The greatest cluster of atrophy was detected in the left inferior frontal gyrus, which is the location of Broca's area involved in the motor–phonological network regulation, as well as in the complex grammatical and syntactic constructions comprehension and production. Atrophy of the described area is one of the major signs of nfvPPA, which correlates with overall severity of aphasia and severity of agrammatisms [21, 22]. The greater degree of atrophy in the left inferior frontal gyrus may be explained by the predominance of patients with this phenotype in the FTD group.

The fusiform gyrus involvement has been reported for all PPA variants and bvFTD [22, 23]. Together with orbitofrontal cortex, amygdala and other temporal association areas, the fusiform gyrus forms a perceptual system responsible for recognition and analysis of others' social signals (for example, understanding of facial expressions) [24], i. e. plays a part in the social behaviour production. Furthermore, it has been shown, that the fusiform gyri atrophy in patients bvFTD correlates with the severity of disinhibition [25].

**Table 1.** Areas of significant (pFWE-corr < 0.05) gray matter volume reduction based on VBM data

| Localization of atrophy | Cluster level | | Peak level | | | MNI peak coordinates (*x*, *y*, *z*), mm |
|---|---|---|---|---|---|---|
| | Degree of atrophy (voxels) | pFWE-corr | T | Z | pFWE-corr (peak level) | |
| **FTD group < control group** | | | | | | |
| Inferior frontal gyrus, S | 8198 | < 0.001 | 11.14 | 6.47 | < 0.001 | –36, 3, 24 |
| | | | 9.66 | 6.05 | < 0.001 | –44, 14, 17 |
| Supramarginal gyrus, S | 350 | < 0.001 | 7.66 | 5.35 | 0.003 | –47, –41, 35 |
| | | | 6.57 | 4.88 | 0.021 | –35, –44, 44 |
| | | | 6.54 | 4.87 | 0.022 | –33, –39, 33 |
| Fusiform gyrus, S | 136 | 0.001 | 7.04 | 5.09 | 0.009 | –54, –8, –27 |
| | | | 6.91 | 5.03 | 0.011 | –57, –17, –23 |
| **nfvPPA group < control group** | | | | | | |
| Middle frontal gyrus, S | 122 | < 0.001 | 9.24 | 5.63 | 0.002 | –30, 38, 33 |
| | | | 7.56 | 5.08 | 0.023 | –30, 47, 23 |
| Inferior frontal gyrus, S | 155 | < 0.001 | 8.46 | 5.39 | 0.006 | –50, 14, 17 |
| | | | 7.95 | 5.21 | 0.013 | –53, 6, 15 |
| | | | 7.89 | 5.19 | 0.014 | –56, –3, 17 |

**Note:** S — left.

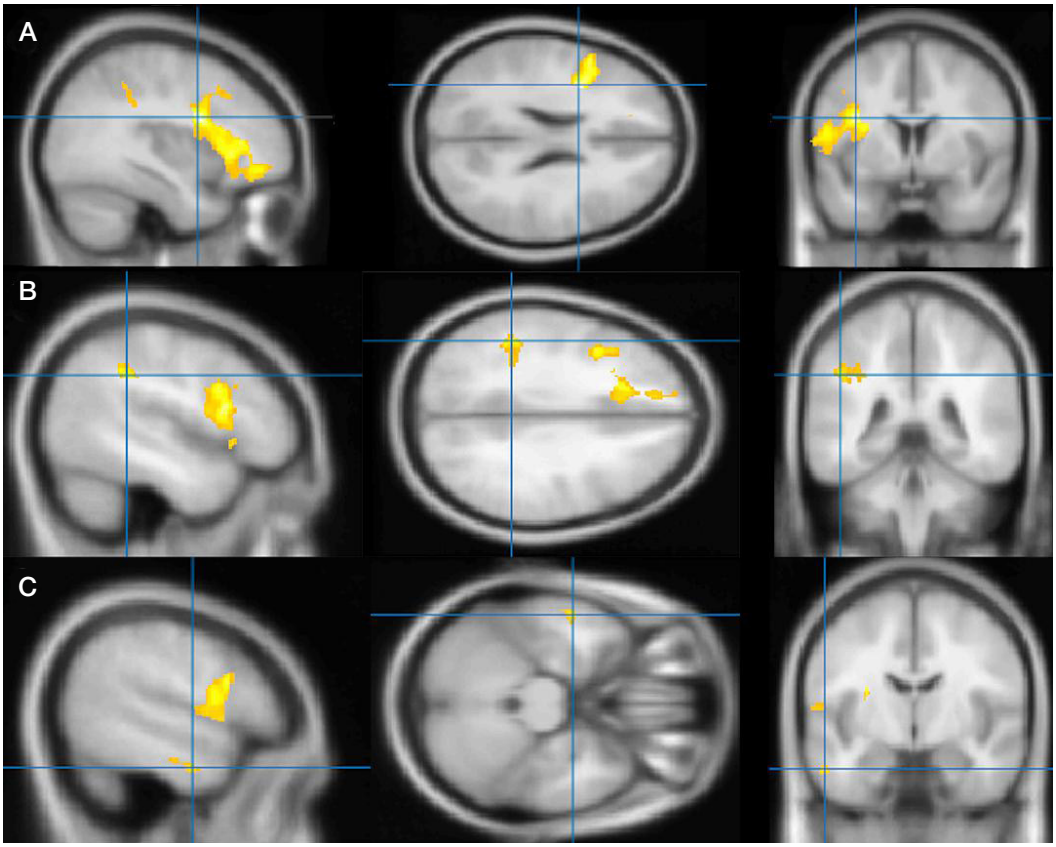


**Fig.** Localization of significant (pFWE-corr < 0.05) GM volume decline in patients with FTD compared to control group. **A**. Lleft inferior frontal gyrus. **B**. Left supramarginal gyrus. **C**. Left fusiform gyrus. From left to right: sagittal, axial and coronal slices

The supramarginal gyrus is involved in phonological short-term memory and phonological speech processing. It also appears to be connected to brain areas responsible for speech motor control [26]. Although the affected supramarginal gyrus is typically considered a neuroimaging sign of lvPPA, some papers report the supramarginal gyrus atrophy in patients with nonfluent agrammatic and semantic variant PPA [7, 27, 28].

Since the nfvPPA phenotype was observed in vast majority of patients of the FTD group, it might be assumed that the GM volume decline in all three areas was associated with nfvPPA, while the other variants' contribution was not of comparable importance. However, VBM in patients with nfvPPA and control group patients revealed the different pattern of atrophy: the affected left inferior frontal and middle frontal gyri. The involvement of left inferior frontal gyrus was less extensive and pronounced compared to the FTD group, while the affected middle frontal gyrus was restricted to that particular group. The similar pattern of atrophy has been previously reported, for example, in the meta-analysis performed in 2007, which revealed reduced volume of opercular part of inferior frontal gyrus, middle frontal gyrus, lentiform nucleus and superior temporal gyri [15], and the other study, which revealed the affected left precentral gyrus in addition to the listed above areas [29]. The decline in inferior frontal gyrus atrophy degree and severity might be associated

**Table 2.** Gray matter volume in patients with FTD and control group

| Gray matter volume | FTD | Control | Significance level, Mann–Whitney *U* test (*p*) |
|---|---|---|---|
| | Median [LQ; UQ] | Median [LQ; UQ] | |
| Total | 508.4 [474.8; 534.2] | 656.2 [597.6; 721.0] | < 0.001 |
| Frontal lobe, S | 33.4 [30.0; 35.4] | 53.4 [47.1; 56.5] | < 0.001 |
| Frontal lobe, D | 40.2 [35.7; 44.6] | 56.6 [48.7; 59.0] | < 0.001 |
| Temporal lobe, S | 33.4 [31.1; 39.4] | 50.0 [43.4; 54.8] | < 0.001 |
| Temporal lobe, D | 41.4 [35.5; 47.2] | 51.9 [46.2; 57.8] | 0.002 |
| Parietal lobe, S | 30.3 [28.1; 35.7] | 46.5 [38.5; 49.3] | < 0.001 |
| Parietal lobe, D | 38.0 [33.6; 41.8] | 49.2 [41.8; 52.9] | 0.003 |
| Occipital lobe, S | 14.8 [13.4; 16.7] | 20.3 [17.4; 23.4] | < 0.001 |
| Occipital lobe, D | 12.4 [11.0; 13.0] | 15.6 [13.2; 16.4] | 0.003 |
| Insular lobe, S | 5.5 [5.2; 6.0] | 7.9 [7.1; 8.4] | < 0.001 |
| Insular lobe, D | 6.3 [5.5; 7.0] | 7.9 [7.2; 8.5] | 0.001 |
| Basal ganglia, S | 8.0 [7.0; 9.4] | 12.1 [10.8; 12.5] | < 0.001 |
| Basal ganglia, D | 10.0 [7.8; 10.4] | 12.0 [10.9; 12.6] | 0.001 |

**Note:** LQ — lower quartile, UQ — upper quartile, S — left, D — right.

with more severe disorder in patients with bvFTD and lvPPA (the average FTD-FRS score was 2.36 among patients with nfvPPA and –0.04 among other patients) given the similar average duration of the disease at the time of assessment (47.5 months in patients with nfvPPA, 48 months in other patients).

Thus, the atrophy of left fusiform and supramarginal gyri detected during the whole group analysis can not be explained by the sampling bias toward the patients with nfvPPA, and is likely to result from the listed areas' lesion in all studied variants of the disease.

The study of correlations of clinical and neuropsychological data with the volume of distinct lobes and subcortical structures revealed only one significant negative correlation between the volume of left temporal lobe and the duration of the disease. The involvement of the left temporal lobe may be explained by the left fusiform gyrus lesion. However, taking into account the prevalence of nfvPPA and greater degree of atrophy in the left inferior frontal gyrus, the correlation of the left frontal gyrus volume with the severity of the disease and speech fluency impairment might be expected, reported in a number of papers. The lack of such correlation may be due to several factors. First, not the volume of distinct gyri, but the volume of entire lobes was taken into account during the analysis. Second, the severity of the disease was assessed using the Frontotemporal Dementia Rating Scale (the questionnaire, which includes the whole range of symptoms), whereas given the predominance of PPA it would be judicious to perform additional assessment of the patients' condition using the aphasia severity scales. Lack of correlations in accordance with the FAB and MoCA scores may be also due to the listed scales specialization: these scales are more focused on the characteristic of bvFTD executive functions impairment detection than on the speech impairment detection.

It should be noted that our study had a number of limitations: small sample size, unequal distribution of patients with distinct FTD variants within the group, and lack of patients with svPPA. We did not compare the distinct variants of the disease (nfvPPA, bvFTD, lvPPA) due to small number of patients with bvFTD and lvPPA. That did not allow us to draw the conclusion about the differences between atrophy patterns characteristic of nfvPPA and other FTD variants. These limitations should be taken into account when planning the further study of the issue.

## CONCLUSION

The VBM study revealed the affected left inferior frontal, supramarginal and fusiform gyri in patients with FTD compared to healthy controls. The limited atrophy pattern which involves left middle frontal and inferior frontal gyri is characteristic of nfvPPA. The results obtained are consistent with the khowlege about the functional anatomy of speech function and social behaviour. Our results are partially consistent with the previous studies reported by foreign authors. However, these studies have revealed the greater involvement of gray matter and the larger number of affected brain areas. Further study of the neuroimaging FTD signs is required in the larger patients' sample to confirm the obtained results.

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# INTERLEUKIN DYNAMICS DURING COGNITIVE STRESS IN PATIENTS WITH CHRONIC CEREBRAL ISCHEMIA

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Neuroimmune interactions represent a highly dynamic mechanism for the regulation of cognitive function in chronic cerebral ischemia (CCI). The aim of this study was to investigate changes in salivary proinflammatory cytokines IL1β and IL6 and anti-inflammatory IL10 in patients with CCI (mean age 65.4 ± 9.1 years) before and after cognitive tests. After cognitive tests, the levels of salivary IL1β and IL6 were significantly elevated by 101.6 ± 19.1 pg/ml ($n$ = 74) and 32.8 ± 6.1 pg/ml ($n$ = 74), respectively. Using one-way ANOVA ana non-parametric statistical methods, we were able to demonstrate associations between changes in salivary interleukins and cognitive performance. In the group of patients with a significant increase in IL1β, some cognitive parameters were lower than in the group with negative or zero dynamics of this cytokine: the patients made more mistakes in the subtraction test ($F$ = 11.5; $n$ = 63; $p$ = 0.001) and performed worse in the Luria test ($F$ = 6.8; $n$ = 65; $p$ = 0.01). For IL6, Spearman's rank correlation coefficient for the number of mistakes in the subtraction test was positive and differed significantly from 0 ($R$ = 0.26; $n$ = 62; $p$ = 0.042). The group with positive IL10 dynamics performed better in N-back test ($F$ = 5.2; $n$ = 67; $p$ = 0.03) and made fewer mistakes in the subtraction test ($F$ = 6.8; $n$ = 63; $p$ = 0.01) in comparison with patients who demonstrated negative IL10 dynamics. Good performance in other cognitive tests was not correlated with interleukin dynamics. The article also discusses possible mechanisms underlying interleukin effects on cognitive function in patients with CCI and applications of the obtained data.

**Keywords:** neuroimmune interactions, vascular encephalopathy, interleukins, IL1β, IL6, IL10, cognitive function

**Author contribution:** Fokin VF performed data analysis and wrote the manuscript; Shabalina AA performed biochemical analysis of cytokines and participated in writing the manuscript; Ponomareva NV collected and analyzed psychometric data, participated in writing the manuscript; Medvedev RB performed clinical examinations, analyzed the literature and proposed the study design; Lagoda OV analyzed clinical data and proposed the study design; Tanashyan MM proposed the study design, summarized clinical data in the context of the obtained results.

**Compliance with ethical standards:** the study was approved by the Ethics Committee of the Research Center of Neurology (Protocol No. 11/14 dated November 19, 2014); all study participants signed informed consent to participate.

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# ИЗМЕНЧИВОСТЬ ИНТЕРЛЕЙКИНОВ ПРИ КОГНИТИВНОЙ НАГРУЗКЕ У БОЛЬНЫХ С ХРОНИЧЕСКОЙ ИШЕМИЕЙ МОЗГА

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Нейроиммунное взаимодействие — один из наиболее активных механизмов влияния на когнитивные функции при хронической ишемии мозга (ХИМ). Целью работы было исследовать динамику провоспалительных интерлейкинов в слюне IL1β и IL6, а также противовоспалительного интерлейкина IL10 до и после выполнения когнитивных тестов больными ХИМ (средний возраст 65,4 ± 9,1 года). После выполнения когнитивных тестов содержание IL1β и IL6 в слюне достоверно увеличилось, соответственно на 101,6 ± 19,1 пг/мл ($n$ = 74) и 32,8 ± 6,1 пг/мл ($n$ = 74). С помощью методов дисперсионного анализа (ANOVA) и непараметрической статистики показана сопряженность изменчивости интерлейкинов с успешностью выполнения когнитивных заданий. Так, в группе со значительным приращением IL1β наблюдалось снижение ряда когнитивных показателей по сравнению с группой с более низкой или отрицательной изменчивостью IL1β: большее число ошибок в тесте вычитания ($F$ = 11,5; $n$ = 63; $p$ = 0,001) и более низкие показатели в тесте Лурия ($F$ = 6,8; $n$ = 65; $p$ = 0,01). Коэффициент ранговой корреляции IL6 с количеством ошибок в тесте вычитания был положительным и достоверно отличался от нуля ($R$ = 0,26; $n$ = 62; $p$ = 0,042). В группе с положительным ростом IL10 наблюдалось более успешное выполнение корректурного теста ($F$ = 5,2; $n$ = 67; $p$ = 0,03), а также меньшее число ошибок в тесте вычитания ($F$ = 6,8; n = 63; $p$ = 0,01), по сравнению с группой больных с отрицательной изменчивостью IL10. Успешность выполнения ряда других когнитивных тестов не была связана с изменчивостью интерлейкинов. Обсуждены возможные механизмы влияния интерлейкинов на когнитивные функции больных ХИМ и практическое использование полученных данных.

**Ключевые слова:** нейроиммунное взаимодействие, хроническая ишемия мозга, интерлейкины, IL1β, IL6, IL10, когнитивные функции.

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Exploration of molecular mechanisms implicated in cognitive disorders is the leading area of neurological research [1]. Progressive cognitive decline culminating in dementia is the main health issue arising from vascular and neurodegenerative diseases, most of which are associated with advancing age. Being a part of normal aging, gradual cerebral ischemia and atrophy in elderly patients can be considered pathologic, as compared with younger age groups, since they also lead to cognitive decline. Vascular disorders and aging are accompanied by chronic inflammation maintained by the

immune system. Neuroimmune interactions are the crucial component of the underlying pathogenetic mechanism as they largely determine the course of the disease and the level of cognitive functioning [2–8].

Understanding the pattern of changes in the salivary concentrations of pro-inflammatory interleukins (IL1β, IL6 and IL10) in response to cognitive stress is an important avenue of contemporary neuroimmunology research. Chronic cerebral ischemia (CCI) is a pathological form of vascular aging, i.e. progredient chronic vascular insufficiency accompanied by non-focal neurological symptoms and cognitive decline caused by cortical or subcortical lacunar infarcts. Ischemia triggers inflammation, production of reactive oxygen species and activation of microglia and other glial cells, thereby stimulating secretion of cytokines including proinflammatory interleukins [9, 10].

Proinflammatory interleukin IL1β plays a role in inflammatory response and other immune processes; its modulatory effects span the nervous, immune and endocrine systems. IL1β and its receptors are present in the brain and especially abundant in the hippocampus. There are reasons to believe that IL1β is involved in the modulation of hippocampal plasticity and memory formation [4]. According to the majority of IL1β studies, elevated IL1β has a negative impact on cognitive function. However, some studies report that IL1β either does not affect or has a beneficial effect on learning and memory. Physiological concentrations of IL1β promote post-tetanic potentiation but its abnormally high levels can be inhibitory and interfere with learning and memory. Furthermore, IL1β, IL6 and a few other cytokines are activated in the brain upon induction of long-term potentiation [5]. It is reported that central administration of IL1β to rats affects inflammatory response and enhances conditioned memory; this cognitive effect is correlated with glucocorticoid levels [6]. In addition, IL1β can interact with the autonomic nervous system [7], which, in turn, may mediate its effects on cognition.

Interleukin 6 (IL6) is one of the major mediators of acute inflammation. The sources of IL6 in the central nervous system are represented by neurons, astrocytes, microglia and endothelial cells. IL6 plays a definitive role in the pathogenesis of inflammatory diseases and the normal homeostasis of nervous tissue [8].

Recently, IL6 has been hypothesized as having an impact on cognitive function. Some researchers applied the analysis of variance to identify factors affecting the levels of IL6. Age, hypertension, diabetes, smoking, moderate consumption of alcohol, total homocysteine, carotid intima-media thickness and body mass index were reported to be positively correlated with IL6 concentrations. In a multidimensional linear regression model, IL6 was negatively correlated with Mini mental state examination scores adjusted for social, economic and vascular risk factors. It is known that IL6 can be expressed by brain cells, including neurons during their depolarization [11]. Perhaps, this process can be activated by cognitive stress.

Elevated IL6 is observed in patients with declining cognitive function, which may be associated with the regulation of post-tetanic potentiation [12, 13]. Similar to IL1β, IL6 interacts with the autonomous nervous system [14].

IL10 is an anti-inflammatory cytokine that inhibits secretion of proinflammatory IL1β, IL6 and tumor necrosis factor alpha (TNFα) and stimulates release of other anti-inflammatory cytokines, including the IL1β receptor antagonist, which exerts anti-inflammatory effects. IL10 reduces IL1β and TNFα levels after traumatic brain injury in rats and improves neurologic recovery [15]. Hyperactive microglial response observed in persistent inflammation is often associated with increased expression of inflammatory IL1β and anti-inflammatory IL10 [16], which often occurs in the setting of sympathetic nervous system activation [17]. IL10 blocks the inhibitory effect of IL1β on post-tetanic potentiation [18].

We hypothesize that interleukin effects on cognitive function are characterized by certain selectivity and mostly target long-term memory.

The aim of this study was to assess associations between cognitive processes and the dynamics of pro- and anti-inflammatory interleukins in patients with CCI.

## METHODS

The study recruited 31 male and 63 female patients with CCI aged 42 to 85 years (the mean age was 65.4 ± 9.1 years). Pathomorphologically, cognitive decline in patients with CCI is characterized by the presence of diffuse and multiple lacunar lesions in the subcortical white matter and the cortex; subcortical defects are often associated with either cerebral atherosclerosis or lipohyalinosis of small penetrating arteries supplying deeper brain regions. Etiologically, CCI has a variety of causes, including atherosclerosis, high blood pressure and hypertensive heart disease, venous insufficiency, diabetic angiopathy, vasculitis of various etiology, hematologic disorders, etc. Our patients with CCI and cognitive decline differed in the extent of memory impairment, ability to work, irritability, brainstem symptoms, etc. The following inclusion criteria were applied: stage I–II dyscirculatory encephalopathy (early or subcompensation stage according to the classification by Levin OS [19–20]); right-handedness; MoCa scores of ≥ 26 (patients who scored < 26 were included in the study if they were not demented and did not need daily care). Exclusion criteria: pronounced dementia (≥ 1 points on the Clinical Dementia Rating Scale); a history of acute cerebrovascular accidents; traumatic brain injury; severe cardiac or metabolic (type 2 diabetes mellitus) pathology; renal insufficiency; uncompensated thyroid dysfunction.

The patients underwent a battery of tests to assess their cognitive function. The tests were performed in strict sequence. The first task was an alphabetic version of the N-back test: the patients were asked to find all occurrences of a specified two-letter combination in a text without space characters in 3 minutes. This test is based on the Kirchner n-back task with $n = 1$. As a rule, healthy subjects are able to complete this task without or with only one mistake.

The N-back test was followed by a verbal fluency test: the patients were asked to name as many words as possible starting with each of the specified Russian letters (C, K and A). The number of words was summarized and averaged.

The next test initially proposed by Luria aimed to assess verbal memory. First, the patients were asked to memorize and recall 10 words (each series of words was repeated 5 times). Then, the patients counted backwards from 100 by sevens. Finally, the patients were asked again to reproduce the memorized words. Immediate and delayed scores were counted. Healthy subjects were able to remember 9–10 words in the immediate recall test, made no mistakes in the subtraction test, and reproduced 8–10 words in the delayed recall test.

Overall cognitive function and patient eligibility for the study were assessed using the Montreal cognitive assessment scale (MoCa).

Salivary interleukins were measured before and after cognitive tests by means of a sandwich ELISA. IL10 was measured using eBioscience reagents (Bender MedSystems; Austria); IL1β and IL6 concentrations were determined using reagents by Vector-Best (Russia). The detection range was from 1 to 2,000 pg/mL. Assay calibrators were purchased from the

**Table 1.** Statistical characteristics of interleukin levels before and after cognitive tests

| Variable | *n* | Mean, pg/ml | Standard deviation, pg/ml | Standard error, pg/ml | *p* |
|---|---|---|---|---|---|
| IL1β, before | 94 | 584.9468 | 275.1798 | 28.38263 | < 0.000001 |
| IL1β, after | 74 | 678.9865 | 272.2785 | 31.65174 | < 0.000001 |
| ILβ, shift | 74 | 101.6216 | 170.5874 | 19.83039 | 0.000002 |
| IL6, before | 94 | 152.5894 | 66.2574 | 6.83393 | < 0.000001 |
| IL6, after | 74 | 191.2527 | 88.2664 | 10.26076 | < 0.000001 |
| IL6, shift | 74 | 32.8459 | 52.4934 | 6.10223 | 0.000001 |
| IL10, before | 92 | 0.9592 | 0.1949 | 0.02032 | < 0.000001 |
| IL10, after | 74 | 0.9159 | 0.2505 | 0.02912 | < 0.000001 |
| IL10, shift | 74 | –0.0177 | 0.2047 | 0.02379 | 0.459261 |

**Note:** *p* — level of statistical significance.

manufacturers of the reagent kits. All assays were performed in duplicates in a VICTOR 2 plate reader (Perken Elmer; USA); samples with low and high analyte content were used for control. Salivary specimens were collected before and after cognitive tests using a previously published protocol [21]. The patients were asked not to drink alcohol in the week preceding saliva collection and to abstain from tea and coffee one hour before saliva collection. Ten minutes before sample collection, the patients rinsed their mouths with water. Saliva was spat into a test tube of at least 1.5 m in volume. Saliva specimens were collected 10 min before cognitive tests and no later than 10 min after the tests were completed. Specimens were tested for blood contamination using ELISA. Contaminated specimens were excluded from the analysis.

The obtained data were processed in Statistica-12 (Dell; USA). Normality of distribution was assessed using the Kolmogorov-Smirnov test. Arithmetic means, standard deviations, standard errors and variance were calculated; one-way ANOVA and correlation analysis were applied. Non-parametric Spearman's rank correlation was applied to compare variables with non-normal distribution.

One-way ANOVA was applied to investigate associations between interleukin reactivity to cognitive stress and patient scores. For that, the patients were divided into groups based on whether changes in interleukin concentrations were below or above the average level. Because average changes in IL10 concentrations did not differ from zero, data on IL10 were arranged into a negative and positive shift groups.

## RESULTS

The dynamics of IL1β, IL6 and IL10 levels did not differ significantly between the two sexes, therefore interleukin response was analyzed in the mixed group of male and female participants. Baseline interleukin concentrations in salivary specimens were correlated with age: $r = 0.25$, $n = 94$, $p = 0.031$ and $r = 0.21$, $n = 94$, $p = 0.041$, respectively. Changes in IL1β and IL6 concentrations following cognitive tests were not correlated with age; for IL10, this correlation was weak: $r = 0.24$, $p = 0.042$, $n = 74$.

Statistical characteristics of interleukin levels is provided in Table 1.

For IL10, the delta value did not differ significantly from zero, in contrast to other mean values.

The Kolmogorov-Smirnov test showed that the distribution of a random variable for IL1β and IL10 did not differ from normal, in contrast to IL6. Data on IL1β and IL10 were subjected to one-way ANOVA. The cumulative data on the dynamics of IL1β and IL10 were arranged in 2 groups: above and below average values. Mean shifts in IL1β and IL10 concentrations in these groups are shown in Table 2. The groups did not differ in terms of age.

Changes in IL1β concentrations in group 1 did not differ significantly from zero, while in other cases the shifts were statistically significant (see Table 2). Differences between the groups were significant for IL1β and IL10: $F = 81.6$ at $p < 0.000001$ and $F = 147.8$ at $p < 0.000001$, respectively.

The next step involved the analysis of the associations between the measured cognitive parameters and shifts in IL1β and IL10 concentrations after cognitive tests.

A positive shift (increase) in IL1β concentrations demonstrated by group 2 was correlated with less successful performance during cognitive tests (Table 3, Fig. 1).

Changes in anti-inflammatory IL10 were also associated with cognitive function, but the established association was opposite to that discovered for proinflammatory IL1β and IL6: the more pronounced response of IL10 was correlated with better performance during cognitive tests (Table 4; Fig. 2).

"Detected patterns" refer to the specified 2-letter combinations found by the patients in the alphabetic N-back test.

Fig. 2 provides a graphic representation of differences in cognitive indicators associated with shifts in IL10 concentrations.

**Table 2.** Shifts in IL1β and IL10 salivary concentrations following cognitive stress in 2 groups of patients

| | Mean IL1β, pg/ml | Standard error IL1β, pg/ml | Mean IL10, pg/ml | Standard error IL10, pg/ml |
|---|---|---|---|---|
| Group 1 (below average) | –0.34 ($n = 44$) | 14.7 ($p = 0,98$) | –0.10 ($n = 40$) | 0.02 ($p < 0,00001$) |
| Group 2 (above average) | 251.2 ($n = 30$) | 26.8 ($p < 0,00001$) | 0.13 ($n = 33$) | 0.02 ($p < 0,00001$) |

**Note:** *p* — level of statistical significance.

**Table 3.** Associations between shifts in IL1β concentrations and cognitive function

| IL1β | *n* | *F* | *p* |
|---|---|---|---|
| Subtraction (100–7) | 63 | 11.49 | 0.001 |
| Delayed recall (based on Luria tests) | 65 | 6.84 | 0.01 |

**Note:** *F* — Fisher's coefficient; *p* — level of significance.

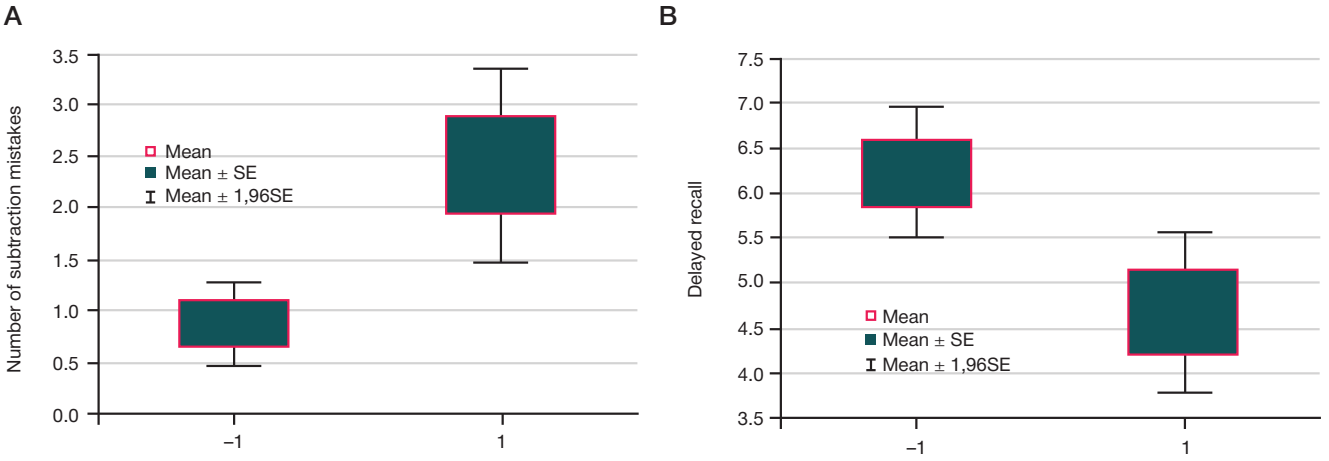


**Fig. 1.** Performance during cognitive tests in groups 1 and 2 of IL1β shifts. –1 — group 1, 1 — group 2. **A**. Number of subtraction mistakes (100–7). **B**. Delayed words recall in the Luria test

Comparison of figures 1 and 2 reveals that cognitive function is negatively correlated with pro- and anti-inflammatory interleukin response.

The Kolmogorov-Smirnov test demonstrated that IL6 shifts did not conform with normal distribution; therefore, associations between IL6 shifts and cognitive function were measured by Spearman's rank correlation used for nonparametric samples. Spearman's correlation coefficient for the subtraction test and IL6 shifts significantly differed from zero ($r = 0.26$; $n = 62$; $p = 0.042$). Similar to IL1β, the higher number of mistakes was associated with a higher increase in IL6 levels.

Some cognitive indicators were insensitive to changes in interleukin concentrations, including MoCa, verbal fluency and immediate recall (Luria) scores.

## DISCUSSION

Reported physiological salivary concentrations of interleukins in general and IL10 in particular vary across studies [22–23]. In people of advancing age, salivary interleukin levels are affected by a multitude of factors, from the past history of diseases to the quality and number of dentures. So, it may be reasonable to analyze and compare relative indicators within one group of patients; some authors rely on the ratios of different proinflammatory cytokines, for example, IL1β to IL10, or measure interleukin response to stress. Proinflammatory interleukins, including IL10, are elevated in the setting of stress [23]. Cognitive tests cause an elevation of IL1β and IL6 levels, but induce no changes in IL10 concentrations. The absence of IL10 dynamics in response to cognitive tests can be explained by weak stress limited to sympathoadrenal activation.

Many proteins originally detected in the immune system are also found in neuronal synapses participating in cognitive processes [24]. Our work demonstrates an increase in salivary interleukins during cognitive tests in patients with CCI. Higher expression of proinflammatory cytokines was correlated with worse performance in cognitive tests. Patients with CCI had more pronounced inflammation in comparison with healthy individuals [25]. In such patients, neuronal activation enhances secretion of proinflammatory interleukins, but the intensity of this enhancement depends on the severity of the disease. It is known that sympathetic neurons secrete IL6 and produce paracrine or autocrine signals in response to the presence of the soluble IL6 receptor [26]. So, hypothetically neuronal activation in patients with CCI will be accompanied by an increase in the levels of proinflammatory interleukins. It is known that proinflammatory cytokines disrupt normal neuronal function in an adult brain by exerting a direct effect on neurons or by triggering mechanisms mediated by non-neuronal cells (like microglia or astrocytes) [27]. Patients with CCI will inevitably experience mild stress during cognitive testing because they perceive cognitive tasks as psychoemotional strain. Stress might trigger cytokine secretion (IL1β, IL6, etc.) On the one hand, physiological levels of IL1β are indispensable to learning and memorizing; on the other hand, elevated IL1β is detrimental to cognitive performance. Increased production of IL6 in the setting of stress exacerbates inflammation by stimulating IL1β secretion in the brain and thus promotes anxiety [28]. Effects of IL1β on memory and learning are often associated with its effects on synaptic mechanisms of long-term potentiation in the hippocampus. IL1β induces hyperpolarization and modulates synaptic inhibition of preoptic and frontal hypothalamic neurons; it also neutralizes long-term depression of synaptic transmission in the hippocampus. The cytokine network consisting of IL1β, IL18, IL6 and TNFa interacts with neurons during long-term potentiation and learning. Blockade of endogenous IL1β is beneficial to memory formation [29].

Effects exerted by IL10 are opposite to those of proinflammatory cytokines. It is known that IL10 is involved in cytokine regulation (feedback loop) and inhibits effects of proinflammatory cytokines. This interleukin demonstrated no significant dynamics during cognitive stress, which might be explained by its low involvement in cognitive processes as such. The impact of IL10 on cognitive function might be mediated by its effects on proinflammatory IL1β and IL6 [30].

Cognitive performance correlated with changes in interleukin concentrations was reflective of memory retention, i.e. plasticity processes. Notably, immediate word recall in the Luria test was not associated with changes in the levels of proinflammatory interleukins, whereas delayed word recall was associated with

**Table 4.** Associations between IL10 response and cognitive function

| IL10 | *n* | *F* | *p* |
|---|---|---|---|
| Subtraction (100–7) | 63 | 6.83 | 0.01 |
| Detected patterns | 67 | 5.16 | 0.026 |

**Note:** *F* — Fisher's coefficient; *p* — level of significance.

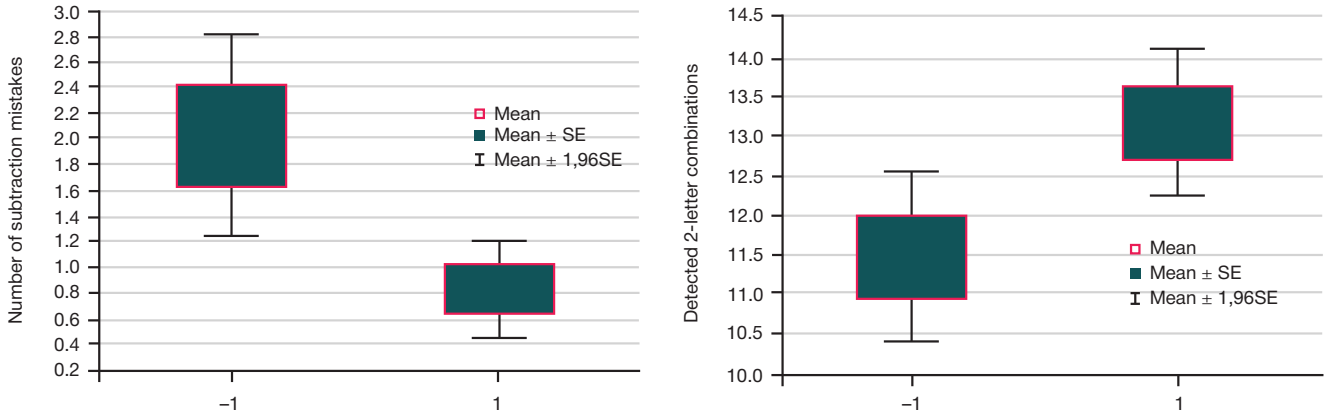


**Fig. 2.** Cognitive test performance in patients with negative or positive IL10 response. The legend is the same as in Fig. 1

changes in IL1β. This confirms the potential role of IL1β in inhibiting post-tetanic potentiation. Perhaps, this mechanism underlies many effects of proinflammatory interleukins.

Our findings may be clinically useful. Current recommendations for people of advancing age struggling with chronic vascular diseases point to the benefits of intense cognitive load (doing crossword puzzles, studying foreign languages, etc.) Our findings suggest that in some cases cognitive load can lead to elevated proinflammatory cytokines and contribute to oxidative stress, which raises questions about the benefits of such recommendations. Further research could be aimed at exploring biological markers of proinflammatory interleukins that could be conveniently used for controlling cognitive load and monitoring the response of proinflammatory cytokines.

## CONCLUSION

The dynamics of pro- and anti-inflammatory interleukins IL1β, IL6 and IL10 were associated with performing cognitive tasks in patients with CCI. Cognitive stress was accompanied by the reliable increase in IL1β and IL6 in the mixed sample of men and women. Changes in the levels of all studied cytokines reflected performance scores. Elevated salivary IL1β and IL6 were associated with worse performance in the subtraction test (100–7); heightened IL1β was associated with poor scores in the delayed recall test. Patients with positive dynamics of salivary IL10 made fewer mistakes in the subtraction test and did better in the N-back test than patients with negative IL10 dynamics. Thus, the significant increase in the levels of salivary proinflammatory cytokines induced by cognitive stress was accompanied by a decline in cognitive function in delayed memory tests, while similar changes in anti-inflammatory IL10 were associated with better cognitive performance. MoCa, verbal fluency and some other scores were not associated with changes in interleukin levels. Further discussion is needed to understand the mechanisms underlying interleukin effects on cognitive function. There is a tight link between the levels of proinflammatory cytokines and some types of mental activity, suggesting that patients with CCI should be monitored for the levels of proinflammatory cytokines during cognitive tasks.

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# A METHOD FOR RAPID GENERATION OF MODEL INTESTINAL BARRIERS *IN VITRO*

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To increase the efficiency of drug development process, it is important to improve performance of preclinical experiments. A major drawback of the currently used *in vitro* intestinal barrier models is that it takes a significant time to obtain functional enterocyte monolayers with formed tight junctions. In this work, we have optimized various parameters such as cell density and different coatings, for a more rapid and efficient producing Caco-2 cell monolayers suitable for further experiments. In vivo microscopy and impedance spectroscopy were used to monitor cells state under various conditions. To determine possible biological mechanisms affected by exposure to various protein substrates, the transcriptomic analysis was applied. It was shown that collagen IV coating of the cell growth substrate significantly increased the rate of proliferation and migration of Caco-2 cells. This effect allows forming a functional monolayer of epithelial cells with tight junctions within 24 hours. Optimally, the initial cell density should be 90,000 to 200,000 cells/cm$^2$. It was observed that collagen IV was poorly expressed by Caco-2 cells while the collagen IV receptor was expressed at a relatively high level in these cells. Laminin-332, another basement membrane component, was found to have no significant effect on times of formation of functional epithelial monolayers. Thus, using the optimal parameters determined in this study allows to significantly improve efficiency of using the *in vitro* intestinal barrier models.

**Keywords:** collagen IV, barrier tissues, laminin-332, TEER, extracellular matrix, Caco-2, impedance spectroscopy

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**Author contribution:** Nikulin SV — cell culture work, preparation of samples for transcriptome analysis, data analysis, manuscript preparation; Poloznikov AA — analysis of transcriptome data, manuscript preparation; Sakharov DA — supervision, manuscript preparation.

**Compliance with ethical standards:** the study complies with the 1964 Helsinki Declaration and its later amendments.

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# МЕТОДИКА УСКОРЕННОГО ПОЛУЧЕНИЯ МОДЕЛЬНЫХ КИШЕЧНЫХ БАРЬЕРОВ *IN VITRO*

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Для повышения эффективности разработки лекарственных препаратов необходимо увеличивать производительность экспериментов, проводимых на доклинической стадии. Существенным недостатком используемых на сегодняшний день *in vitro* моделей кишечного барьера является скорость образования функционального монослоя энтероцитов со сформировавшимися плотными контактами. Целью работы было провести комплексный подбор параметров (различные покрытия и плотность клеток) для быстрого и эффективного получения пригодного к проведению экспериментов монослоя клеток Caco-2. Для оценки состояния культуры клеток при различных условиях применяли прижизненную микроскопию и импедансную спектроскопию. Для определения возможного биологического механизма действия различных белковых субстратов на энтероциты использовали транскриптомный анализ. Показано, что покрытие субстрата для роста клеток коллагеном IV существенно повышает скорость пролиферации и миграции клеток линии Caco-2. Такое воздействие позволяет в течение 24 ч сформировать функциональный монослой эпителиальных клеток с плотными контактами. С целью получения пригодного для проведения экспериментов кишечного барьера *in vitro* в течение 24 ч начальная плотность клеток должна лежать в диапазоне 90–200 тыс. клеток на 1 см$^2$. Обнаружено, что клетки Caco-2 слабо экспрессируют коллаген IV, при этом рецепторы к коллагену IV у данных клеток экспрессированы на достаточно высоком уровне. Показано также, что еще один компонент базальной мембраны ламинин 332 не оказывает заметного влияния на скорость формирования функционального монослоя эпителиальных клеток. Таким образом, в работе были определены оптимальные параметры, позволяющие существенно повысить производительность экспериментов с *in vitro* моделями кишки.

**Ключевые слова:** коллаген IV, барьерная ткань, ламинин 332, TEER, внеклеточный матрикс, Caco-2, импедансная спектроскопия

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Intestine is an important organ where food is digested, nutrients and drugs are absorbed into the blood, and interaction of microorganisms with host cells occurs. One of the main intestine functions is the barrier function. Intestinal barrier dysfunction has been implicated in numerous health conditions including inflammatory and autoimmune diseases [1]. Cancer chemotherapies often lead to gut barrier dysfunction [2].

The pathways for transporting molecules through the intestinal barrier include active transport, passive diffusion through the cell membrane, and passive diffusion through the intercellular spaces in between epithelial cells. Epithelial tight junctions are the key structures regulating paracellular trafficking of molecules [3]. Tight junctions are multi-protein complexes located close to the apical surfaces of the epithelial cells

and consisting of different cytoplasmic and transmembrane proteins such as occludin and claudins. Due to dynamic changes of tight junctions, intestinal permeability can change rapidly [4]. The tight junction protein complexes are dynamically modulated by various signaling molecules such as kinases (c-Src, c-Yes, etc.) and cytokines (PHOα, interferron γ, etc.) [5]. Since tight junctions play an important role in the functioning of the intestine both in normal and in pathology, it is crucial to develop appropriate *in vitro* models to evaluate various effects on tight junctions [6, 7].

There are a few different techniques for measuring the paracellular permeability reflecting the integrity of tight junctions. In particular, measurements of molecular marker concentrations on both sides of the barrier are widely used [8, 9]. However, this method is time-consuming and not easy to use. Alternatively, transepithelial electrical resistance (TEER) can be used to monitor changes in epithelial cell culture integrity [10]. Using this quantitative technique takes researchers less time and is well suitable for the high-throughput screening; TEER measurement accuracy can be increased by applying impedance spectroscopy [11, 12].

Currently, Caco-2 cell line is widely used to model the intestinal barrier *in vitro* [13]. The Caco-2 cells were originally derived from a colon adenocarcinoma — the cells turned out to be able to spontaneously differentiate into a monolayer of cells with many properties typical of the small intestine epithelium [14, 15]. Caco-2 cells are known to form tight junctions as they grow and differentiate, and the cell density of their tight junctions is higher than in normal colon [14, 15]. This makes Caco-2 cell line a valuable transport model system for studying tight junction processes. However, for a wider application of this cell model, it is necessary to improve experiment performance. This can be partially achieved by cell culture automation, e.g. using microfluidic chips [16–18] however, it would be very helpful to optimize cultivation conditions to make it possible to have ready for use cell models more rapidly.

Our study was aimed at determining the optimal cultivation conditions for Caco-2 cells that would allow obtaining functional cell monolayers with tight junctions as quickly as possible.

## METHODS

Caco-2 cells were obtained from the Institute of Cytology of the Russian Academy of Sciences (Russia). The cells were cultured in MEM (Gibco; USA) supplemented with 20% fetal bovine serum (Gibco; USA) and 1% penicillin-streptomycin (Gibco; USA). Cells were maintained at 37 °C in an incubator with 5% $CO_2$. Subcultivation was performed every 2 or 3 days according to the standard procedure using a trypsin EDTA solution (PanEco; Russia). Cell counts were performed using Automated Countess Cell Counter (Gibco; USA) according to the manufacturer's recommendations.

Cells were seeded onto 1.0 μm pore-size polyester inserts (PET) HTS Transwell-96 (Corning; US). Before seeding, a part of membranes was coated with laminin-332 (BioLamina; Sweden) and another part was coated with type IV collagen (Imtek; Russia): 30 μl of proten in DPBS solution with 10 μg/ml concentration was added to each membrane insert. Then, 96-well plates with membrane inserts were incubated at 4 °C for 24 h. After incubation, protein solutions were removed from all wells, and each well was washed 3 times with 100 μl of DPBS solution.

Right before cell seeding, HTS membrane inserts were filled with culture medium (50 μl into the upper chamber and 235 μl in the lower chamber) and incubated in a cell incubator for one hour. Then different quantities of cells were added to each membrane insert (6,250, 12,500 or 25,000 cells per well) to 50 μl of the culture medium to achieve initial cell density of 43,700, 87,400 and 174,800 cells per cm2, respectively. Each experiment was carried out in three repeats. 96-well plates with membrane inserts were incubated in a cell incubator throughout the experiment.

To determine TEER values in 24 h and 48 h from the onset of the experiment, impedance spectra measurements were made using an impedance spectrometry system (BioClinicum; Russia) and original electrodes (BioClinicum; Russia). TEER values were calculated following the previously described equivalent electrical circuit [19] using CEISA Impedance fitting (BioClinicum; Russia). Statistical analysis of obtained data was carried out using the programming language R 4.0 with the integrated development environment RStudio 1.1 (RStudio PBC; USA). To assess the statistical significance of the observed TEER differences, we used a three-factor (substrate type, initial cell density, and time from the onset of the experiment) Analysis of Variance (ANOVA) Tukey-adjusted for multiple comparisons. Differences were considered significant at $p < 0.05$.

To obtain microscopic images of Caco-2 cells on various substrates, some of the wells in 96-well plates (Corning; US) was coated with laminin-332 (BioLamina; Sweden) and type IV collagen (Imtek; Russia) using the protocol similar to membrane inserts coating. 50 μl of protein solution per well was used for coating. Each well was then loaded with 100 μL of cell suspension in a complete culture medium with concentration of 100,000 and 200,000 cells per 1 ml (corresponding to the initial cell densities 31,300 and 62,600 per $cm^2$). The plates were further incubated in a cell incubator. Microscopic images were obtained using PrimoVert inverted microscope (Carl Zeiss; Germany).

To obtain fully differentiated Caco-2 cells, culturing was carried out according to the procedure described in [11, 19]. Analysis of gene expression levels in differentiated and undifferentiated Caco-2 cells was carried out using microchips GeneChip Human Genome 1.0 ST (Affymetrix; USA) [20]. Cells were lysed with QIAzol lysis buffer (Qiagen; Germany). Total RNA was isolated using miRNeasy Mini Kit (Qiagen; Germany) according to the manufacturer's protocol. Concentration of isolated total RNA was measured using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific; USA). RNA quality was assessed using the Experion system (Bio-Rad; USA). For hybridization on microchips, 500 ng of each RNA sample was used. The experiment was carried out in three repeats.

The results were processed using TAC 4.0 software (Thermo Fisher Scientific; USA). The statistical significance of differences in expression levels between differentiated and undifferentiated Caco-2 cells was assessed by single factor variance analysis (ANOVA) adjusted with Benjamini–Hochberg. The significance threshold was 0.05. Genes with an expression level less than 6.0 on the Affymetrix logarithmic scale were considered not expressed.

## RESULTS

Based on TEER measurements (Fig.1) for membrane inserts made 24 h after the cell seeding, TEER was shown to increase as initial cell density increased. E.g., for uncoated control membrane inserts, TEER values were found to be about 118 $\Omega.cm^2$ higher for the maximum initial cell density compared to the minimum initial cell density ($p < 0.001$). No significant differences were found between intermediate and minimum

initial cell densities ($p$ = 1). Similar results were observed for the laminin-332 coated wells. In case of laminin-332 coating, no significant TEER differences were found as compared to uncoated control wells for all tested initial cell densities ($p$ = 1).

In case of type IV collagen coating, the results differed significantly. Considerable differences were observed between the minimum tested initial cell density of 43,700 cells per cm$^2$ and 87,400 cells per cm$^2$ (an increase of 147 Ω.cm$^2$; $p$ < 0.001), as well as between the minimum tested initial cell density of 43,700 cells per cm$^2$ and 174,800 cells per cm$^2$ (an increase of 208 Ω.cm$^2$; $p$ < 0,001). Thus, in case of type IV collagen coating, the dependence of TEER values on initial cell densities was more significant. For the initial densities 87,400 cells per cm$^2$ and 174,800 cells per cm$^2$, a strong increase in TEER (188 Ω.cm$^2$ and 142 Ω.cm$^2$, respectively) was also found in type IV collagen coated wells as compared to control polyester wells ($p$ < 0,001 in both cases).

After 48 h from the beginning of the experiment, TEER values in the uncoated control wells increased significantly for the 87,400 initial cell density and for the 174,800 initial cell density compared to the measurements taken 24 h after cell seeding (202 Ω.cm$^2$ and 110 Ω.cm$^2$, respectively; $p$ < 0.001 and $p$ = 0.002, respectively). At the same time, in case of minimal 43,700 initial cell density, there was no significant difference between TEER values for 48 h measurements and 24 h measurements ($p$ = 1).

In the case of laminin-332 coating, similar dynamics was observed comparing 48 h vs. 24 h TEER values: for 43,700 cells/cm$^2$ initial density, no significant difference was found ($p$ = 0.1); for 87,400 cells/cm$^2$ initial density, TEER values significantly increased by 165 Ω.cm$^2$ ($p$ < 0.001) however, for the 174,800 cells/cm$^2$ initial density, the observed growth by 83 Ω.cm$^2$ was not statistically significant ($p$ = 1). On the other hand, in case of type IV collagen coated wells, a significant TEER increase (175 Ω.cm$^2$) was reported in case of the minimum initial cell density only ($p$ < 0.001) while no significant differences between related 48 h and 24 h measurements were found for 87,400 cells/cm$^2$ and 174,800 cells/cm$^2$ initial densities ($p$ = 0.2 and $p$ = 1, respectively).

Interestingly, for uncoated wells and for laminin-332 coated wells, 48 h TEER values were still dependent on the initial cell densities. In case of uncoated wells, 48 h TEER values were higher as compared to the 43,700 cells/cm$^2$ initial density for both 87,400 initial density (172 Ω.cm$^2$; $p$ < 0.001) and 174,800 cells/cm$^2$ initial density (188 Ω.cm$^2$; $p$ < 0,001). Similar results were obtained in case of laminin-332 coating. At the same time, 48 h TEER values in the wells coated with type IV collagen were not dependent significantly on the initial cell density ($p$ = 1 in all cases) and were more than 200 Ω.cm$^2$.

In order to evaluate the effect of type IV collagen/laminin-332 substrate coating on cell morphology and growth rate, live-cell imaging of Caco-2 cells was performed in 24 hours after cell seeding (Fig. 2). It turned out that for control uncoated wells and laminin-332 coated wells, 100% confluence was not achieved at the considered initial cell densities. However, in case of type IV collagen coating, about 80% of surface was covered in wells with 31,300 cells/cm$^2$ initial density, and a monolayer was formed in wells with 62,600 cells/cm$^2$ initial density. In type IV collagen coated wells, an increased amounts of elongated spindle shaped cells were observed compared with control wells and laminin-332 wells.

Transcriptomic analysis showed that in both differentiated and undifferentiated Caco-2 cells, expression of all type IV collagen chains was at a relatively low level (< 7 according to Affymetrix logarithmic scale) (see Table). Moreover, in the process of cell differentiation a slight decrease in expression of COL4A1 and COL4A6 genes was observed.

Integrins α1β1 and α2β1 are known to be the main receptors of type IV collagen [21]. Transcriptomic analysis showed that *ITGB1* gene (β1-integrin chain) was expressed at a sufficiently high level in both differentiated and undifferentiated Caco-2 cells (10.0 and 10.1 in Affymetrix logarithmic scale respectively), and its expression did not significantly change during the process of differentiation ($p$ = 0.4). ITGA1 gene (α1-chain integrin) was also expressed in both differentiated and undifferentiated Caco-2 cells (8.6 and 9.0 in Affymetrix logarithmic scale, respectively) however, its expression slightly decreased in differentiated cells (1.3 times lower; $p$ = 0,002).

Similar results were obtained for ITGA2 gene (α2 integrin chain): the average expression values were 9.2 for differentiated cells and 9.5 for undifferentiated cells while a small 1.3 time decrease of expression in differentiated cells was statistically significant ($p$ = 0,04).

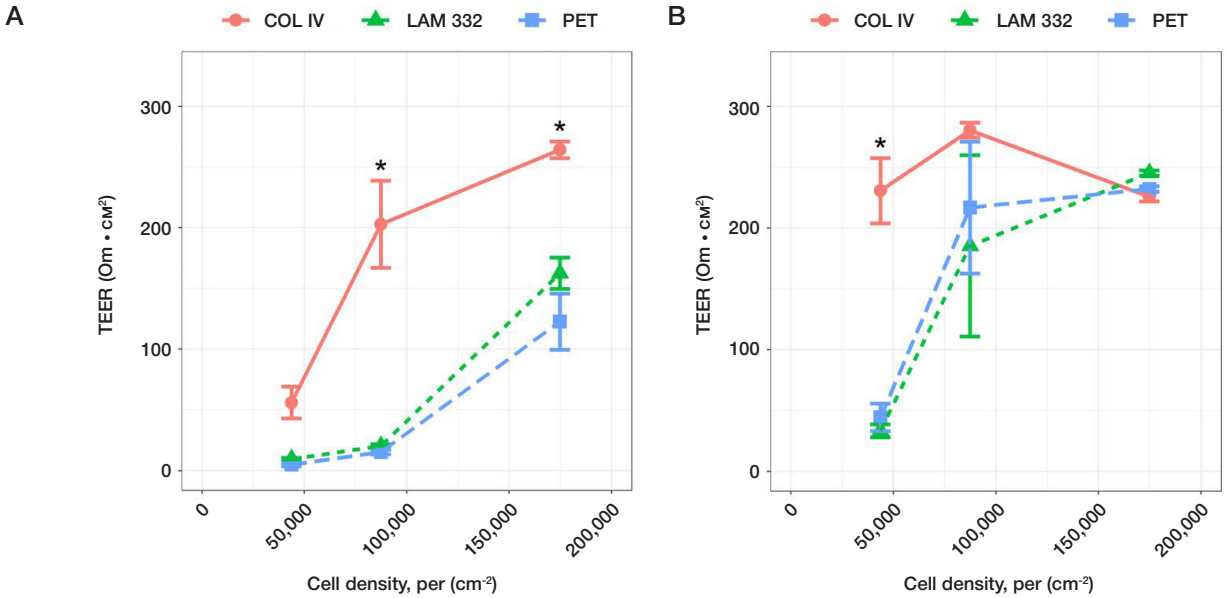


**Fig. 1.** TEER values in 24 hours (**A**) and 48 hours (**B**) from the onset of experiment. Statistically significant differences of coated membranes compared to uncoated membranes are marked with * COL IV — type IV collagen; LAM 332 — laminin-332; PET — uncoated

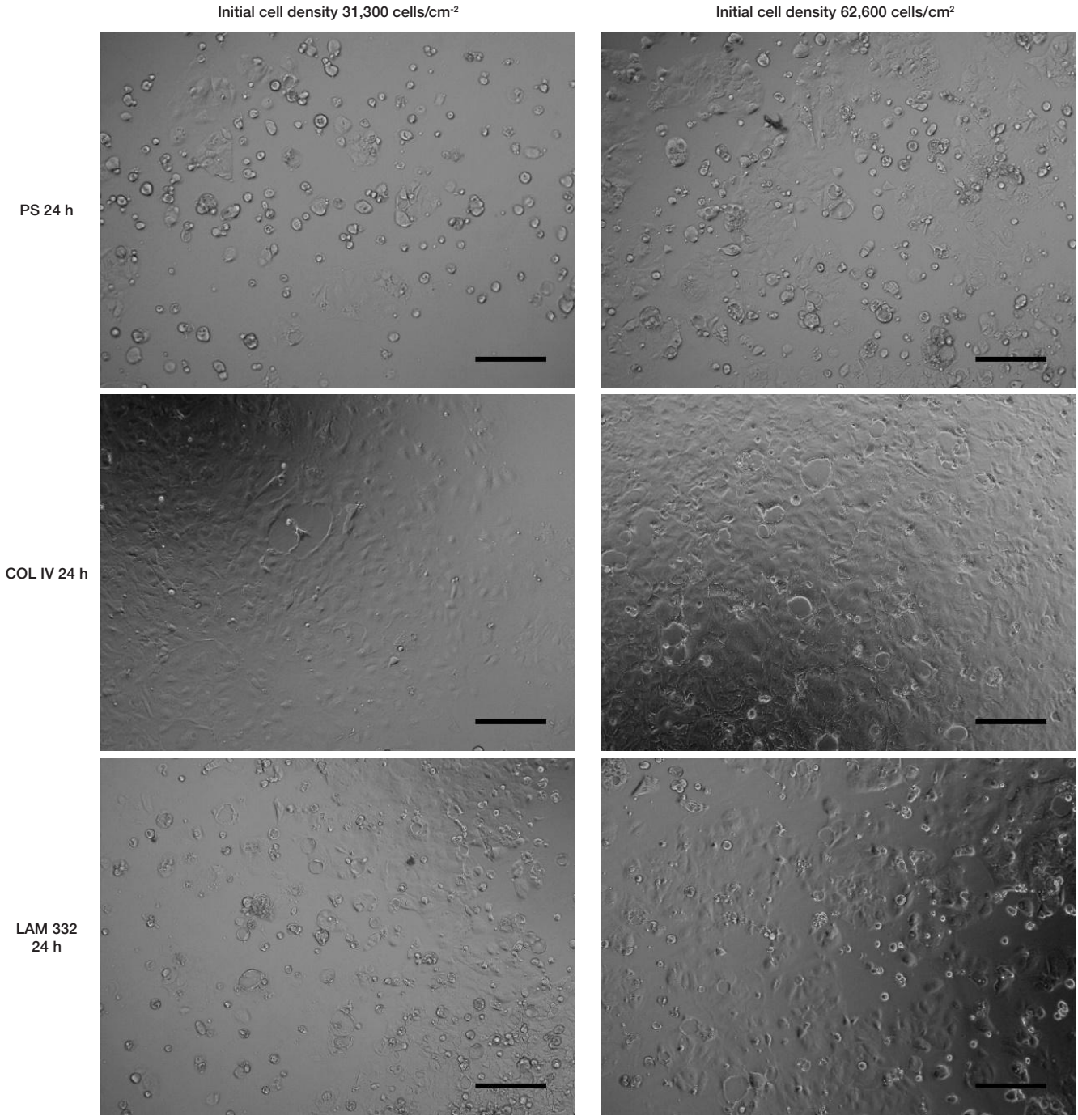


**Fig. 2.** Caco-2 monolayer images in 24 h (scale segment bar is 200 μm) COL IV — type IV collagen; LAM 332 — laminin-332; PET — uncoated

## DISCUSSION

The experiment showed that type IV collagen was the most effective substrate significantly accelerating the process of formation of functional epithelial intestinal barrier. Some chains of type IV collagen (α1, α2, α5 and α6) are known to be involved in formation and development of the intestine, and the proteins can be synthesized in both epithelial and mesenchymal cells [22]. Based on the results of transcriptomic analysis, Caco-2 cells cannot sufficiently synthesize type IV collagen while at all stages, the cells express receptors for type IV collagen which indicates that type IV collagen may have effect on the cell processes.

Based on the obtained cell images, it can be concluded that type IV collagen promotes both proliferation and migration of Caco-2 cells. To date, an extended data has been accumulated showing that type IV collagen stimulates both adhesion and migration of Caco-2 cells [23-25]. Type IV collagen is also known to stimulate proliferation of other epithelial cell types [26, 27]. Thus, the obtained results are well consistent with the data available from earlier studies.

Effect of type IV collagen on Caco-2 monolayer TEER values has already been studied, and it was found that a few days after cell seeding, TEER values were significantly higher in the wells coated with type IV collagen however, the dynamics of TEER changes during the first few days of cell culturing was not determined [28]. In the present study, it was shown that type IV collagen affects not only the TEER values but also the time periods when TEER values (about 200 Ω.cm$^2$) [9] become sufficient for barrier model experiments — it can be really achieved within 24 h period. The obtained results can be easily

**Table.** Type IV collagen expression levels in differentiated and undifferentiated Caco-2 cells (Affymetrix logarithmic scale)

| Gene | Average expression level in differentiated Caco-2 cells | Average expression level in undifferentiated Caco-2 cells | Difference | FDR *p* |
|---|---|---|---|---|
| *COL4A1* | 6.15 | 6.39 | –1.18 | 0.0235 |
| *COL4A2* | 6.7 | 6.74 | –1.02 | 0.3724 |
| *COL4A3* | 5.92 | 5.96 | –1.03 | 0.3166 |
| *COL4A4* | 5.66 | 5.67 | –1.01 | 0.5068 |
| *COL4A5* | 6.54 | 6.74 | –1.15 | 0.0904 |
| *COL4A6* | 5.91 | 6.29 | –1.3 | 0.0302 |

applied both to other static *in vitro* models of barrier tissues and to dynamic microfluidic systems [29, 30].

## CONCLUSION

In our experiments, we found that using the type IV collagen coated substrate for cell growth significantly increased the rate of proliferation and migration of Caco-2 cells. This made it possible to obtain functional monolayers of epithelial cells with tight junctions contacts within 24 hours. The optimal initial cell densities were also determined. For obtaining an intestinal barrier model *in vitro* during 24-hour period, the initial cell density should be 90,000 to 200,000 cells per $cm^2$. Type IV collagen was found to be poorly expressed by Caco-2 cells while the expression levels of type IV collagen receptor proteins were relatively high in the cells. Another basement membrane component laminin-332 was shown to have no significant effect on the rate of formation of functional epithelial cell monolayers.

The findings can be used to further improve the performance of experiments involving *in vitro* intestinal barrier models both in static conditions and in microfluidic systems.

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## LOCAL ANTIOXIDANT EFFECT OF ORIGINAL DERMAL FILM WITH MELATONIN IN THERMAL INJURY

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Oxidative stress (OS) escalation associated with thermal trauma (TT) and pleiotropic effects of melatonin (MT) suggest a study of protective properties of the latter when applied as part of a novel dermal film (DF) to skin burns. This work aimed to assess the content of OS markers in the skin subjected to experimental TT and treated with DF with MT. Third A degree TT (area of 3.5%) were modeled by immersing a patch of skin in boiling water. Twelve cm$^2$ of DF with 5 mg/g of MT were applied daily for 5 days. The parameters calculated were wound's area and epithelializatiohon rate. The products monitored in the burn wound were lipid peroxidation (LPO) products in heptane and isopropanol phases of the lipid extract and protein oxidative modification (POM) products, the modification being spontaneous and metal-dependent. With TT in the wound, the content of secondary and end LPO products in heptane and isopropanol phases increased on the 5th and 10th days; the total content of POM products grew on the 5$^{th}$ day (primary products, neutral) and on the 10$^{th}$ day (primary and secondary products, neutral). Application of DF to a TT wound reduced the burn area, increased the epithelialization rate (by the 10$^{th}$ day, the median went from 1.90% to 6.57%; $p < 0.05$), reduced the content of secondary and end LPO products in isopropanol phase (by the 10$^{th}$ day, the median went from 0.007 to 0.004 u.o.i; $p < 0.05$), reduced the total content of OMP products, namely that of primary neutral products — on the 5$^{th}$ day, of primary and secondary neutral products — on the 10$^{th}$ day. With TT present in the context of MT application, the burn area showed presence of secondary LPO products in heptane and isopropanol phases, LPO end products in isopropanol phase, POM products in the wound (basic and neutral primary/secondary POM products).

**Keywords:** thermal trauma, oxidative stress, melatonin, dermal film

**Author contribution:** MV Osikov — study concept and design, integrated analysis of the data obtained, authoring, manuscript editing; EV Simonyan — experimental material collection, analysis of the data obtained; AA Ageeva — experimental material collection, statistical processing and analysis of the data obtained, authoring; YuI Ageev — experimental material collection, statistical processing and analysis of the data obtained; AA Fedosov — analysis of the results, manuscript editing; AI Sinitsky — experimental material collection, manuscript editing.

**Compliance with ethical standards:** the study was approved by the Ethics Committee of the South Ural State Medical University, Chelyabinsk (Minutes #10 of November 15, 2019), carried out in standard vivarium conditions with strict adherence to the requirements for animal keeping and care, as well as withdrawal of animals from the experiment and subsequent disposal in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental or Other Scientific Purposes (ETS № 123 of March 18, 1986, Strasbourg), EC Recommendations 2007/52/EC of June 18, 2007 outlining procedures of keeping and care for animals used for experimental and other scientific purposes, as well as the European Parliament and EU Council Directive 2010/63/EU of September 22, 2010 on protection of animals used for scientific purposes as governed by the rules of humane treatment of animals, guidelines for their withdrawal from experiments and euthanasia.

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## ЛОКАЛЬНЫЙ АНТИОКСИДАНТНЫЙ ЭФФЕКТ ОРИГИНАЛЬНОЙ ДЕРМАЛЬНОЙ ПЛЕНКИ С МЕЛАТОНИНОМ ПРИ ТЕРМИЧЕСКОЙ ТРАВМЕ

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Эскалация оксидативного стресса (ОС) при термической травме (ТТ) и плейотропные эффекты мелатонина (МТ) являются предпосылкой для изучения его протекторных свойств в составе новой дермальной пленки (ДП) при ожогах кожи. Цель работы — оценить содержание маркеров ОС в коже при экспериментальной ТТ и применении ДП с МТ. ТТ IIIA степени площадью 3,5% моделировали погружением участка кожи в кипящую воду. ДП площадью 12 см$^2$ с МТ в концентрации 5 мг/г наносили ежедневно в течение 5 сут. Вычисляли площадь раны и скорость ее эпителизации. В ожоговой ране определяли продукты пероксидного окисления липидов (ПОЛ) в гептановой и изопропанольной фазах липидного экстракта, продукты окислительной модификации белков (ОМБ) в спонтанном и металл-зависимом режимах. При ТТ в ране на 5-е и 10-е сут. увеличилось содержание вторичных и конечных продуктов ПОЛ в гептановой и изопропанольной фазах, суммарное содержание продуктов ОМБ за счет первичных продуктов нейтрального характера на 5-е сут., первичных и вторичных продуктов нейтрального характера на 10-е сут. Применение ДП при ТТ уменьшило площадь ожога, увеличило скорость эпителизации раны (на 10-е сут. по медиане с 1,90% до 6,57%; $p < 0,05$), снизило содержание в изопропанольной фазе вторичных и конечных (на 10-е сут. по медиане с 0,007 до 0,004 е.и.о.; $p < 0,05$) продуктов ПОЛ, снизило суммарное содержание продуктов ОМБ на 5-е сут. за счет первичных продуктов нейтрального характера, на 10-е сут. за счет первичных и вторичных продуктов нейтрального характера. При ТТ в условиях применения МТ площадь ожога ассоциирована с содержанием в ране вторичных продуктов ПОЛ в гептановой и изопропанольной фазах, конечных продуктов ПОЛ в изопропанольной фазе, суммарным содержанием продуктов ОМБ, содержанием первичных, вторичных, основного и нейтрального характера продуктов ОМБ.

**Ключевые слова:** термическая травма, окислительный стресс, мелатонин, дермальная пленка

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**Соблюдение этических стандартов:** исследование одобрено этическим комитетом Южно-Уральского государственного медицинского университета г. Челябинск (протокол № 10 от 15 ноября 2019 г.), выполнено в стандартных условиях вивария при строгом соблюдении требований по уходу и содержанию животных, а также выводу их из эксперимента с последующей утилизацией в соответствии с Европейской конвенцией о защите позвоночных животных, используемых для экспериментов или в иных научных целях (ETS № 123 от 18 марта 1986 г., Страсбург), Рекомендациями Европейской комиссии 2007/526/EC от 18 июня 2007 г. по содержанию и уходу за животными, используемыми в экспериментальных и других научных целях, а также Директивой 2010/63/EU Европейского парламента и совета Европейского союза от 22 сентября 2010 г. по охране животных, используемых в научных целях в соответствии с правилами гуманного отношения к животным, методическими рекомендациями по их выведению из опыта и эвтаназии.

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There are about 250,000 burn cases registered in the Russian Federation annually. About 80% of them are associated with thermal trauma (TT) [1]. The most common causes of TT are hot liquids and flame; two-thirds of patients have the burn area smaller than 10% of the body surface [2]. Studying pathophysiology of burn wounds allows finding ways to halt their progression and develop new, pathogenetically valid methods of safe necrectomy and wound closure, modern substitutes. Burn wound healing rate, adverse outcome and development of TT-related complications depend not only on the injury's area and depth, severity of inflammation, acute phase response and immune reactions, but also on the local redox status [3]. For free radicals, the key targets in the skin are lipids and proteins. The "attacks" produce metabolites of lipid peroxidation (LPO) and protein oxidative modification (POM), respectively. As reported earlier, LPO products contribute to DNA damage, mutagenic and carcinogenic effects, modification of membrane proteins, enzymes, signaling molecules [4]. In the TT pathogenesis, the relationship between inflammation and redox status under the OxInflammation concept describes the prooxidative potential of the lesion with positive feedback from inflammatory process events, cross-interactions of inflammatory mediators and redox status with possible progressions into SIRS [5]. Among the biomarkers of OxInflammation are lipid hydroperoxides 4-hydroxy-2-nonenal and malonic dialdehyde, carbonyl derivatives of 3-nitrotyrosine proteins, hydroxyguanosine etc. In pathological situations, identification of aldehyde- or ketone-containing carbonyl derivatives of proteins (POM products) and LPO products (as markers of oxidative stress and performance of antioxidants) is of great interest [6].

Currently, there is a number of types of wound dressings applied as treatment of localized thermal skin injuries. Such dressings ease pain during bandaging, make the wound moist and thus promote cell differentiation and effective intercellular interaction, and shorten treatment time. There are absorbent dressings, atraumatic (usually tulle) dressings of natural fibers, powder (xerogel) dressings, spongy dressings made of porous material, hydrogel dressings made of insoluble polymers, hydrocolloidal dressings made of gel-forming substances, film dressings made of semipermeable polymer materials, etc [7]. No dressing is universal; its choice depends on the wound's stage, the degree of exudation and complications, if any. About 80% of wound dressings are made by foreign manufacturers, which makes research and development of an original dermal film (DF) relevant and promising. The films may incorporate various pharmacologically active substances (antimicrobial, antiseptic, analgesic, etc.); endogenous regulators of homeostasis with pleiotropic properties are particularly interesting [8–11]. In addition to its capacity to regulate rhythms of sleep and wakefulness, melatonin (MT) can produce multitropic effects and possesses antioxidant, pro- and anti-inflammatory, immunomodulatory, antiapoptogenic, cell proliferation and differentiation regulating, anti-aging and other properties, which make it attractive as a therapeutic agent [12]. There are no papers published and available that mention local use of MT as a part of DF applied to TT.

This study aimed to assess the content of oxidative stress markers in the skin subjected to experimental TT and treated with original DF with MT.

## METHODS

The experiment involved 88 male Wistar rats weighing 200–240 g. The animals were randomly divided into three groups: group 1 ($n$ = 20) — intact control; group 2 ($n$ = 36) — animals with TT; group 3 ($n$ = 32) — animals with TT and DF with MT applied to the wound. Third degree A TT burns with a relative area of 3.5% were imposed in the interscapular area by immersion in purified water at 98–99 °C for 12 s. Depth of the burn was verified by morphological methods. Experimental model relying on hot water is most often considered by researchers as the TT standard. Zoletil 100 (tiletamine, zolazepam) (Virbac Sante Animale; France), dose of 20 mg/kg, was the anesthetic used in the experiment. Subjects of the 3rd group received a 12 $cm^2$ patch of the film with MT immediately after TT was inflicted. The film was fixed with an aseptic bandage, changed daily for 5 days. Preliminary studies yielded DF composition based on sodium carboxymethylcellulose (poly-1,4-β-O-carboxymethyl-D-pyranosyl-D-sodium glycopyranose). The film was enriched with MT (5 mg/g), after which its pharmacological and technological parameters were evaluated, namely: organoleptic indicators (type, color, transparency, elasticity, presence of impurities and microcracks), adhesiveness, mechanical tensile strength, thickness (patent application #2020118766). A Nikon Coolpix S2800 camera (Nikon; China) and Microsoft Office Visio software package (Microsoft; USA) enabled digital planimetry that allowed establishing the wound area 24 hours later, on the 5$^{th}$ and 10$^{th}$ days after TT infliction. The epithelialization rate ($VS$) was calculated using the following formula: $VS = S – Sn / t$, where $S$ is the initial wound area before treatment (hereinafter, area at previous measurement); $Sn$ is the area at subsequent measurement; $t$ is the number of days between measurements. The area of the wound at subsequent measurements was determined in %, with 100% being the area before treatment. The results of the measurement were expressed in %/day.

To prepare 10% skin homogenate, the burn wound was excised, immersed in a cooled 0.1 M phosphate buffer solution (pH 7.4), after that about 40 mg of tissue was homogenized in a glass mechanical homogenizer (1 : 10 ratio) for 3 min at 4 °C, which finally yielded 1 ml of the homogenate. SF-56 spectrophotometer (LOMO-Spectr; St. Petersburg) was used to determine the content of LPO products in the homogenate;

**Table 1.** Influence of MT as part of DF on the parameters of healing of the wound, with experimental TT (Me ($Q_{25}$; $Q_{75}$))

| Indicator | Group 2 TT, 5$^{th}$ day ($n$ = 16) | Group 2 TT, 10$^{th}$ day ($n$ = 20) | Group 3 TT + MT, 5$^{th}$ day ($n$ = 16) | Group 3 TT + MT, 10$^{th}$ day ($n$ = 16) |
|---|---|---|---|---|
| Burn area, $cm^2$ | 11.66 (11.50; 11.94) | 9.48 (9.28; 9.93)* | 10.33 (10.17; 10.56)# | 8.34 (8.19; 8.51)# |
| Relative area, % | 3.34 (3.25; 3.39) | 3.17 (3.10; 3.29)* | 3.36 (3.23; 3.42) | 3.02 (2.91; 3.13)# |
| Epithelialization rate, %/day | 0.89 (0.86; 0.89) | 1.90 (1.88; 1.95)* | 1.33 (1.29; 1.35)# | 6.57 (5.92; 6.93)# |
| Wound area shrinkage, % | 2.61 (2.59; 2.64) | 3.68 (3.53; 4.23)* | 9.80 (9.64; 10.08)# | 16.10 (14.62; 17.73)# |

**Note:** * — statistically significant ($p < 0.05$) differences with group 2 on the 5$^{th}$ day; # — with group 2 on the corresponding day.

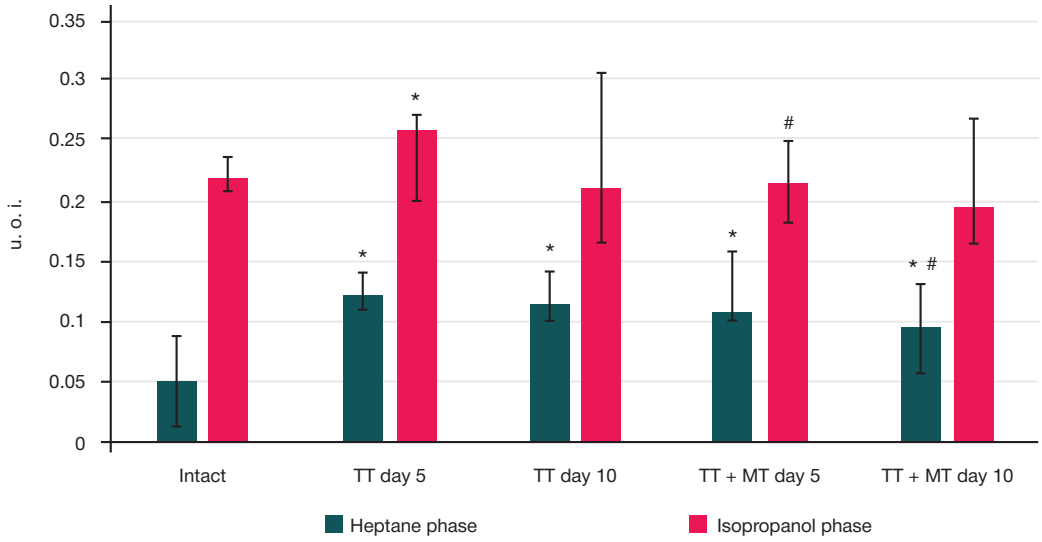


**Fig. 1.** Effect of MT as part of DF on the content of ketodienes and conjugated trienes in the heptane and isopropanol phases of skin homogenate, experimental TT (Me ($Q_{25}$; $Q_{75}$)). * — statistically significant ($p < 0.05$) differences with group 1; # — with group 2

the extraction-spectrophotometric method applied was described in earlier works [13]. Optical density was the parameter measured in the heptane and isopropanol phases of the lipid extract. The measurement wavelengths were 220 nm (content of isolated double bonds), 232 nm (content of diene conjugates, DC), 278 nm (content of ketodienes and conjugated trienes, KT and CT), 400 nm (Schiff bases, SB). The relative content of LPO products was expressed in units of oxidation indices (u.o.i): E232/E220 (DC), E278/E220 (KD and CT), and E400/E220 (SB). The POM products in the homogenate were determined by the reaction between protein carbonyl derivatives and 2,4-dinitrophenylhydrazine in spontaneous and metal-dependent modes, according to the Fenton reaction, with the following step being registration of aldehyde dinitrophenylhydrazones (ADNPH) and ketondinitrophenylhydrazones (KDNPH) in UV and visible parts of the spectrum [14]. The results were expressed in optical density units per 1 mg of protein (c.u./mg) or in relative values (%). The reserve adaptive potential (RAP, %) was calculated as the ratio of difference between the total content of POM products in the induced and spontaneous modes to the content of POM products in the metal-dependent mode. IBM SPSS Statistics 19 software (SPSS: An IBM Company; USA) was used for statistical processing of the data. The indicators were presented as median (Me) and quartiles ($Q_1$; $Q_3$). Kruskell–Wallis, Mann–Whitney, Wald–Wolfowitz tests enabled assessment of significance of differences between the groups. Spearman's correlation coefficient (R) allowed identification of relationship between the studied parameters. The differences were considered significant at $p < 0.05$.

## RESULTS

Assessment of the burn wound healing parameters revealed that its absolute and relative areas decreased, with the result of this decrease greater on the 10th day compared to the 5th day. This process boosts the monitored indicators, i.e. wound epithelialization rate and proportion of decrease of its area (Table 1). On the 5th and 10th days, the content of LPO products registered in the wound skin homogenate changed (Fig. 1, 2). Thus, on the 5th day the content of ketodienes, conjugated trienes and Schiff bases increased significantly in the heptane and isopropanol phases. It is predominantly reserve lipids (triacylglycerides) that accumulate in the heptane phase, while those accumulating in the isopropanol phase are membrane phospholipids. Assessment of the LPO product

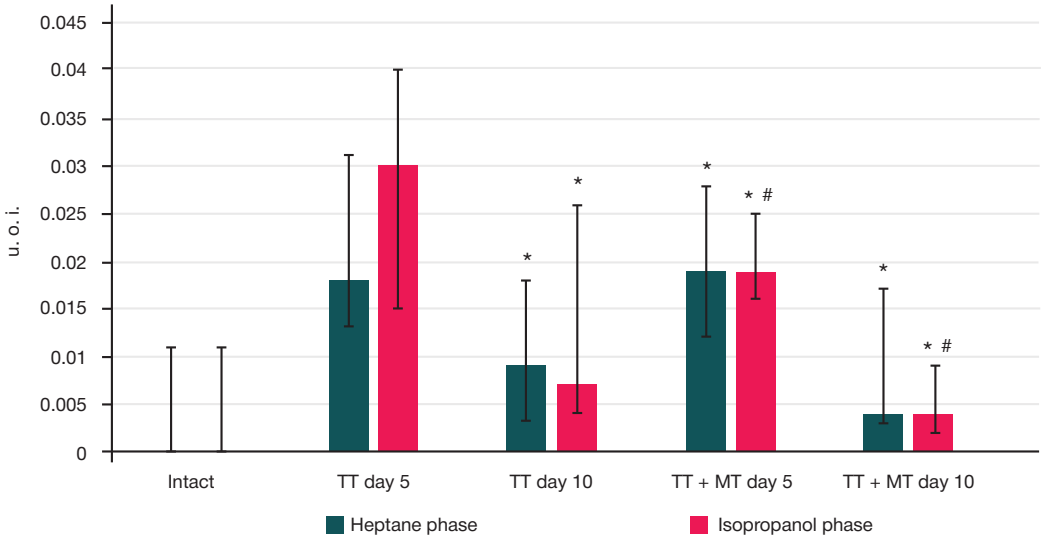


**Fig. 2.** Effect of MT as part of DF on the content of Schiff bases in the heptane and isopropanol phases of skin homogenate, experimental TT (Me ($Q_{25}$; $Q_{75}$)). * — statistically significant ($p < 0.05$) differences with group 1; # — with group 2

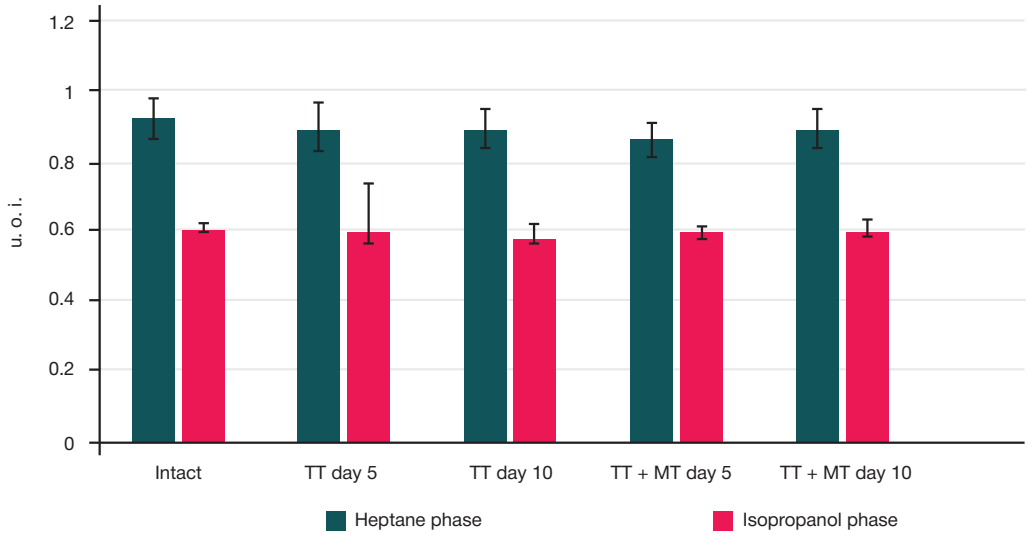


**Fig. 3.** Effect of MT as part of DF on the content of diene conjugates in the heptane and isopropanol phases of skin homogenate, experimental TT (Me ($Q_{25}$; $Q_{75}$))

content on the 10th day of TT revealed a significant increase in ketodienes and conjugated trienes and Schiff bases in the lipid extract's heptane phase, while in the isopropanol phase only the Schiff bases have grown. As for the primary LPO products (diene conjugates), their content in the heptane and isopropanol phases of the burn wound skin homogenate's lipid did not change significantly by the 5th and 10th days (Fig. 2). With TT changing over time, the content of LPO products in the burn wound decreases: on the 10th day, as compared with the 5th day, the share of Schiff bases was smaller in the lipid extract's heptane phase ($p < 0.01$), and in the isopropanol phase ($p < 0.01$) the content of ketodienes, conjugated trienes and Schiff bases went down.

Table 2 shows the results of examination of POM products in the burn wound skin homogenate. The total amount of POM products increased on the 5th and 10th days of the experiment. Considered in the context of TT dynamics, the total content of POM products increased on the 10th day in comparison with that registered on the 5th day ($p < 0.01$). Analysis of POM components in the spontaneous mode burn wound skin homogenate revealed that on the 5th day the share of ADNPH increased significantly in the UV part of the spectrum, which is also true for the total ADNPH content and the overall amount of POM products. As for KDNPH, their content did not change significantly in the burn skin wound homogenate on the 5th day (as verified in both UV and visible part of the spectrum). On the 10th day of TT existence, there were registered ambiguous changes in the shares of various OMP products: the amount of ADNPH and KDNPH as seen in the UV part of the spectrum have grown, while that in the visible part of the spectrum has decreased significantly. Accordingly, on the 10th day the total content of ADNPH and KDNPH has increased, as did the total content of POM products in the UV part of the spectrum, and the total content of POM products in the visible part of the spectrum has gone down. It should also be noted that the total content of POM products in the visible part of the spectrum on the 10th day is significantly ($p < 0.01$) higher than that on the 5th day.

RAP of the burn wound skin homogenate was assessed through calculation of the difference between the content of POM products in the metal-induced and spontaneous modes and its share in the content of POM products in the metal-induced mode (see Table 2). This stage required studying the content of POM products in the burn wound skin homogenate after induction of protein oxidation by the components of reaction mixture containing $Fe^{2+}$ and $H_2O_2$, with the reaction also yielding formation of a highly reactive radical OH — in the Fenton reaction. The approach allows estimation of uplift in carbonyl derivatives *in vitro*, as influenced by the Fenton's reagent, and calculation of the ratio of results of measurement of spontaneous oxidation and induced oxidation. It was found that on the 5th and 10th days, POM indicators in the burn wound skin homogenate (metal-induced mode) have grown in relation to the total content of POM products, content of ADNPH and KDNPH, with the assessment carried out for both UV and visible parts of the spectrum. In addition, the total content of ADNPH and KDNPH in the UV and visible spectra, as well as the total content of POM products therein, have increased. The changes in POM indicators peculiar to the metal-induced mode largely align with the POM indicator changes registered for the spontaneous mode on the 5th day. With the exception of POM products in the visible part of the spectrum, same is true for the 10th day of TT existence. The total RAP, assessed by the total content of POM products, increased significantly on the 5th day and did not change on the 10th day. The change of the 5th day results from the RAP's growth in relation to ADNPH and KDNPH in the UV and visible spectra, with ADNPH growing predominantly in the visible part of the spectrum, and KDNPH in both UV and visible spectra. On the 10th day, the overall RAP was significantly ($p < 0.01$) lower than on the 5th day of the experiment.

It was established that, in absolute figures, application of DF with MT to a TT wound decreases the burn wound significantly on the 5th and 10th days, and in relative values — on the 10th day (see Table 1). The wound epithelialization rate and the relative wound area shrinking process accelerated on the 5th and 10th days of observation. On the 5th day of TT existence, the absolute area of the burn surface decreased by 11% (median). The maximum change was recorded on the 10th day, when the absolute area of the wound decreased by 12% and the epithelialization rate have grown by 246% (median) relative to the group that had no DF with MT applied after TT infliction. Dynamics of experimental TT have shown that on the 10th day, as compared to the 5th day, the burn area has decreased significantly ($p < 0.01$), and the rate of wound epithelialization and relative wound area shrinkage have increased. As shown in Table 2, we have registered changes in LPO products content in the burn wound skin homogenate's lipid extract in the group that had DF with MT applied to the TT (see Table 2). On the 5th day of observation, the content of ketodienes and

**Table 2.** Effect of MT as part of DF on the content of POM products in skin homogenate, with experimental TT (Me ($Q_{25}$; $Q_{75}$))

| Indicators | Group 1 Intact ($n$ = 20) | Group 2 TT, 5th day ($n$ = 16) | Group 2 TT, 10th day ($n$ = 20) | Group 3 TT + MT 5th day ($n$ = 16) | Group 3 TT + MT 10th day ($n$ = 16) |
|---|---|---|---|---|---|
| $S_{ADNPH}$ uv (sp), c.u./mg | 29.85 (24.69; 32.84) | 51.49 (48.03; 55.81)* | 60.50 (52.95; 93.13)* | 32.87 (31.04; 48.35)# | 53.71 (45.11; 59.16)*# |
| $S_{ADNPH}$ vs (sp), c.u./mg | 6.93 (5.32; 8.71) | 6.91 (5.72; 9.75) | 3.53 (2.09; 5.07)* | 7.09 (5.17; 12.27) | 5.24 (3.10; 7.17)*# |
| $S_{KDNFH}$ uv (sp), c.u./mg | 8.19 (7.37; 10.59) | 7.79 (7.34; 9.43) | 15.19 (9.05;25.63)* | 9.29 (7.56; 16.39) | 8.22 (6.48; 12.61)# |
| $S_{KDNFH}$ vs (sp), c.u./mg | 0.89 (0.69; 1.14) | 0.91 (0.69; 1.41) | 0.50 (0.35; 0.66)* | 0.79 (0.66; 1.42) | 0.45 (0.27; 0.77) |
| S POM (sp), c.u./mg | 47.83 (41.94; 55.40) | 66.87 (60.56; 76.11)* | 79.30 (62.59; 122.34)* | 49.97 (41.94; 79.07)# | 64.65 (56.91; 76.64)*# |
| $S_{ADNFH}$ (sp), c.u./mg | 38.54 (30.64; 41.39) | 59.19 (52.29; 62.31)* | 65.04 (54.51; 96.45)* | 40.77 (35.11; 64.89)# | 57.90 (48.93; 64.39)*# |
| $S_{KDNFH}$ (sp), c.u./mg | 10.12 (8.23; 11.31) | 8.81 (8.09; 10.67) | 15.49 (9.56; 26.11)* | 10.28 (8.23; 18.01) | 8.97 (6.76; 13.15)# |
| S uv (sp), c.u./mg | 38.47 (34.05; 45.31) | 59.10 (53.57; 68.66)* | 74.97 (60.81; 118.45)* | 40.94 (36.99; 64.74)# | 61.28 (52.69; 70.97)*# |
| S vs (sp), c.u./mg | 7.87 (6.02; 9.73) | 7.81 (6.41; 11.16) | 4.05 (2.35; 5.85)* | 7.88 (5.84; 13.69) | 5.67 (3.37; 7.89)# |
| $S_{ADNFH}$ uv (ind), c.u./mg | 90.68 (70.81; 94.67) | 386.82 (279.79; 542.03)* | 178.71 (128.45; 239.17)* | 260.81 (94.29; 50.90)*# | 211.60 (140.76; 235.78)* |
| $S_{ADNFH}$ vs (ind), c.u./mg | 18.81 (16.01; 21.09) | 156.21 (90.34; 244.15)* | 37.79 (29.25; 60.75)* | 91.49 (19.62; 264.02)*# | 68.35 (45.15; 100.18)*# |
| $S_{KDNFH}$ uv (ind), c.u./mg | 28.73 (24.72; 37.55) | 208.12 (128.85; 320.23)* | 76.51 (45.82; 94.77)* | 125.89 (29.35; 333.99)*# | 93.56 (60.57; 127.56)*# |
| $S_{KDNFH}$ vs (ind), c.u./mg | 2.07 (1.62; 2.32) | 14.53 (8.45; 22.58)* | 3.84 (2.93; 6.02)* | 8.84 (2.32; 26.09)*# | 6.61 (4.68; 9.76)*# |
| S POM (ind), c.u./mg | 140.38 (113.93; 155.46) | 771.61 (507.38; 1128.99)* | 312.84 (207.79; 393.90)* | 487.04 (145.58; 1175.01)*# | 378.89 (262.70; 476.87)*# |
| $S_{ADNFH}$ (ind), c.u./mg | 109.49 (87.20; 115.77) | 548.02 (370.12; 786.18)* | 230.51 (157.87; 301.83)* | 352.30 (113.90; 814.93)*# | 277.53 (197.90; 338.84)* |
| $S_{KDNFH}$ (ind), c.u./mg | 30.82 (26.73; 39.69) | 223.59 (137.26; 342.81)* | 80.12 (48.56; 101.26)* | 134.74 (31.67; 360.08)* | 99.88 (64.40; 138.03)*# |
| S uv (ind), c.u./mg | 119.47 (95.53; 132.23) | 594.94 (408.64; 862.26)* | 252.79 (175.39; 344.84)* | 386.71 (123.64; 884.89)*# | 304.79 (218.89; 363.53)*# |
| S vs (ind), c.u./mg | 20.91 (17.63; 23.23) | 170.73 (98.74; 266.73)* | 41.63 (32.13; 66.99)* | 100.33 (21.94; 290.12)*# | 74.96 (49.83; 109.94)*# |
| RAP, % | 61.81 (53.98; 72.55) | 91.66 (86.44; 94.78)* | 69.22 (62.76; 74.76) | 83.77 (71.19; 96.33)* | 81.15 (74.35; 86.09)*# |

**Note:** * — statistically significant ($p < 0.05$) differences with group 1; # — with group 2.

conjugated trienes, as well as Schiff bases, in the isopropanol phase of the lipid extract has decreased significantly. As for the content of diene conjugates, ketodienes and conjugated trienes, as well as Schiff bases in the heptane phase, and Schiff bases in the isopropanol phase of the lipid extract, these did not differ significantly from the same indicators registered in the group of animals that had TT inflicted but no DF with MT applied. On the 10th day of the experiment, we have identified a significant decrease in ketodienes and conjugated trienes in the heptane phase, Schiff bases in the isopropanol phase of the burn wound skin homogenate's lipid extract. The data obtained indicate that the effect MT produces on TT as part of DF is maximal in the isopropanol phase of the burn wound skin homogenate's lipid extract. As for the content of secondary and end LPO products in the heptane and isopropanol phases, it was lower on the 10th day ($p < 0.01$) than on the 5th day of TT evolution, which is same as registered in the group that did not receive DF with MT.

The use of MT in the composition of DF applied to TT leads to a change in the content of POM products in the burn wound (see Table 2). On the 5th day of the experiment, in the spontaneous mode, the content of ADNPH in UV spectrum, the total content of ADNPH, the total content of POM products in UV spectrum have decreased; consequently, the total content of POM products has grown down. It should be noted that the values of these indicators do not differ from the values captured in the group of intact animals, which allows stating their complete recovery. On the 10th day, in the spontaneous mode, the total content of POM products seen in the TT groups has decreased and did match the values peculiar to the group of intact animals, i.e. the recovery was only partial. Against this background, the content of ADNPH and KDNPH in the UV region, the total content of ADNPH and KDNPH, POM products in the UV region have decreased and completely recovered in relation to KDNPH in the UV region, and POM products in the UV region. When assessing the RAP, its decrease on the 5th day was found to depend on the approximately equivalent contribution of the studied primary and secondary POM products in the visible and UV light range. On the 10th day, RAP

**Table 3.** Correlation between burn area (cm²) and FRO indicators in the skin homogenate, experimental TT, application of DF with MT

| Indicators | 5th day | 10th day |
|---|---|---|
| DC (heptane phase), u.o.i | R = 0.21 | R = 0.18 |
| KD and CT (heptane phase), u.o.i. | **R = 0.34** | **R = 0.52** |
| SB (heptane phase), u.o.i | R = 0.17 | R = 0.27 |
| DC (isopropanol phase), u.o.i. | R = 0.17 | R = 0.15 |
| KD and CT (isopropanol phase), u.o.i. | **R = 0.68** | R = 0.21 |
| SB (heptane phase), u.o.i | **R = 0.53** | **R = 0.51** |
| S POM (spont. mode), c.u./mg | **R = 0.74** | **R = 0.67** |
| S POM (ind. mode), c.u./mg | **R = 0.53** | R = –0.25 |
| S ADNPH (spont. mode), c.u./mg protein | **R = 0.51** | **R = 0.39** |
| S KDNFH (spont. mode), c.u./mg protein | R = 0.07 | **R = 0.47** |
| S uv (spont. mode), c.u./mg protein | **R = 0.57** | **R = 0.37** |
| S vs (spont. mode), c.u./mg protein | R = 0.18 | **R = 0.51** |

**Note:** significant ($p < 0.05$) connections are highlighted in bold.

has increased in the DF with MT group, mainly driven by the RAP growing in relation to ADNPH and KDNPH in the visible spectrum, KDNPH in the UV spectrum.

## DISCUSSION

We found that in the locus of thermal damage to the skin, the amount of secondary and end LPO products increases in the heptane and isopropanol phases of lipid extract. With TT, the total amount of carbonyl derivatives of proteins goes up, and they are irreversible products of oxidative stress formed due to the oxidation of several amino acid residues, as well as interactions with LPO products and reducing sugars. Since neutral carbonyl derivatives accumulate in the UV spectrum, and basic carbonyl derivatives accumulate in the visible part of the range, a relative analysis of the total content of carbonyls in the UV and visible spectra allows assessment of the nature of the products formed against the background of TT dynamics. On the 5th and 10th days of TT existence, the POM products accumulating predominantly are neutral, as evidenced by an increase in the total amount of products in the UV region of the spectrum. In the TT groups, the total ADNPH content in the skin increases on the 5th and 10th days, that of KDNPH — on the 10th day. This fact allows registering primary POM products accumulation on the 5th and 10th days, these products being the early markers of oxidative protein destruction, with another phenomenon registered being predominant aggregation of proteins under the influence of OH. On the 10th day, it is the secondary POM products that accumulate, which are late markers of oxidative protein destruction, and the second phenomenon registered is predominant fragmentation of proteins under the influence of the combined action of $OH^{\cdot}$ and $O_2^{\cdot-}$ radicals. Protein fragments are highly resistant to proteolysis, have toxic properties and can initiate apoptosis or cell necrosis, expanding the zone of secondary alteration. Skin is the largest organ with intensive LPO and POM processes, and the LPO and POM products formed therein can have a local and distant cytotoxic effect [15]. Oxidative stress in burns is registered not only in the locus, but also in the heart, lungs, kidneys and other organs, where protein, lipid and DNA damage products are found [16]. The main inducers of oxidative stress after TT are activated neutrophils, monocytes/macrophages, endotheliocytes with known systems for generating reactive oxygen species (ROS): NADPH oxidase and MPO, xanthine oxidase, NO synthase [17]. Increased production of endogenous glucocorticoids plays an important part in escalation of oxidative stress in the presence of TT [18]. The depletion of the antioxidant defense system contributes to the pathogenesis of oxidative stress with TT — the content of reduced glutathione, activity of SOD, catalase, and glutathione peroxidase (GPO) decrease [19]. We demonstrated a decrease in the body levels of zinc and copper, which are part of SOD, due to their loss with urine and burn wound exudate, as well as a deficiency of selenium, HPO component, due to a decrease in the intake through the gastrointestinal tract associated with burns [20]. We also demonstrated the role of iron in the activation of oxidative metabolism in the presence of TT. ROS in a burn wound are associated not only with tissue destruction and negative consequences: they also participate in activation of the pro-matrix metalloproteinase and skin repair after TT [21]. ROS play an important role in the modification of components of the extracellular matrix of connective tissue — glycosaminoglycans, collagens, and non-collagen glycoproteins. Also, they contribute to wound healing through activation of signaling pathways in stem cells [22]. ROS generated during inflammation associated with TT induce local synthesis of pro- and anti-inflammatory cytokines.

According to the results of the study, the use of MT in the composition of the DF applied to a TT injury accelerates healing of the burn wound, reduces its area and reduces the content of oxidative stress metabolites — LPO and POM products. On the 5th day of TT existence, the content of secondary and end LPO products in the lipid fraction of phospholipids decreases; on the 10th day, the content of secondary LPO products in the lipid fraction of triglycerides and end LPO products in the phospholipid fraction decrease. MT limits POM: on the 5th, this process relies on the neutral amino acid residues of the primary POM products and on the protein fragmentation decrease, on the 10th day it relies on the neutral amino acid residues of primary and secondary POM products and a decrease in protein fragmentation and aggregation. We believe there is a number of mechanisms that drive the decrease of content of LPO and POM products in a burn wound with experimental TT after application of DF with MT. MT can be rapidly distribute inside the cell and in the intercellular fluid through passive diffusion, as well as with the help of glucose transporters (GLUT1) and oligopeptides (PEPT1/2) [23, 24]. MT1 receptor is found in keratinocytes and fibroblasts of the skin, hair follicle cells; MT2 receptor is found mainly in the eccrine glands and blood vessels of the skin, melanocytes; RORα nuclear receptor for MT was identified in keratinocytes, fibroblasts, melanocytes [25–27]. In the skin, MT can absorb ROS (OH, $H_2O_2$) directly, and one MT molecule is capable of

binding up to four or more ROS. MT's antioxidant effect is more pronounced than that of an equivalent dose of vitamins C and E [28]. There is also indirect antioxidant effect, which is the result of an increase in glutathione synthesis, activation of glutathione peroxidase, glutathione reductase, glutathione-S-transferase, SOD, catalase, hemoxidase-1, and a decrease in the activity of quinone reductase-2, NOS-1 [29]. Moreover, MT realizes its antioxidant effect by maintaining the mitochondrial membrane potential and increasing oxidative phosphorylation, production of ATP and not ROS [30]. Apparently, the antioxidant effect of MT in the TT locus, lighter damage to proteins and lipids that make up cytoplasmic membranes and cell organelles limit secondary alteration, reduce the term of vascular and exudative reactions, help activate reparative reactions earlier and reduce healing time of a burn wound. Correlation analysis of the burn surface area and content of LPO and POM products in the TT locus with DF with MT applied (Table 3) has shown that on the 5th day of TT existence there is direct average strength connection between the absolute area of the burn and content of secondary and end LPO products in lipid extract's isopropanol phase in the burn wound, as well as weak connection with the content of secondary LPO products in the heptane phase, direct average strength connection with the total content of POM products in spontaneous and induced modes, and the total content of primary POM products and the total content of neutral POM products. On the 10$^{th}$ day of TT existence, there was established a direct average strength connection between the content of secondary LPO products in the heptane phase and end POM products in the isopropanol phase of the lipid extract, total content of basic POM products, content of POM products in spontaneous mode, a weak direct connection with total content of primary and secondary POM products, neutral POM products in spontaneous mode.

## CONCLUSION

The study allowed achieving the goal set and establishing that with the evolution of TT, a burn wound sees growth of content of secondary and end LPO products in heptane and isopropanol phases of the lipid extract, same as the content of predominantly neutral primary and secondary POM products. It was demonstrated that application of DF with MT (original composition) to TT reduces absolute and relative areas of the burn, boosts wound epithelialization, and reduces the content of LPO and OMP products in the burn wound. When TT has DF with MT applied to it, the area of the burn is associated with the content of LPO products and OMP products in the burn wound. The results obtained expand the existing understanding of the role of redox status changes in TT pathogenesis, serve as a prerequisite for further studies of FRO in the skin of admitted patients with burns (clinical studies) in order to designate LPO and OMP products as diagnostic markers and predictors of complications, as well as indicators the effectiveness of therapy. The antioxidant and reparation stimulating effect of MT as part of DF, which we have demonstrated at the preclinical stage, is a prerequisite for further study of the action mechanism and the effectiveness of MT application in clinical settings.

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# BUCCAL URETEROPLASTY FOR RECURRENT EXTENDED STRICTURES AND OBLITERATIONS OF DISTAL URETER

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At the current stage of development of urology, selection of the surgical method for cases of severe obstructive diseases of the upper urinary system remains a challenge. This study aimed to explore the results of application of a buccal graft (BG) to remedy extended recurrent strictures and obliterations of the distal ureter. Seven patients with the mentioned diseases had undergone surgery: for six of them, the method of choice was complete BG ureteroplasty, one had onlay ureteroplasty. One intervention was laparoscopic, the remaining surgeries were open. The length of the replaced ureteral defect was 5–8 cm. In five cases, the flap was additionally vascularized with the iliac muscle, in one we used omentum tissue, in another — both the iliac muscle and the omentum. There were no fatalities registered, nor severe complications as per the Clavien–Dindo classification. The patients were followed-up for 4–18 months; as of today, no recurrence cases were identified. Control examinations showed complete patency of the neoureter and good vascularization of the BG. Thus, this method can be an option in cases disallowing distal ureter restoration with tissues of the patient's own urinary tract or segments of the gastrointestinal tract.

**Keywords:** buccal mucosa graft, ureteral obstruction, ureteral obliteration, ureteral stricture, distal ureter

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**Compliance with ethical standards:** the study was approved by the ethics committee of the Hospital for War Veterans (Minutes #1 of February 6, 2018). All participants submitted the signed informed consent forms confirming their consent to participate in the study.

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# БУККАЛЬНАЯ УРЕТЕРОПЛАСТИКА ПРИ РЕЦИДИВНЫХ ПРОТЯЖЕННЫХ СТРИКТУРАХ И ОБЛИТЕРАЦИЯХ ДИСТАЛЬНОГО ОТДЕЛА МОЧЕТОЧНИКА

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Выбор метода хирургического лечения тяжелых обструктивных заболеваний верхних мочевых путей является сложной проблемой и на современном этапе развития урологии. Целью исследования было изучить результаты применения буккального графта (БГ) при протяженных рецидивных стриктурах и облитерациях дистального отдела мочеточника. Прооперировано семь пациенток с данными заболеваниями: у шести пациенток была выполнена полная заместительная уретеропластика БГ, у одной — заместительная onlay уретеропластика. Лапароскопическим доступом оперировали в одном случае, в остальных использовали открытый. Протяженность замещенного дефекта мочеточника составила 5–8 см. Лоскут дополнительно васкуляризировали подвздошной мышцей в пяти случаях, в одном — тканью сальника, и у одной пациентки использовали подвзошную мышцу и сальник. Тяжелые осложнения по классификации Clavien–Dindo, а также летальные исходы отсутствовали. Период наблюдения за пациентами составил 4–18 месяцев, рецидива заболевания у оперированных на сегодняшний день нет. Контрольные исследования показали полную проходимость неоуретера и хорошую васкуляризацию БГ. Таким образом, данный способ может служить одной из дополнительных методик при невозможности восстановления дистального отдела мочеточника с использованием тканей собственных мочевых путей или сегментов желудочно-кишечного тракта.

**Ключевые слова:** буккальный графт, обструкция мочеточника, облитерация мочеточника, стриктура мочеточника, дистальный отдел мочеточника

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Extended strictures and obliterations of the ureters result from iatrogenic injuries incurred during surgeries on pelvic organs and abdominal cavity, following radiotherapy and such diseases as tuberculosis, urolithiasis, Ormond's disease, ureteral tumors, etc. [1]. There are three main groups of ureteral strictures by their origin: post-traumatic, post-inflammatory, and post-radiation [2]. Thirteen percent of patients diagnosed with iatrogenic ureter injury following urinary system surgery developed a post-traumatic stricture [3].

In most cases, obstruction of the lower third of the ureter caused by its strictures and obliterations is a result of endourological interventions aimed at urolithiasis [3–5]. Also, such obstruction may be a delayed complication of pelvic radiotherapy [6] most commonly aimed at bladder, cervix or uterine body cancer. It is a known fact that post-radiation complications in the form of urinary tract lesions may reach up to 28% in cervical cancer patients [7]. The irradiation degrades vascularization and regeneration capabilities of the subject

**Table 1.** Patient group characteristics

| № | Diagnosis | Etiology | Comorbidities |
|---|---|---|---|
| 1 | Recurrent stricture of the lower third of the ureter | Radiation therapy | Sigmoid colon cancer. Type 2 diabetes mellitus. Class 3 obesity |
| 2 | Obliteration of the lower third of the ureter | Endourological operations | Bladder tumor. ICD. Essential hypertension III degree. Risk 4 |
| 3 | Obliteration of the lower third of the ureter | Radiation therapy | Cervical cancer |
| 4 | Obliteration of the lower third of the ureter | Radiation therapy | Cervical cancer. Class 3 obesity. ICD |
| 5 | Recurrent stricture of the lower third of the ureter | Post-tuberculous ureteritis | Spinal tuberculosis. Urinary system tuberculosis. Type 2 diabetes mellitus. Class 3 obesity |
| 6 | Obliteration of the lower third of the ureter | Radiation therapy | Cervical cancer |
| 7 | Recurrent stricture of the lower third of the ureter | Endourological operations | ICD. Type 2 diabetes mellitus. Class 3 obesity |

tissues, which rids conservative treatment options of chances to be successful [8]. Currently, there is no distal ureter post-radiation obstruction surgery method that is considered to be a standard. This type of obstruction is difficult to manage: with the ureter wall ischemia in the background, it is often impossible to use its own tissues for reconstruction purposes. Ureteroplasty variations that make use of various segments of the gastrointestinal tract entail a significant number of complications, and the success rate of such surgeries does not exceed 80% [9, 10]. Thus, development of the new methods for restoring patency of the distal ureter remains relevant. Most researchers believe that buccal mucosa graft (BG) shows the greatest promise in the context considered. For over 20 years now such grafts have been used as reconstruction material in surgeries remedying strictures and obliterations of the proximal and middle ureter [11].

This study aimed to explore the efficacy or using a BG to treat extended recurrent strictures and obliterations of the distal ureter.

## METHODS

From 2018 to 2020, seven patients with extended strictures/obliterations of the lower third of the ureter have undergone buccal flap replacement ureteroplasty in the surgical center of the Rostov Region Hospital for War Veterans. The mean age of the patients was 52.5 + 9.0 years (36–67 years). One (14.3%) patient was diagnosed with recurrent ureteral stricture against the background of a long-standing urolithiasis and several corrective surgical interventions; another patient (14.3%) had recurrent post-radiation ureteral stricture after treatment of an intestinal tumor. In one case (14.3%), the obliteration of the ureter's lower third was pronounced a result of several surgeries performed on the ureter; in three cases (42.9%), the ureter obliteration was post-radiation by nature, an after-effect of complex treatment of cervical cancer; and in one case (14.3%), the recurrent stricture was a consequence of the tuberculous inflammatory process.

All patients in this group had a pronounced background of comorbidities, a history of various tumor and severe systemic diseases (Table 1). In the past, patients of the analyzed group had undergone extensive surgical procedures: three (42.9%) had malignant cervical cancer removed through extended extirpation of the uterus with appendages, which was followed by radiotherapy; three (42.9%) had abdominal surgery, one (14.3%) in the form of hemicolectomy targeting a malignant tumor and followed by radiotherapy, two (28.6%) — as traditional appendectomy for destructive forms of acute appendicitis. Besides, one patient (14.3%) had thoracolumbophrenotomy and vertebral resection for spinal tuberculosis, another (14.3%) — supravaginal uterine amputation (see Table 1).

Before buccal ureteroplasty, this group of women had undergone 21 different operations on the upper urinary system (UUS), including reconstructive, endoscopic and drainage interventions. Considering the outcomes of the said interventions, it should be noted that for almost all patients (85.3%) they ended in persistent kidney drains, which signals of a need for alternative methods of surgical treatment. Two (28.6%) patients were offered nephrectomy, which they refused, two more (28.6%) had nephrostomes for more than two years.

At the time of surgery, two (28.6%) patients already had lesions of the contralateral kidney, which led to the development of chronic kidney disease (CKD) (Table 2). In one case, blood urea was at 13.1 mmol/l, blood creatinine at 259.2 μmol/l, GFR as per the CKD-EPI formula — 18 ml/min/1.73 $m^2$. In another case, blood urea was at 11.6 mmol/l, blood creatinine at 174.4 mmol/l, GFR as per the CKD-EPI formula — 27 ml/min/1.73 $m^2$.

At the time of admission and at other timepoints post surgery, all patients underwent a comprehensive urological examination that included interviewing for complaints and history compilation, laboratory diagnostics, kidney ultrasonography with triplex scanning of the renal arteries, retrograde ureteropyelography, cystoscopy and ureteroscopy, computed tomography with contrast, intravenous urography, radionuclidography (as indicated), morphological studies.

**Table 2.** Types of previous surgical interventions

| № | Past surgeries on UUS | Contralateral kidney | CKD | Persistent drainage |
|---|---|---|---|---|
| 1 | Boari operation (BO) | Norm | No | No |
| 2 | Transurethral bladder resection; ureter orifice endotomy + ureterorenoscopy with contact ureterolithotripsy and ureterolithoextraction (URS) + ureter stenting (US); URS + US; ureter orifice endotomy + US; URS + US; URS + US; percutaneous nephrostomy (PN) | ICD. Chronic pyelonephritis | Yes | Nephrostomy |
| 3 | BO, PN | Norm | No | Nephrostomy |
| 4 | PN | Norm | No | Nephrostomy |
| 5 | PN; URS + US; IN; URS + US; BO; permanent ureter stenting | ICD. Chronic pyelonephritis | Yes | Ureteral stent |
| 6 | PN | Norm | No | Nephrostomy |
| 7 | URS + US; URS + US; permanent ureter stenting | Norm | No | Ureteral stent |

**Table 3.** Characteristics of BG ureteroplasty in the patient group

| № | Type of surgery involving a BG | Method | BG length, cm | Additional BG vascularization | Blood loss, ml |
|---|---|---|---|---|---|
| 1 | Graft onlaying onto the lower third of the ureter | Open | 5 | IM | 200 |
| 2 | Complete replacement of the lower third of the ureter and its orifice | Open | 6 | IM | 150 |
| 3 | Complete replacement of the lower third of the ureter | Open | 7 | IM | 170 |
| 4 | Complete replacement of the lower third of the ureter | Open | 7 | IM | 250 |
| 5 | Complete replacement of the lower third of the ureter | Open | 8 | IM + omentum strand | 200 |
| 6 | Complete replacement of the lower third of the ureter and its orifice | Laparoscopic | 6 | Omentum strand, attached | 300 |
| 7 | Complete replacement of the lower third of the ureter and its orifice | Open | 7 | IM | 200 |

Ureteroscopy was performed with an 8 Ch rigid ureteroscope (Karl Storz; Germany), cystoscopy — with 19–22 Ch rigid cystoscopes (Karl Storz; Germany). We used an endoscopic stand with equipment and accessories for endovideosurgery (Karl Storz; Germany), Image 1 SCONNECT, IMAGE 1S H3-LINK, IMAGE 1S H3-Z, PowerLED 175 SCB, Radiance 32, ENDOFLATOR 40, HAMOUEndomat, VIO 300 D. Ultrasound examination (US) of the kidneys and their vessels was performed with Philips EPIQ 5 Elite (Philips; the Netherlands), an expert class ultrasound system; intravenous urography was performed with a TeleKoRD-MT (MTL Company; Russia), a telecontrolled X-ray diagnostic complex. Philips Brilliance 64 tomograph (Philips; the Netherlands) enabled spiral computed tomography (SCT) of the retroperitoneal space. It was done in the scanning mode, with slices 2 mm thick and subsequent reconstruction by 0.75 mm and bolus enhancement with Omnipak-300 solution (100 ml).

Six (83.3%) patients had a buccal flap surgically placed as a complete replacement in the lower third of the ureter and its orifice, and in one (14.3%) patient it was used as an onlay in the lower third of the ureter. Of the seven women, one (14.3%) had the operation performed laparoscopically, and for the remaining six patients (83.7%) the surgery was open. The length of the ureteral defect requiring replacement, as established intraoperatively, was 5–8 cm (median — 6.5 cm) (Table 3).

Pre-surgery, the patients had their oral cavities prepared. The process included an oral cavity checkup and sanitation, a month long cigarette-free period and antiseptic mouthwash application for a week before the operation.

The presence of persistent drains and a chronic inflammatory process in the urinary system made preventive antibiotics unpromising in these patients. Post-surgery, they were prescribed long-term antibiotic therapy based on the results of bacteriological examination of urine.

In all cases, BG was additionally vascularized. For example, in five (71.4%) of them it was attached to the iliopsoas muscle (IM). In a patient that had ureteroplasty done laparoscopically (14.3%), the flap was wrapped with an omentum strand attached to the BG at several points; in one case (14.3%), the vascularization was combined, it included both the IM and the omentum strand fixed in position.

Of the six patients with tubularized BG, three (50%) had neoureterocystoanastomosis done following the antireflux technique (formation of a submucosal tunnel), and three more (50%) had a direct anastomosis formed between the tubularized BG and the bladder.

In all cases, the intraoperative blood loss was minimal and did not exceed 300 ml (see Table 3). The surgeries lasted 220–350 min (mean time — 280 min).

The surgeries were considered successful if the disease did not recur within 4–18 months post-surgery and there was no need for ureteral stenting (US) or percutaneous nephrostomy (PN).

## RESULTS

We continue monitoring the state of all the patients. The ureteral stent was removed routinely six weeks after surgery. The observation period for the patients is 4–18 months. Up to this moment, none of cases presented recurrence of the disease and surgery complications. Only one (14.3%) patient with CKD has residual hydronephrosis persisting for 11 months (Table 4).

There were no intraoperative complications registered in the analyzed group. The immediate post-surgery complications were assessed as per the Clavien–Dindo classification. It should be recognized that in this cohort of patients we did not observe severe post-surgery complications. One patient with chronic anemia needed transfusion of one dose of erythrocyte mass to correct the blood gas transport function. On the fourth day after the operation, another patient was diagnosed with an incarceration of a postoperative ventral hernia, which necessitated surgical correction under general anesthesia that was free of complications (see Table 4).

**Table 4.** Surgery results

| № | Complications as per Clavien-Dindo, degree/type | Observation, months | Residual hydronephrosis | Recurrent stricture/obliteration |
|---|---|---|---|---|
| 1 | No | 18 | No | No |
| 2 | No | 14 | No | No |
| 3 | No | 10 | No | No |
| 4 | II/blood transfusion | 12 | No | No |
| 5 | IIIb/bowel loop incarceration | 11 | Yes | No |
| 6 | No | 4 | No | No |
| 7 | No | 5 | No | No |

Control studies confirmed successful engraftment of the BG in all cases: cystoscopy and ureteroscopy revealed the neoureters had pink mucous membranes and their orifices were sound. Control retrograde ureteropyelography established full patency of the anastomosis in all patients. In the first case, the CKD patients' laboratory test reports returned the following figures: blood urea — 7.8 mmol/l, blood creatinine — 181.4 μmol/l, GFR as per the CKD-EPI formula — 26 ml/min/1.73 $m^2$. The figures for the second case were: blood urea — 8.9 mmol/l, blood creatinine — 141.6 μmol/l, GFR as per the CKD-EPI formula — 34 ml/min/1.73 $m^2$. The figures reflect a moderate positive trend.

To date, none of the patients needs a kidney drain.

The following case reflects the degree of efficacy of buccal ureteroplasty.

Patient T., 54 years old, was admitted on 05.07.2019 to the surgical center of the Rostov Region Hospital for War Veterans for complaining about a nephrostome.

In June 2014, the patient had undergone cystoscopy for hematuria in the clinic at her place of residence. The procedure revealed villous formation at the right ureteral orifice. A transurethral resection targeting the formation followed. Histological examination revealed a benign bladder tumor. In January 2015, the patient developed lower back pain in the right side; during the examination, she was diagnosed with hydroureteronephrosis and calculi in the lower third of the right ureter. Cystoscopy revealed no right ureter orifice but a scar in its place. The procedures that followed were bougienage of right ureter orifice, cold-knife endoureterotomy, ureterorenoscopy (URS) with right side urethrolithotripsy, ureter stenting. In May 2015, URS and US were repeated. In December 2015, the disease recurred and was managed with wide cold-knife endotomy of the right ureter orifice and ureter stenting. In 2016 and 2017, the patient has undergone URS and stenting of the right ureter. In April 2019, she developed acute right-side pyelonephritis and was diagnosed with right-side hydroureteronephrosis (Fig. 1). Cystoscopy failed to reliably identify orifice of the right ureter, which was found to be impassable for the ureteral catheter (Fig. 2). The patient has undergone percutaneous nephrostomy. She was admitted to the surgical center for surgery.

Diagnosis: obliteration of the lower third of the right ureter.

On 06.07.2019, the patient had a complete replacement of the lower third of the ureter with a buccal graft.

The affected and healthy tissue interface was determined intraoperatively. The ureter, found in a conglomerate of scar-altered tissue, was resected within healthy tissues and removed along with the tissue. The next step was ligation of the ureteral stump at the site of its entry into the bladder.

The length of the removed section of the ureter was 8 cm. The calculated length of the graft allowing anastomosis without tension was 6.5 cm. The second team of surgeons collected a buccal mucosa graft measuring 2.2 — 7.0 cm; the flap started on the right cheek's surface (4.5 cm) and stretched to the mucosa of the lower lip, as per the generally accepted rules. The flap was tubularized on a stent and additionally anchored to the iliopsoas muscle (IM) and bladder wall with monocryl 4/0 suture material. The orifice of the neoureter was formed using the antireflux technique, with shaping of a tunnel on the superior lateral wall of the bladder.

The post-surgery period was uneventful. The backup drain was removed on the 4$^{th}$ day; no urine leakage was detected. The nephrostome was removed on the 5$^{th}$ day. The patient was discharged on the 7$^{th}$ day in a satisfactory condition.

The stent was removed after 6 weeks. Kidney US scanning made 1, 3, 6, 9 months after the operation and excretory urography made 3 and 6 months after the operation showed regression of hydronephrosis on the right side.

A control examination performed 12 months after (kidney US scanning and SCT) revealed no right-side hydronephrosis and complete patency of the neoureter (Fig. 3). The results of the complex endourological examination were as follows: cystoscopy revealed a newly formed slit-shaped orifice that periodically let through urine (Fig. 4); retrograde ureterography showed complete patency of the replaced part of the ureter, which was moderately dilated after injection of the contrast

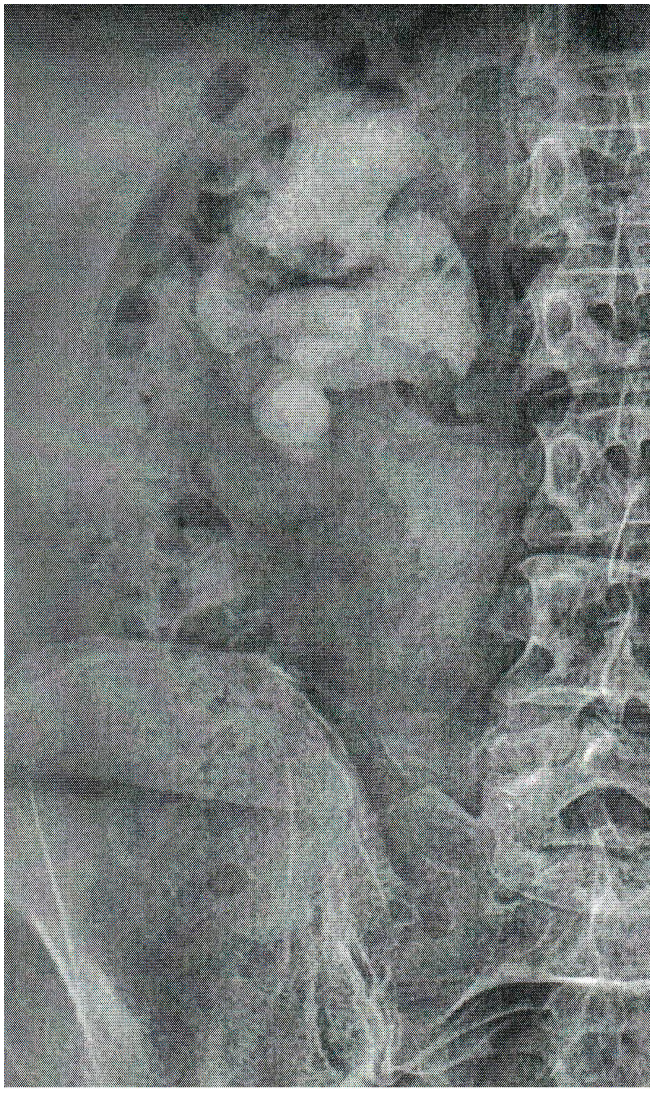

**Fig. 1.** Intravenous urography. Right-side hydronephrosis identified on the 60$^{th}$ minute of the examination

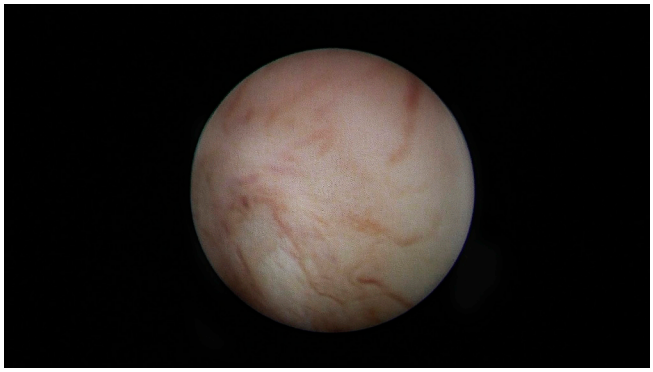

**Fig. 2.** Cystoscopy, pre-surgery. The orifice of the right ureter cannot be found

agent (Fig. 5); cystography uncovered no evidence of vesicoureteral reflux; ureteroscopy showed that the neoureter's mucosa was pink, vascularization of the BG good and the lumen unobstructed (Fig. 6).

Thus, in the presented clinical case, reconstruction of the distal ureter with a BG led to a complete restoration of the kidney function and eliminated the need for persistent kidney drain.

## DISCUSSION

Since 1999, when J. H. Naude used a buccal mucosa graft to reconstruct the ureter with strictures and obliterations for the first time, the number of papers dedicated to the subject has been growing steadily. Most of them describe use of a buccal flap to remedy defects in the upper third of the ureter and the pelvic-ureteric segment, i.e. in its proximal sections [12–14].

The reports covering ureter's lower third reconstruction with a BG are scarce [15–17]. According to some authors, there are standard approaches to restoration of the distal ureter in case of its obstruction, which renders application of BG for the purpose unnecessary [18]. Indeed, tissues of the patient's own UUS are considered an ideal material for restoration of patency of the system's tracts. However, the reconstruction-related intervention options are scarce due to the limited volume of healthy tissues available, especially after past ureter inflammations and surgeries [19].

Thus, a direct ureterocystoanastomosis is a viable option only with the ureter obliterations or strictures measuring less than 5 cm [20]. The psoas-hitch operation, which involves mobilization of the bladder and its attachment to the psoas muscle, allows restoring UUS defects measuring up to 10 cm, but it needs a significant strand of muscle and practically the entire lateral wall of the bladder, which can trigger a number of complications and is a challenge technically [21]. The Boari bladder flap, although effective for ureter defects up to 15 cm long, significantly decreases the volume of the bladder and is quite traumatic, especially when done after previous operations on the pelvic organs. Moreover, this operation is not always possible in case there are post-radiation changes in the small pelvis, when finding a well-perfused bladder flap can be a problem [22, 23].

Intestinal plastic surgery is the optimal approach for extended strictures/obliterations of the distal ureter in somatically intact patients, however, this technique entails a significant number of early and late postoperative complications, including their severe metabolic varieties, the risk of development of which is directly related to the presence of CKD, diabetes mellitus and other systemic diseases. Enteroplasty interventions imply significant intraoperative trauma; they are complicated urological operations with numerous contraindications, including concomitant radiation enteritis [24–26]. Buccal mucosa is particularly well-suited for transplantation into the urinary system: it is hairless, easily accessible for harvesting, survives well in a moist environment and has its own subepithelial vasculature that facilitates graft revascularization. Moreover, harvesting a buccal mucosa graft for urological reconstruction is associated with a low number of reported complications [27].

The use of BG minimizes bladder wall allocation, eliminates the need for traumatic mobilization of the neighboring organs from post-radiation conglomerates, allows avoiding excessive mobilization of the ureter and kidney and reduces the scale of the surgery, its duration and intraoperative blood loss. Although today it is believed that onlay ureteroplasty delivers better late-stage results than ureter reconstruction with a tubularized BG

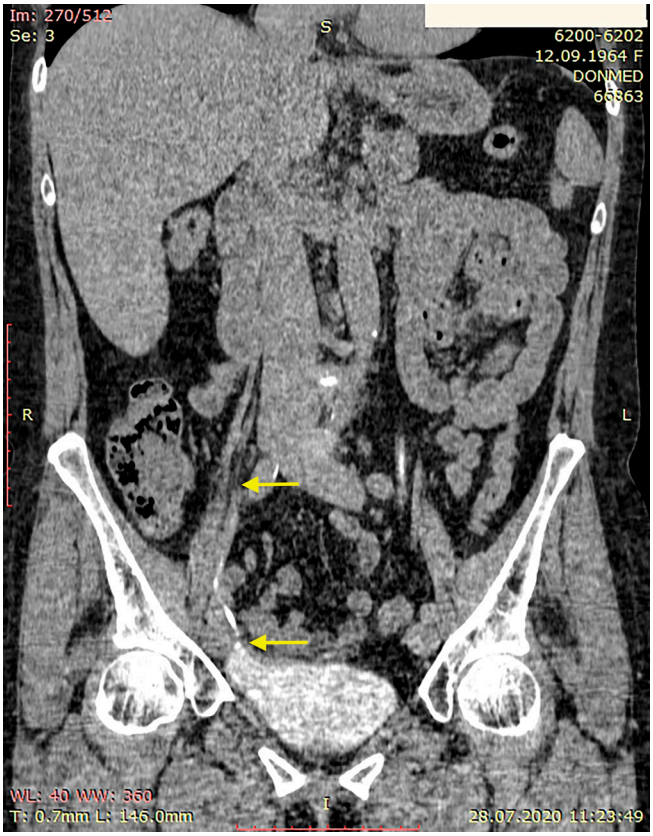


**Fig. 3.** Contrast enhanced computed tomography, post-surgery. Arrows mark the boundaries of the replaced ureter segment

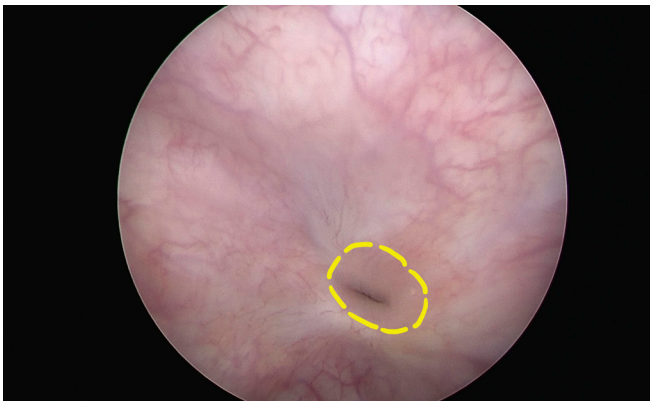

**Fig. 4.** Cystoscopy, post-surgery. Dotted line marks the interface between BG and mucous membrane of the bladder

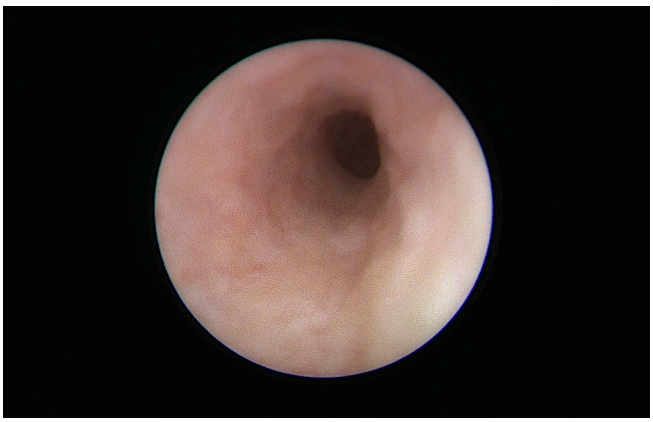

**Fig. 5.** Ureteroscopy, post-surgery. Visible: successful BG engraftment, well-vascularized pink mucosa

[28], the number of reports supporting this belief is limited and disallows comparative studies.

The patients from the group considered had either extensive obliteration of the distal ureter or an extended recurrent stricture with minimal lumen. The tissues were cicatricial, with no signs of any blood circulation in them. We believe that onlaying a BG onto tissue made fibrous by prolonged specific or nonspecific inflammation, repeated surgical interventions or repeated exposure to radiation can lead to necrosis of this BG and recurrence of the disease. At the same time, there is no need to isolate the entire affected part of the ureter; it is enough to prepare a section of the bladder for formation of the new orifice.

As stated in the literature, the main materials enabling vascularization of a BG are omentum flap and perinephric fat [29, 30]. In our case series, we used IS to support vascularization. It also acted as a kind of framework and feeding bed for the neoureter. Simply enveloping a tubularized BG in an omentum flap may be insufficient for neoangiogenesis. Moreover, it translates into a significant expansion of the replaced part of the ureter and can also cause circular stenosis of the implantation site due to omentum scarring [31].

## CONCLUSION

The modest experience we have in using a BG to reconstruct distal ureter in patients with extended strictures and obliterations thereof shows that this technique is a viable option. In our opinion, it can be the technique of choice when using the patient's own urinary or intestinal segments is impossible or poses a high risk, which is especially relevant for patients with post-radiation injuries of pelvic organs. We hope that further modification of the surgical technique and analysis of long-term results registered in a significant number of patients will lead to standardization of the said technique.

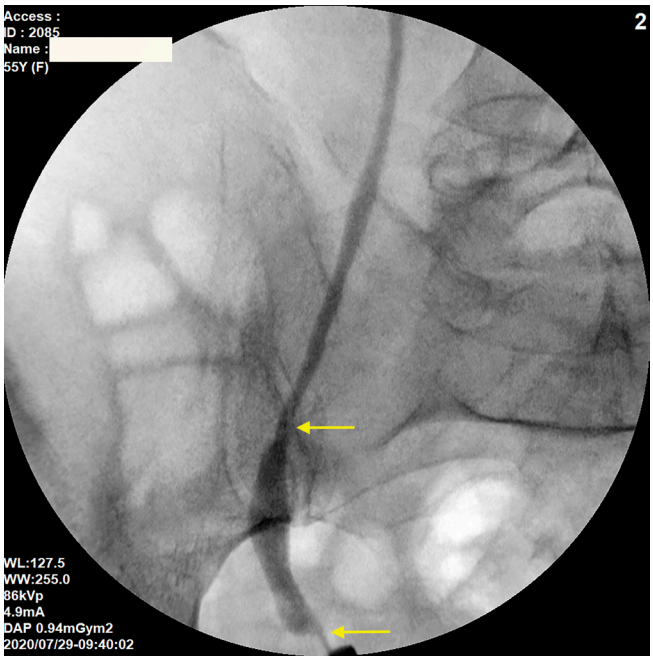


**Fig. 6.** Ascending ureterography, post-surgery. Arrows mark the boundaries of the replaced ureter segment

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# DISSOMIC DISORDERS ASSOCIATED WITH JUVENILE RHEUMATOID ARTHRITIS: IMPACT ON QUALITY OF LIFE

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Dyssomnic disorders (DD) associated with juvenile rheumatoid arthritis (JRA) are some of the most common conditions that are difficult to endure and that lead to deconditioning. This study aimed to assess prevalence and structure of DD, their relationship with clinical picture peculiarities and contribution to deterioration of the quality of lives of JRA patients. At the 1st stage, we assessed prevalence of DD in a continuous sample of JRA patients and healthy children aged 8–16 years. At the 2nd stage, we assessed DD structure, features associated with gender and age, connections to the key clinical characteristics of JRA and quality of life of the patients. In the context of the study, we used the SDSC sleep quality scale, the PedsQL 4.0 quality of life model, and the Ritchie index. DD develop in JRA patients 3.3 times more often than in healthy children (in 178 (72.3%) and 93 (22.2%) children, respectively). The DD registered were sleep initiation and maintenance disorders (54 cases, 22.0%), respiratory disorders (32 cases, 13.0%), sleep-to-wakefulness transition disorders (31 cases, 12.6%), excessive sleepiness disorders (38 cases, 15.4%), combinations thereof (23, 9.3%). Girls had sleep initiation and maintenance disorders more pronounced ($p = 0.003$), boys were more prone to excessive sleepiness (p = 0.008). The severity of DD increases with patients' age ($r = 0.69$; $p = 0.001$) and JRA onset age ($r = 0.71$; $p = 0.001$); they are also more severe in polyarticular JRA patients ($r = 0.32$; $p = 0.048$). We have clarified the relationship between DD and indicators of inflammatory ($r = 0.56$; $p = 0.001$) and Ritchie indices ($r = 0.44$; $p = 0.005$), duration of morning stiffness ($r = 0.49$; $p = 0.029$). The proven connection between DD and JRA entails the need for routine checks for DD in such patients, and, when discovered, DD should call for personalized therapeutic and diagnostic approach rather than be regarded as one of the JRA syndromes.

**Keywords:** juvenile rheumatoid arthritis, dyssomnic disturbances, quality of life

**Author contribution:** AA Elezarov, AS Kucheryavyy — data collection, analysis and interpretation; LN Gumenyuk — research concept and design; LE Sorokina, SR Arifdzhanova, OYu Gerbali — article preparation.

**Compliance with ethical standards:** the study was approved by the ethical committee of the S.I. Georgievsky Medical Academy of Vernandsky Crimean Federal University (Federal State Autonomous Educational Institution), minutes protocol #9 of October 14, 2019); it was planned and conducted in accordance with the Declaration of Helsinki, with parents of all JRA patients and healthy children included in the study signing a voluntary informed consent.

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# ДИССОМНИЧЕСКИЕ РАССТРОЙСТВА ПРИ ЮВЕНИЛЬНОМ РЕВМАТОИДНОМ АРТРИТЕ: ВЛИЯНИЕ НА КАЧЕСТВО ЖИЗНИ

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Диссомнические расстройства (ДР) при ювенильном ревматоидном артрите (ЮРА) — одно из наиболее распространенных, тяжело переносимых и дезадаптирующих состояний. Цель исследования — оценить распространенность и структуру ДР, их взаимосвязь с клиническими особенностями, вклад в снижение качества жизни у больных ЮРА. На I этапе оценивали распространенность ДР в сплошной выборке больных ЮРА и здоровых детей 8–16 лет. На II этапе у больных ЮРА с верифицированными ДР оценивали их структуру, гендерно-возрастные особенности, взаимосвязь с основными клиническими характеристиками ЮРА, качеством жизни больных. Применяли шкалу для оценки качества сна SDSC, опросник качества жизни — PedsQL 4.0, индекс Ричи. У больных ЮРА ДР встречаются в 3,3 раза чаще, чем у здоровых детей (у 178 (72,3%) и у 93 (22,2%) соответственно). Спектр ДР был представлен расстройствами инициации и поддержания сна — 54 (22,0%), расстройствами дыхания — 32 (13,0%), расстройствами перехода сна в бодрствование — 31 (12,6%), расстройствами чрезмерной сонливости — 38 (15,4%) и их сочетаниями — 23 (9,3%). У девочек больше выражены расстройства инициации и поддержания сна ($p = 0,003$), у мальчиков — расстройства чрезмерной сонливости ($p = 0,008$). Тяжесть ДР нарастает по мере увеличения возраста больных ($r = 0,69$; $p = 0,001$) и возраста дебюта ЮРА ($r = 0,71$; $p = 0,001$), при полиартикулярном варианте ($r = 0,32$; $p = 0,048$). Уточнена связь ДР с показателями воспалительного ($r = 0,56$; $p = 0,001$), суставного индексов ($r = 0,44$; $p = 0,005$) и продолжительностью утренней скованности ($r = 0,49$; $p = 0,029$). Наличие взаимосвязи ДР и ЮРА диктует необходимость проведения рутинной диагностики ДР у данной категории больных, при этом для ведения ДР необходимо применять скорее персонифицированный лечебно-диагностический подход, чем расценивать его в качестве одного из синдромов ЮРА.

**Ключевые слова:** ювенильный ревматоидный артрит, диссомнические расстройства, качество жизни

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Dyssomnic disorders (DD) associated with juvenile rheumatoid arthritis (JRA) are some of the most common conditions. They are difficult to endure and lead to deconditioning [1], which makes addressing them an important task from both medical and social points of view. DD entails significant aggravation of the course of underlying disease, modification of the clinical response to treatment and rehabilitation effectiveness deterioration [2]. In addition, DD worsen the child's daytime quality of life: the disorders contribute to the manifestation of or intensify daytime sleepiness, aggressiveness, chronic fatigue, attention deficit, and hamper performance at school [3]. Consequently, the quality of life of the patients deteriorates, they are less likely to stay committed to therapy, and see the associated expenditures going up. At the same time, the connection between existing DD and JRA manifestations, as well as the effect the combination has on the patients' quality of life, are often underestimated in clinical practice.

It is a known fact that JRA patients also suffer from DD, yet the data on their prevalence are contradictory. A number of published papers report DD developing in JRA patients 2.8 times more often than in healthy children of the same age [4, 5]. Other data available point to comparability of DD occurrence in JRA patients to the general population indicators [6].

According to the study reports available, the predominant DD are night wakefulness and anxiety, parasomnia, periodic limb movements, respiratory arrest, early morning awakenings and daytime sleepiness [5]. However, it should be noted that most studies only compare DD in healthy children and JRA patients [7], and the influence of gender and age on the predominance of types of DD in this cohort of patients remains practically unexplored.

The pathophysiology of DD against the background of JRA is multifactorial, and its mechanisms are being studied. The available literature mainly discusses issues related to the cause-and-effect relationships between DD and pain [7]. A few studies consider interconnections DD have with the disease's duration and clinical characteristics [1]. However, the results presented are fragmentary and insufficient for unambiguous conclusions, the probable reasons for that being differences in samples and methodological approaches to research setup.

The effect JRA onset age has on DD type prevalence and manifestation severity has not been investigated in any of the studies. The question of how DD relates to the key characteristics of articular syndrome remains pressing and relevant. There are research reports stating DD influences the quality of life of JRA patients [2, 8], yet the data are isolated and conflicting.

The problem of DD in JRA patients is not adequately covered in the current scientific literature by Russian authors.

This study aimed to assess degree of occurrence and prevalence of types of DD, analyze their relationship with clinical picture peculiarities and contribution to deterioration of the quality of lives of JRA patients.

## METHODS

The study included two stages. At the first stage, we assessed the prevalence of DD in a continuous sample of JRA patients and healthy children. The second stage was a one-stage prospective study of JRA patients with comorbid dyssomnic disorders. The goal was to assess structure, gender and age characteristics of patients with DD, the connections these aspects have with the key historical data and clinical characteristics of JRA, as well as the quality of life of patients.

The study's continuous sample was comprised of 246 JRA patients (mean age of 12.6 ± 2.4 (10.2; 15.0) years old), including 140 girls (56.9%) and 106 boys (43.1%), and 420 healthy children (average age of 12.6 ± 2.4 (10.2; 15.0) years old), including 252 girls (60.0%) and 168 boys (40.0%) that met the inclusion criteria. The groups were comparable in gender ($p = 0.95$; $\chi^2$) and age ($p = 0.91$; $\chi^2$).

The inclusion criteria for JRA patients were: verified JRA; age 8 to 16 years; 1 year or more of the underlying disease course; informed consent to participate in the study.

The exclusion criteria were: concomitant chronic inflammatory diseases; mental disorders; systemic or intra-articular administration of glucocorticoids four weeks before the start of the study; refusal to participate in the clinical trial.

The inclusion criteria for healthy children were: age 7 to 16 years; absence of chronic diseases and allergic reactions; absence of infectious and acute diseases for two months before the study.

For the 2nd stage of the study, 178 patients with DD verified at the 1st stage were selected, with DD determined using the Sleep disturbance scale for children, SDSC. The patients were grouped by the type of DD: sleep initiation and maintenance disorders ($n = 54$), respiratory disorders ($n = 41$), sleep-to-wakefulness transition disorders ($n = 36$), excessive sleepiness disorders ($n = 47$). The groups were analyzed individually, depending on the type of DD identified. In order to level the effect combined DD types may have on the studied parameters, the analysis did not include patients who scored above the threshold on two or more subscales of SDSC.

JRA diagnosis, nature of its course and the activity status were established as prescribed in ICD-10 (class 8) [9], ARA [10] and EULAR [11] recommendations. The Ritchie index was used to assess the articular syndrome. SDSC enabled assessment of sleep disorders [12], while Pediatric quality of life inventory (PedsQL 4.0, form for children) allowed establishing the patients' quality of life (QOL) [13].

STATISTICA 8.0 software package (StatSoft Inc.; USA) was used to statistically process the data obtained. The values distribution was checked for conformity with the standard normal distribution with the help of Kolmogorov–Smirnov test. Since the majority of quantitative characteristics did not meet the normal distribution requirements, they were described using the median (Me) and quartiles (Me (25%; 75%)). Mann–Whitney $U$ test enabled comparison of quantitative characteristics of independent groups, while for qualitative characteristics we relied on the analysis of contingency tables using the $\chi^2$ (chi-square) test and the Fisher's exact test. Comparison of quantitative characteristics registered in dependent groups was enabled by the Wilcoxon test. The differences were considered significant at $p < 0.05$. Spearmen's rank-order correlation backed assessment of relationships between the characteristics.

## RESULTS

Two hundred and eleven (85.7%) JRA patients complained about sleep problems, while in the group of healthy children this figure was 140 (33.4%). With the difference being significant, parents of JRA patients reported the following more often than parents of the participating healthy children: frequent nocturnal awakenings ($p = 0.009$), shorter total sleep duration ($p < 0.001$), apnea episodes ($p = 0.001$), periodic limb movements ($p = 0.006$), bruxism ($p = 0.009$), daytime sleepiness ($p < 0.001$). SDSC allowed diagnosing DD in 178 (72.3%) JRA patients and in 93 (22.2%) healthy children ($p < 0.001$). In JRA patients, the DD were sleep initiation and maintenance disorders, respiratory disorders, sleep-to-wakefulness transition disorders, excessive sleepiness, as well as combinations thereof (Fig.).

Compared to patients without sleep initiation and maintenance disorders (SI and MD) (mean SI and MD subscale score — 20.2 (16.0; 22.0)), those suffering from SI and MD (mean SI and MD subscale score — 25.7 (23.0; 28.0)) were significantly older, had JRA onset at a later time, suffered from the disease for a significantly longer period of time, had it in polyarticular form more often, had more affected joints, slept considerably less, exhibited bruxism episodes more often, with bruxism being more severe, suffered from postsomnic fatigue, had more severe nocturnal awakenings and excitement disorder (ED) manifestations, more severe pain, lower QOL emotional component (EC) and integral (IS) scores (Table 1).

Patients with respiratory disorders (RD) (mean score on the RD subscale — 10.6 (9.0; 12.5)), compared to patients without RD (mean score — 4.9 (3.0; 6.7)), were significantly younger, had the disease for a shorter period of time, took longer to fall asleep, had more severe nighttime restlessness and snoring, suffered from daytime sleepiness (its acute forms) more often, had excessive sleepiness disorders (ESD) more severe and scored lower on the QOL physical component (PC) scale (Table 2).

Patients with sleep-to-wakefulness transition disorders (SW TD) (mean score on the SW TD subscale — 26.5 (25.0; 27.0)), compared to the participants not suffering the condition (mean score — 18.2 (16.0; 20.0)) had the following distinctive characteristics: older age, significantly more frequent occurrence of oligoarticular variant of JRA, more frequent and severe difficulties with laying down to sleep, manifestations of problems with falling asleep, more frequent occurrence and higher severity of bruxism, nightmares, excessive sweating, difficult awakening, greater severity of periodic limb movements and frequent pose changes (PLM and FPC), severe excitement disorders and excessive sleepiness, more acute pain experienced, lower QOL social component (SC) score (Table 3).

Patients with ESD (mean score on the ESD subscale —23.1 (21.0; 25.0)), compared with the study participants without ESD (mean score — 14.4 (12.0; 18.0)), were predominantly male, older, had the JRA onset earlier and life and lived with the condition for a longer period of time, woke up at night more frequently, slept considerably less, woke up during the night more frequently and more often, stayed awake longer after falling asleep, exhibited more episodes of snoring, suffered more acute postsomnic asthenia, scored lower on the sleep quality scale, experienced pain more acutely, performed less well in school and scored lower on the integral QOL scale (Table 4).

In connection with the dependence of the course of many diseases on gender, the JRA patients' DD structural features and severity were analyzed with sexual dimorphism factored in. According to the data obtained, girls, compared to boys, were more prone to suffer from shorter sleep duration (73.7 (66.4%) cases), nightmares (44 (39.9%) cases) and postsomnic asthenia (79.0 (71.2%) cases), while the respective figures reflecting same conditions in boys are 29 (42.8%) cases (at $p = 0.029$), 16 (23.6%) cases (at $p = 0.048$) and 30 (44.9%) cases (at $p = 0.001$). The difference is significant. Girls have also shown a significantly greater number of nocturnal awakenings episodes — 7.0 (5.2; 7.8) versus 5.0 (3.1; 6.4) in boys ($p = 0.002$), — and bruxism episodes — 4.5 (3.0; 5.0) versus 3.3 (2.0; 4.0) in boys ($p = 0.019$). Significant gender-driven differences were also registered in the prevalence of SI and MD (25.1 (23; 28) in girls versus 23.0 (21; 24) in boys, $p = 0.003$), and ESD (24.2 (22; 26) in boys versus 21,6 (20; 23) in girls ($p = 0.008$).

Investigating the effect of age on the DD type prevalence, we established that the combination of SI and MD and SW TD symptoms was significantly more often registered in patients aged 15.7 (14.1; 16.0) compared to patients 10.9 (7.0 ; 14.0) years old; the scores were 37 (36.1%) points versus 12 (16.4%) points, respectively ($p = 0.007$). The integral sleep quality score (ISQS) was significantly lower in patients aged 8.6 (6.0; 10.4) compared with patients 13.7 (10.1; 16.0) years old, the difference in points being 26.7 (22.4; 28.0) versus 32.2 (29.6; 35.8), respectively ($p < 0.001$). The correlations between age and frequency of nocturnal symptoms ($r = 0.55$; $p = 0.001$) and DD manifestation intensity ($r = 0.69$; $p = 0.001$) were found to be significant.

We have established a correlation between the JRA onset age and DD manifestation intensity: the values reflecting the

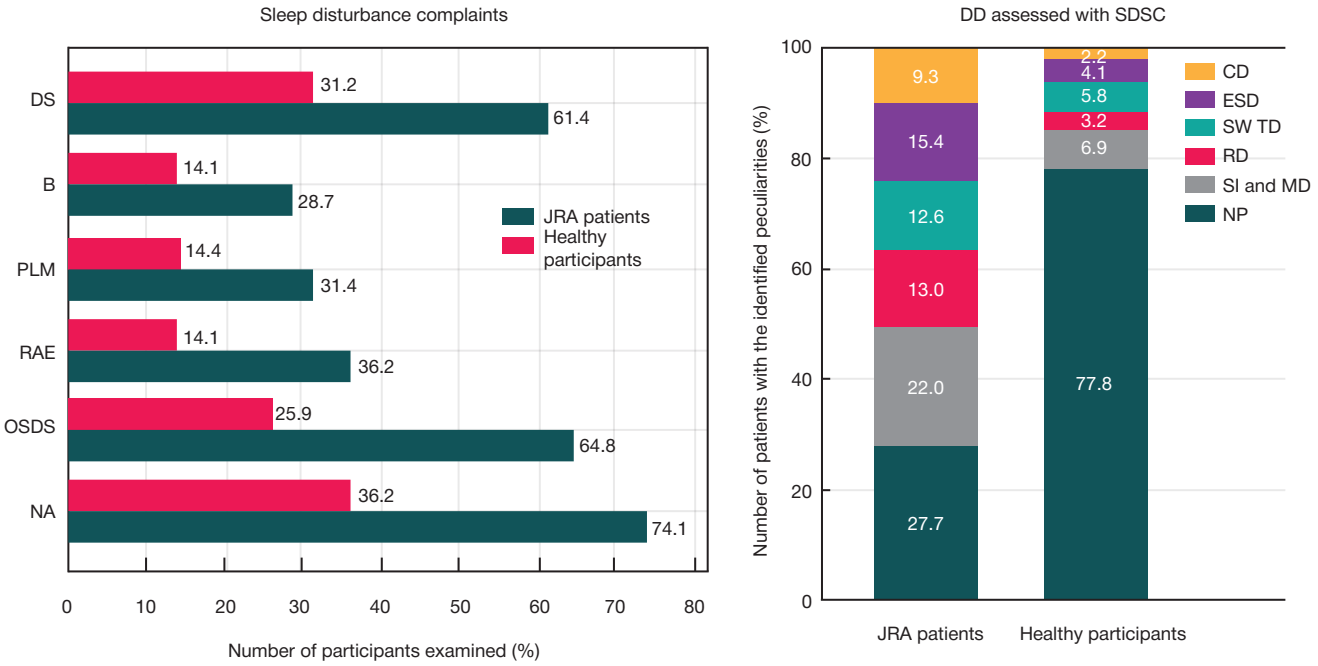


**Fig.** Structure of sleep disorders, JRA patients and healthy children. DD — dyssomnic disorders; NA — nocturnal awakenings; OSDS — overall sleep duration shortening; RAE — respiratory arrest episodes; PLM — periodic limb movements; B — bruxism; DS — daytime sleepiness; NP — no peculiarities; SI and MD — sleep initiation and maintenance disorders; RD — respiratory disorders; SW TD — sleep-to-wakefulness transition disorders; ESD — excessive sleepiness disorders; CD — concomitant disorders

**Table 1.** Comparative analysis of patients with and without sleep initiation and maintenance disorders

| | With SI and MD $n = 44$ | Without SI and MD $n = 96$ | $p$ |
|---|---|---|---|
| Age | 14.2 (13.3; 16.0) | 8.1 (7.0; 13.2) | $p = 0.063^{\bullet}$ |
| JRA onset (age) | 11.4 (6.0; 15.0) | 3.4 (1.0; 5.0) | $p < 0.001^{***}$ |
| JRA duration (years) | 6.2 (5.8; 7.1) | 3.3 (1.0; 4.6) | $p = 0.006^{**}$ |
| Polyarticular variety (%) | 62.4 | 33.2 | $p = 0.008^{\bullet\bullet}$ |
| Over 4 affected joints (%) | 71.4 | 42.6 | $p = 0.044^{\bullet}$ |
| SDSC 1 Sleep duration (hours) | 5.4 (5.0; 7.3) | 7.1 (6.5; 8.2) | $p = 0.001^{**}$ |
| SDSC 10 Nocturnal awakenings (points) | 4.1 (4.0; 5.0) | 3.4 (2.0; 4.0) | $p = 0.002^{**}$ |
| Nocturnal awakenings (times per night) | 6.1 (4.8; 7.4) | 4.2 (3.2; 5.2) | $p = 0.003^{**}$ |
| SDSC 11 Wakefulness after falling asleep (points) | 4.2 (3.0; 5.0) | 3.1 (2.0; 4.0) | $p = 0.011^{*}$ |
| SDSC 19 Bruxism (points) | 3.5 (2.0; 5.0) | 2.9 (1.0; 4.0) | $p = 0.007^{**}$ |
| SDSC 23 Postsomnic sleep fatigue (points) | 4.1 (3.0; 5.0) | 2.8 (2.0; 4.0) | $p = 0.013^{**}$ |
| Postsomnic fatigue, yes/no (%) | 78.9/21.1 | 61.4/38.6 | $p = 0.006^{\bullet\bullet}$ |
| SDSC 24 Motor limitations during awakening (points) | 3.6 (3.0; 4.0) | 1.8 (1.0; 2.0) | $p = 0.012^{*}$ |
| Motor limitations during awakening, yes/no (%) | 42.2/57.8 | 12.1/87.9 | $p < 0.001^{\bullet\bullet\bullet}$ |
| SDSC PB subscale (points) | 7.8 (6.0; 9.0) | 4.2 (1.0; 6.0) | $p = 0.001^{**}$ |
| SDSC Integral indicator (points) | 54.4 (52.0; 61.0) | 45.4 (41.0; 50.0) | $p = 0.009^{**}$ |
| Pain index (points) | 3.1 (2.0; 4.2) | 1.5 (1.2; 1.8) | $p = 0.034^{*}$ |
| EC QOL (points) | 55.6 (50.9; 61.0) | 66.8 (60.0; 72.5) | $p = 0.004^{**}$ |
| IA QOL (points) | 59.8 (54.8; 64.7) | 67.1 (62.1; 71.4) | $p = 0.003^{**}$ |

**Note:** the values are given at $p < 0.05$; * — $p < 0.05$ (significance of differences estimated with Mann–Whitney $U$ test); ** — $p < 0.01$ (significance of differences estimated with Mann–Whitney $U$ test); *** — $p < 0.001$ (significance of differences estimated with Mann–Whitney $U$ test); • — $p < 0.05$ (significance of differences estimated with $\chi^2$ test); •• — $p < 0.05$ (significance of differences estimated with $\chi^2$ test); ••• — $p < 0.05$ (significance of differences estimated with $\chi^2$ test); n is the number of observations.

state of SI and MD, ESD, ISQS were significantly higher in patients that saw the onset of JRA at an older age (11.2 (10.0; 15.0) years) compared with the patients in whom the onset of JRA happened earlier (5.6 (1.0; 10.0) years), with the scores being 31.2 (26.2; 33.0), 25.1 (23.0; 27.4) and 49.7 (45.0; 52, 1) points versus 24.6 (22.1; 26.0) ($p = 0.008$), 18.9 (16.0; 20.2) ($p = 0.006$) and 35.9 (32.0; 38.1) points ($p = 0.001$), respectively. The dependence of DD severity on the JRA onset age was confirmed by the significant correlation between the JRA onset age and the ISQS score established with the help of SDSC ($r = 0.71$; $p = 0.001$).

The longer a patient had JRA, the more severe were sleep initiation and maintenance and excessive sleepiness disorders suffered by that patient: the SI and MD score in patients who had JRA for 8.3 (6.1; 9.7) years was 32.2 (29.0; 34, 1) points, which is significantly higher than the same score shown by patients that had JRA for 2.2 (1.0; 3.0) years, who scored 24.8 (23.0; 26.1) points ($p = 0.001$), and those that suffered from the condition for 4.5 (3.1; 6.0) years, the score of which was 28.4 (24.0; 30.0) points ($p = 0.008$). As for the ESD, the scores were 22.9 (20.0; 23.4) points, 19.2 (15.0; 20.9) points ($p = 0.048$) and 16.5 (14.4; 20.1) points ($p = 0.005$), respectively. Regardless of the types of DD registered and the manifestation intensity of the disorders, patients with JRA affecting their lives for 8.3 years exhibited a significantly shorter sleep duration compared to those who were living with the diseases for 2.2 (1.0; 3.0) years, the figures being 6.6 (5.0; 7.0) hours versus 8.6 (8.0; 9.0) hours, respectively ($p = 0.001$). However, we found no correlation between ISQS score and duration of the disease: patients that had JRA for 2.2 (1.0; 3.0) years scored

**Table 2.** Comparative analysis of patients with and without respiratory disorders

| Parameters | With RD $n = 32$ | Without RD $n = 135$ | $p$ |
|---|---|---|---|
| Age (years) | 9.4 (7.0; 11.6) | 15.8 (11.7; 16.0) | $p = 0.008^{\bullet\bullet}$ |
| JRA duration (years) | 3.1 (1.0; 4.2) | 5.7 (3.0; 6.6) | $p = 0.034^{*}$ |
| SDSC 2 Time needed to fall asleep (minutes) | 38.1 (30.0; 42.4) | 51.4 (47.2; 55.5) | $p = 0.004^{**}$ |
| SDSC 5 Nocturnal anxiety/fears (points) | 4.3 (3.0; 5.0) | 3.4 (2.0; 5.0) | $p = 0.011^{*}$ |
| SDSC 14 Respiratory arrest (points) | 4.2 (4.0; 5.0) | 2.2 (2.0; 3.0) | $p = 0.003^{**}$ |
| SDSC 15 Snoring (points) | 3.5 (3.0; 5.0) | 2.1 (1.0; 3.0) | $p = 0.008^{**}$ |
| SDSC 25 Daytime sleepiness (points) | 4.2 (3.0; 5.0) | 2.8 (2.0; 4.0) | $p = 0.003^{**}$ |
| Daytime sleepiness, yes/no (%) | 62.5/37.5 | 44.6/55.4 | $p = 0.046^{\bullet}$ |
| SDSC ESD subscale (points) | 16.4 (13.0; 18.0) | 11.6 (10.0; 14.0) | $p = 0.009^{**}$ |
| FC QOL (points) | 58.4 (55.7; 60.0) | 63.1 (61.1; 66.7) | $p = 0.007^{**}$ |

**Note:** the table shows values at $p < 0.05$; * — $p < 0.05$ (significance of differences estimated with Mann–Whitney $U$ test); ** — $p < 0.01$ (significance of differences estimated with Mann–Whitney $U$ test); *** — $p < 0.001$ (significance of differences estimated with Mann–Whitney $U$ test); • — $p < 0.05$ (significance of differences estimated with $\chi^2$ test); •• — $p < 0.05$ (significance of differences estimated with $\chi^2$ test); n is the number of observations (here and in tables 2–4).

33.9 (31.2; 36.8) points, those that had it for 4.5 (3.1; 6.0) years scored 32.4 (30.0; 35.8) points and patients that suffered from JRA for 8.3 (6.1; 9.7) years showed the score of 35.1 (32.4; 36.9) points.

Investigating the relationship between JRA type and DD peculiarities, we discovered that patients with polyarticular JRA tended to wake up at night more frequently and had SI and MD manifesting more intensely than those who had oligoarticular JRA, the figures being 6.5 (3.8; 7.3) awakenings per night, 38.4 (35.2; 40.1) points versus 5.7 (3.4; 6.6) awakenings per night, 37.6 (33.9; 39.2) points, respectively. However, the difference was not significant. The daytime sleepiness index in patients with four or more affected joints was significantly higher than in patients with one to three joints affected: 4.4 (3.0; 5.0) points versus 3.1 (2.2; 4, 0) points, respectively ($p$ = 0.004). Correlation analysis revealed a relationship between daytime sleepiness manifestation intensity and the number of affected joints: the more intense the former, the greater the number of the latter ($r$ = 0.32; $p$ = 0.048).

More intense JRA symptoms were associated with more pronounced DD manifestations, but ISQS did not differ significantly depending on JRA intensity: children with JRA in remission had this indicator at 32.2 (30.0; 34.1) points, those with the I degree JRA — at 33.1 (30.9; 34.8) points, with the II degree — at 34.2 (30.0; 35.2) points, with the III degree JRA — at 34.4 (31.6; 36.0) points. The results of correlation analysis confirmed there is no relationship between the degree of JRA and the severity of DD.

Investigating the effect DD has of the JRA patients' QOL, we discovered the following: lower physical functioning scores correlated with shorter sleep time ($r$ = 0.56; $p$ = 0.001), growing postsomnic fatigue ($r$ = –0.49; $p$ = 0.028), RD ($r$ = –0.46; $p$ = 0.001) and ESD ($r$ = –0.73; $p$ = 0.0001), as well as lower ISQS ($r$ = –0.56; $p$ = 0.001). Emotional functioning scale score depended on the duration of sleep ($r$ = 0.71; $p$ = 0.008), frequency of nocturnal awakenings ($r$ = –0.74; $p$ = 0.001), severity of bruxism ($r$ = –0.81; $p$ = 0.0001), SI and MD ($r$ = –0.77; $p < 10^{-6}$) and ISQS ($r$ = –0.71; $p$ = 0.0001). Social functioning scale score correlated with the frequency of apnea episodes ($r$ = –0.44; p = 0.012), postsomnic fatigue ($r$ = –0.55; $p$ = 0.001), daytime sleepiness ($r$ = –0.79; $p$ = 0.028), SI and MD ($r$ = –0.44; $p$ = 0.006), SW TD ($r$ = –0.68; $p$ = 0.012). School performance scores reflected the frequency of apnea episodes ($r$ = –0.67; $p$ = 0.001), severity of daytime sleepiness ($r$ = –0.88; $p$ = 0.006) and ESD ($r$ = –0.92; $p$ = 0.008). Decreasing Integral Indicator of Quality of Life correlated with the growing SI and MD indicators ($r$ = –0.91; $p$ = 0.001), ISQS ($r$ = –0,91; $p < 10^{-6}$).

## DISCUSSION

It is a known fact that JRA patients also suffer from DD, yet the data on their prevalence and typical structure are scarce and fragmentary, and assumptions about their connection with the disease controversial. A number of studies report that, compared to healthy pees, JRA patients largely suffer from nocturnal wakefulness, restless sleep, respiratory disorders, periodic limb movements in sleep, and daytime sleepiness [5–7, 14]. At the same time, the figures that are given as reflecting the occurrence of DD in JRA patients are comparable to those peculiar to the population in general [6]. Thus study shows that JRA patients have DD 3.3 times more often than their healthy peers (72.3 versus 22.2%). Our results are consistent with the data from earlier studies that established a high prevalence of DD among JRA patients (from 40 to 71.4%) [4, 5] compared with the same age population groups not having JRA (25%) [4].

According to our data, in JRA patients, the occurrence of periodic limb movements during sleep, nocturnal awakenings and daytime sleepiness exceeds the indices of healthy peers

**Table 3.** Comparative analysis of patients with and without sleep-to-wakefulness transition disorders

| Parameters | With SW TD<br>$n$ = 26 | Without SW TD<br>$n$ = 114 | $p$ |
|---|---|---|---|
| Age (years) | 15.2 (14.0; 16.0) | 11.6 (7.4; 13.9) | $p$ = 0.048• |
| Oligoarticular variety, $n$ (%) | 64.2/35.8 | 32.1/67.9 | $p$ = 0.006•• |
| SDSC 3 Difficulties with putting to bed (points) | 3.8 (3.0; 5.0) | 1.9 (1.0; 3.0) | $p$ = 0.004** |
| Difficulties with putting to bed, yes/no (%) | 64.4/35.6 | 22.9/77.1 | $p$ = 0.001•• |
| Difficulties with falling asleep, yes/no (%) | 72.8/27.2 | 42.5/57.5 | $p$ = 0.001• |
| SDSC 12 PLM and FPC (points) | 4.1 (2.0; 5.0) | 2.2 (1.0; 4.0) | $p$ = 0.007** |
| SDSC 16 Excessive sweating (points) | 3.4 (2.0; 4.0) | 2.5 (1.0; 3.0) | $p$ = 0.011* |
| SDSC 16 Excessive sweating, yes/no (%) | 46.6/53.4 | 14.2/85.8 | $p$ < 0.001••• |
| SDSC 19 Bruxism (points) | 4.3 (3.0; 5.0) | 2.9 (1.0; 3.0) | $p$ = 0.003** |
| SDSC 19 Bruxism, yes/no (%) | 88.6/11.4 | 14.2/85.8 | $p$ < 0.001••• |
| SDSC 21 Nightmares (points) | 4.2 (3.0; 4.0) | 2.8 (2.0; 3.0) | $p$ = 0.015* |
| Nightmares, yes/no (%) | 51.1/48.9 | 33.4/66.6 | $p$ = 0.019• |
| SDSC 22 Difficulties with waking up points | 4.5 (4.0; 5.0) | 2.4 (1.0; 3.0) | $p$ = 0.005** |
| Difficulties with waking up, yes/no (%) | 81.7/18.3 | 35.4/64.6 | $p$ = 0.006•• |
| SDSC PB subscale (points) | 10.1 (6.0; 12.0) | 8.1 (4.0; 10.0) | $p$ = 0.009** |
| SDSC ESD subscale (points) | 19.2 (16.0; 20.0) | 14.4 (12.0; 18.0) | $p$ = 0.006** |
| Pain index (points) | 3.4 (2.0; 4.5) | 2.5 (2.2; 3.6) | $p$ = 0.021* |
| EC QOL (points) | 55.6 (50.9; 61.0) | 66.8 (60.0; 72.5) | $p$ = 0.005** |
| SF QOL | 54.4 (51.2; 57.8) | 66.4 (61.1; 70.7) | $p$ = 0.001** |

**Note:** the table shows values at $p < 0.05$; * — $p < 0.05$ (significance of differences estimated with Mann–Whitney $U$ test); ** — $p < 0.01$ (significance of differences estimated with Mann–Whitney $U$ test); *** — $p < 0.001$ (significance of differences estimated with Mann–Whitney $U$ test); • — $p < 0.05$ (significance of differences estimated with $\chi^2$ test); •• — $p < 0.05$ (significance of differences estimated with $\chi^2$ test); ••• — $p < 0.05$ (significance of differences estimated with $\chi^2$ test); n is the number of observations.

and confirms the viewpoint of a number of other researchers [5]. We did not confirm the high incidence of presomnic disorders (namely, difficulties with falling asleep) in JRA patients, which was reported in other studies [15]. According to our data, the figures reflecting prevalence of frequent nocturnal awakenings and their association with more pronounced daytime sleepiness are close to those obtained in two other studies [5, 16]. In contrast to the already published results [1, 6, 14], which make parasomnia and respiratory disorders during sleep the most frequent manifestations of DD in JRA patients, we have discovered that such patients often sleep less in total, i.e. their overall sleep duration is shorter. Compared to their healthy peers, children with JRA and their parents report a significantly higher incidence of parasomnias in the form of nightmares and sleepwalking [7]. We have discovered that the predominant parasomnia in JRA patients is bruxism.

This study shows the connection between DD and patient gender. Girls were registered to more often suffer from shortened sleep duration, nightmares and post-sleep asthenia, they wake up and have bruxism episodes more often and suffer from more acute SI and MD, while boys tend to have ESD. These differences may be the result of both biological and psychosocial factors. Taking into account the results obtained and the therapy personalization trend, the question of the specificity of intergender differences in the manifestation and pathophysiological basis of DD associated with JRA needs further investigation. We have discovered how age affects the specifics of DD: the older the patients, the more often they have polymorphic variety of the disorder and the more severe are the dyssomnic manifestations. This result of our study disagrees with the report of one research effort that states inverse correlation between patients' age and sleep disorders [17].

Duration of the disease and clinical and anatomical features (variety, number of affected joints, process intensity) are considered to be the potential prerequisites that determine peculiarities of sleep disorders in JRA patients [1]. However, the data on the role played by these factors in sleep pattern changes are ambiguous.

It was established that patients with ESD score highest on the inflammatory index scale and have the longest periods of morning stiffness. JRA patients that had the disease onset later in their adolescence, between the ages of 11 and 15, suffer from more severe forms of SI and MD, ESD. The severity of DD increases together with the age at which the patient had the JRA onset, which is proved by a significant strong correlation between the age of JRA onset and ISQS as determined with SDSC. We did not find this fact reported in any similar work, therefore, it needs additional investigation.

This study has established that sleep duration decreases and SI and MD, ESD manifestation intensity increases as JRA matures. However, the relationship between ISQS and JRA duration has not been confirmed. The data obtained are close to the results indicating no or weak link between ISQS and the duration of the disease [18].

We have confirmed there are no differences in the intensity of DD depending on the suffered JRA variety, which was reported earlier [1]. Polyarticular JRA was established to be associated with the growing frequency of nocturnal awakenings and more intense SI and MD, but the association did not reach statistical significance. Some research reports confirm the link between DD types and the number of affected joints, but these results are contradictory [18]. In JRA patients, a clear relationship was established: the severity of daytime sleepiness increases as the number affected joints grows. The findings are consistent with the results of a study that reported higher PDSS (pediatric daytime sleepiness scale) scores shown by patients with polyarthritis [19]. Lack connection between the intensities of DD and JRA discovered by us is consistent with

**Table 4.** Comparative analysis of patients with and without excessive sleepiness disorders

| Parameters | With ESD $n = 38$ | Without ESD $n = 129$ | $p$ |
|---|---|---|---|
| Gender, male/female (%) | 56.8/21.2 | 33.2/66.8 | $p = 0.021^{\bullet}$ |
| Age (years) | 13.9 (12.0; 16.0) | 9.2 (7.0; 11.9) | $p = 0.018^{\bullet}$ |
| JRA duration (years) | 5.8 (3.5; 7.6) | 3.1 (1.0; 4.4) | $p = 0.016^{*}$ |
| JRA onset age | 3.9 (1.0; 8.1) | 9.1 (6.6; 16.0) | $p = 0.002^{**}$ |
| Activity degree I, *n* (%) | 34.6 | 55.2 | $p = 0.027^{\bullet}$ |
| Activity degree III, *n* (%) | 62.5 | 39.5 | $p = 0.009^{\bullet\bullet}$ |
| SDSC 1 Sleep duration (hours) | 6.9 (6.0; 7.5) | 8.4 (8.0; 9.8) | $p = 0.004^{**}$ |
| SDSC 10 Nocturnal awakenings (points) | 4.6 (4.0; 5.0) | 3.1 (2.0; 4.0) | $p = 0.003^{**}$ |
| Nocturnal awakenings (times per night) | 7.3 (5.2; 9.4) | 4.2 (2.8; 5.6) | $p = 0.003^{**}$ |
| Wakefulness after falling asleep (minutes) | 22.4 ± 3.4 | 10.2 ± 1.8 | $p < 0.001^{***}$ |
| SDSC 14 Respiratory arrest, yes/no (%) | 68.6/31.4 | 39.5/60.5 | $p < 0.001^{\bullet\bullet\bullet}$ |
| SDSC 15 Snoring (points) | 3.6 (3.0; 5.0) | 2.2 (1.0; 3.0) | $p = 0.005^{**}$ |
| Snoring, yes/no (%) | 56.7/43.3 | 32.6/67.4 | $p = 0.006^{\bullet\bullet}$ |
| SDSC 23 Postsomnic sleep fatigue (points) | 4.6 (4.0; 5.0) | 3.3 (2.0; 4.0) | $p = 0.012^{*}$ |
| SDSC Integral indicator (points) | 63.1 (60.0; 66.0) | 53.2 (50.0; 60.0) | $p = 0.001^{**}$ |
| Inflammatory index (points) | 0.8 (0.5; 0.9) | 0.6 (0.3; 0.7) | $p = 0.018^{*}$ |
| Morning stiffness (minutes) | 48.6 (44.2; 52.0) | 39.1 (36.3; 40.2) | $p = 0.001^{**}$ |
| SF QOL | 54.9 (52.0; 58.5) | 62.2 (58.4; 65.0) | $p = 0.015^{*}$ |
| IA QOL (points) | 58.0 (56.8; 60.7) | 66.1 (64.0; 68.9) | $p = 0.006^{**}$ |

**Note:** the table shows values at $p < 0.05$; * — $p < 0.05$ (significance of differences estimated with Mann–Whitney *U* test); ** — $p < 0.01$ (significance of differences estimated with Mann–Whitney *U* test); *** — $p < 0.001$ (significance of differences estimated with Mann–Whitney *U* test); • — $p < 0.05$ (significance of differences estimated with $\chi^2$ test); •• — $p < 0.05$ (significance of differences estimated with $\chi^2$ test); ••• — $p < 0.05$ (significance of differences estimated with $\chi^2$ test); *n* is the number of observations.

the data obtained in the context of study [1], which has shown no correlation between intensity of the disease activity and severity of sleep disorders as registered with CSHQ.

We have shown the multifactorial nature of DD in JRA patients and clarified the relationship of their manifestations with the main parameters of the articular syndrome. A number of studies interprets pain experienced by JRA patients as a predictor of sleep disorders [7, 8], but there is no evidence of a direct connection between pain and dyssomnic disorders. Most authors note that conceptual models of sleep disorders and pain imply complex, bi-directional relationships [14]. At the same time, it was shown that pain-dependent sleep quality deterioration in JRA patients is of greater prognostic significance, unlike the bi-directional influence in these relationships [20]. In our study, we have demonstrated that patients with SI and MD experience the most pronounced increase in the pain syndrome. We have also established a strong correlation between shortening of the overall sleep duration and aggravation of pain. This link was to be expected, since shorter sleep reinforces central sensitization, which any sensitivity threshold decrease is based on. The results obtained confirm the findings of the previous studies, which point out that self-assessment of the severity and frequency of pain largely (significantly) depends on the sleep structure and quality and distortions thereof [1, 6, 7]. However, the reported significant correlation between daytime sleepiness and pain severity [19] was not confirmed in our study. We have shown that ESD patients have the highest inflammatory index and morning stiffness duration figures. We did not find this fact stated in any similar work, which means it should be studied further.

An important result of this study is the clarification of the influence of DD manifestations on the JRA patients' quality of life parameters. It should be noted that studies covering the effect various JRA manifestations have on the quality of life provide varying assessments of DD's contribution thereto, with some naming them one of the key factors [2] and other discounting such disorders as the factors of low significance [8].

The inconsistency of data may be the result of heterogeneity in the population characteristics of the patients examined, as well as differences in the methodological approaches to setup of the studies. In our study, we have shown that JRA patients with concomitant SI and MD score poorly in terms of emotional functioning, those that have RD lack in physical functioning, patients with SW TD do not do well socially and those suffering from ESD underperform in school. We have also confirmed the relationship between DD intensity and the integral assessment of QOL. The significant negative effect DD has on the QOL indicators of JRA patients is the basis for development of the adequate remedial techniques.

## CONCLUSION

Thus, the proven connection between DD and JRA entails the need for routine checks for DD in such patients. DD should call for personalized therapeutic and diagnostic approach rather than be regarded as one of the JRA syndromes. Further study of the relationship between DD and JRA should go beyond registration of epidemiological data and include investigation of the mechanisms of pathogenesis of these comorbidities.

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# QUALITY OF LIFE IN STROKE PATIENTS IN RESIDUAL STROKE PERIOD AND ITS DETERMINANTS

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Stroke remains one of the leading causes of disability; therefore, it is important to investigate factors that might affect the quality of life of stroke patients and refine rehabilitation technologies for better functional gains. The aim of this paper was to study possible factors that determine the quality of life in the residual ischemic stroke period. The MOS SF-36 health survey was completed by 210 patients undergoing early rehabilitation at a stroke care unit. The study revealed a significant decline in some quality of life indicators correlated with advancing age and severity of the condition (correlation coefficient –0.5; $p < 0.01$). Both physical and mental component summary scores were lower in women than in men ($p < 0.01$ and $p < 0.001$, respectively). High scores on the majority of the applied subscales were observed in the patients with a vertebrobasilar stroke, as compared with those who had suffered a carotid stroke ($p < 0.05$). The early rehabilitation regimen complemented with acupuncture in the acute stroke period and the subsequent rehabilitation program at the Rehabilitation Hospital significantly contributed ($p < 0.05$) to improving the quality of life of stroke patients in the residual stroke period.

**Keywords:** quality of life, ischemic stroke, rehabilitation, acupuncture

**Author contribution:** Molchanova EE — study design; data acquisition and analysis; manuscript preparation; Polunina VV, Polyaev BA, Plotnikov VP, Lobov AN, Parastaev SA — study concept and manuscript preparation. All authors read and approved the final version of the manuscript.

**Compliance with ethical standards:** the study was approved by the Ethics Committee of Amur State Medical Academy (Protocol № 10 dated November 20, 2019); the study conformed with the guidelines for the medical research involving human subjects. Voluntary informed consent was obtained from all the participants.

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# КАЧЕСТВО ЖИЗНИ ПАЦИЕНТОВ В РЕЗИДУАЛЬНОМ ПЕРИОДЕ ИШЕМИЧЕСКОГО ИНСУЛЬТА И ОПРЕДЕЛЯЮЩИЕ ЕГО ФАКТОРЫ

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Инсульт остается одной из основных причин серьезной инвалидизации, поэтому изучение факторов, влияющих на качество жизни постинсультных больных, и совершенствование реабилитационных технологий, способных улучшить его, сохраняет актуальность. Целью исследования было изучить возможные факторы, определяющие качество жизни больных, перенесших ишемический инсульт, в резидуальном периоде. Анкетирование проводили с помощью опросника MOS SF-36 у 210 пациентов, проходивших курс ранней реабилитации в условиях первичного сосудистого отделения. Исследование показало достоверное ухудшение ряда показателей качества жизни с увеличением возраста пациентов и тяжести инсульта (с коэффициентом корреляции –0,5; $p < 0,01$). У женщин оказались более низкими как физический ($p < 0,01$), так и психический ($p < 0,001$) компоненты качества жизни. Более высокие значения большинства субшкал качества жизни ($p < 0,05$) получены в группе пациентов, перенесших вертебробазилярный инсульт, по сравнению с пациентами, у которых была каротидная локализация очага. Оптимизация ранней реабилитации включением методов рефлексотерапии в остром периоде инсульта и продолжение реабилитационного процесса в условиях «Больницы восстановительного лечения» способствовали повышению ($p < 0,05$) качества жизни пациентов в периоде отдаленных последствий.

**Ключевые слова:** качество жизни, ишемический инсульт, реабилитация, рефлексотерапия, акупунктура

**Вклад авторов:** Е. Е. Молчанова — дизайн исследования, сбор и обработка материалов, анализ полученных данных, написание текста; В. В. Полунина, Б. А. Поляев, В. П. Плотников, А. Н. Лобов, С. А. Парастаев — концепция и редактирование текста; все авторы прочли и одобрили финальную версию статьи.

**Соблюдение этических стандартов:** исследование одобрено этическим комитетом Амурской государственной медицинской академии (протокол № 10 от 20 ноября 2019 г.), выполнено с соблюдением этических принципов проведения научных медицинских исследований с участием человека; всеми участниками подписано добровольное информированное согласие на участие в исследовании.

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Long-lasting disability after stroke is increasingly common [1]. The growing incidence of cardiovascular disease and cerebrovascular accidents is one of the grave consequences of population ageing [2]. Advances in acute stroke therapy have improved survival of stroke patients with pronounced neurological deficits. Poststroke disability poses a serious scientific and clinical challenge and is a significant social and economic burden [3]. The expanding arsenal of pharmacological interventions and rehabilitation technologies has reduced mortality among stroke patients but created a large population of people struggling with the aftermath of stroke. Currently, there are over 2 million stroke survivors in Russia [4]. The overwhelming majority of these people have to adapt to the acquired functional limitations and accept the profound impact of their disability on the family, professional and social life [5]. Therefore, the quality of life (QOL) after stroke and the effectiveness of poststroke rehabilitation cannot be measured by the survival rate alone [4]. This raises the need for research

into factors that affect QOL in stroke patients as QOL is one of the most reliable criteria used to analyze the effectiveness of new diagnostic and therapeutic techniques and prevention strategies [6–8].

So far, a few factors that might affect QOL in stroke patients have been studied, including sex [9, 10], age [11], severity of stroke [12], family status, occupational status, and duration of rehabilitation [13]. Earlier studies also looked into the effect of ischemic lesion location on the patient's QOL, but it is difficult to draw any definitive conclusions from those publications due to their scarcity. Some authors point to more pronounced cognitive and emotional impairments in patients with right hemisphere ischemic or hemorrhagic strokes, suggesting that right hemispheric lesions might be predictors of unfavorable outcome [14–16]. At the same time, according to another study the prevalence of anxiety and depression is by 20–30% higher in patients with left hemisphere ischemic stroke (IS) [12]. Patients with a left hemisphere stroke have higher physical component summary scores ($p < 0.05$) and higher general health (GH) scores on the SF-36 scale in the acute st and early rehabilitation period [10]. Patients with a verterobasilar (VB) stroke demonstrate better (20–50%; $p < 0.05$) physical function than patients with hemispheric IS, regardless of the involved side [12]. It is reported that physical decline after stroke is more dramatic than mental decline, regardless of the lesion site [17].

Rehabilitation programs complemented by non-pharmacological interventions in the early rehabilitation period improve QOL twofold, allowing 72.7% of patients to return to work within 6 months after stroke [18, 19]. There are publications discussing the advantages of poststroke rehabilitation in a specialized healthcare facility (sanitarium) vs. an outpatient clinic [20, 21].

Thus, studies investigating the factors that affect QOL in poststroke patients produce conflicting data. In addition, there is a paucity of information on the long-term impact of stroke on QOL. Insufficient data about a patient and their condition is a setback for the refinement of rehabilitation programs and prevention strategies. Identifying the predictors of gains will allow to improve the quality of rehabilitation [14, 16]. Therefore, it is important to investigate factors that affect QOL of stroke patients and refine rehabilitation programs for better functional gains.

The aim of this study was to investigate factors that determine QOL in the residual stroke period (3 years after the acute cerebrovascular accident).

## METHODS

Our study was conducted between 2013 and 2017 and recruited 210 patients (110 females and 100 males) aged 40–78 years (the mean age was 64.4 ± 1.05 years) undergoing neurorehabilitation at the stroke care unit of Blagoveshchensk City Clinical Hospital. Ischemic stroke was confirmed by CT and/or MRI findings. The majority of the participants (140; 66.7%) had a middle cerebral artery stroke (right-sided lesion was diagnosed in 79 (37.6%) patients; left-sided lesion was detected in 63 (30%) patients). A VB stroke was diagnosed in 68 (32.4%) patients. According to the classification of acute ischemic stroke subtypes (TOAST, 1993), 120 patients (57.1%) had large-artery atherosclerosis, 53 patients (25.2%) had cardioembolism, 12 (5.7%) had small-vessel occlusion, and 25 (11.9%) had a stroke of undetermined etiology.

In the acute stroke period, all patients were receiving a standard pharmacological therapy and non-pharmacological rehabilitation. For 140 patients, the rehabilitation program was complemented with acupuncture (AP); another 70 patients had physiotherapy and exercise without AP. Assignment to the AP or no AP groups was done through randomization. After being discharged from the hospital, 63 patients continued their rehabilitation at the Rehabilitation Hospital (RH); the remainder participants continued rehabilitation at an outpatient clinic.

The following inclusion criteria were applied: voluntary informed consent; IS confirmed by CT/MRI; age of 35–80 years; no contraindications for physiotherapy, AP and therapeutic exercise. Exclusions criteria: severe cognitive or mental impairment, pronounced aphasia or motor impairment that could prevent patients from completing the survey.

QOL was evaluated using an 8-scale MOS SF-36 health survey, which the participants completed independently [22] in the acute stroke period before being discharged home and 3 years after the cerebrovascular accident. The results were assessed on the scale from 0 to 100 points. A higher score meant a better QOL. SF-36 has two summary scales consisting of 4 subscales each: the physical component summary and the mental component summary. QOL was compared between the groups comparable in terms of sex, age, lesion site and rehabilitation modalities applied during the acute stroke period. To study the effects of different rehabilitation programs (with or without AP) on QOL in the residual stroke period, gains were compared between the groups of patients comparable in terms of sex, age, lesion site, and stroke pathogenesis. We also compared the outcomes between the patients who continued their rehabilitation at the outpatient clinic and at the specialized RH. These 2 groups were based on the case-control principle and were identical in terms of age, sex, severity of the condition on admission, lesion site, stroke pathogenesis, and the applied rehabilitation modalities in the acute stroke period.

Additionally, the psychological and emotional state of the patients was evaluated using Beck's Depression Inventory and the Spielberg–Khanin anxiety scale.

Normally distributed variables are presented below as a mean ± the standard error of the mean (M ± *m*) or as a mean ± a standard deviation (M ± SD). Significance of differences was assessed using Student's *t* test. Pearson's correlation coefficient r was used to determine the strength and direction of correlations between the studied variables. Differences were considered significant at $p < 0.05$.

## RESULTS

The SF-36 survey revealed a decline in the mental (56.5 ± 2.8 points) and physoemotional (59.8 ± 2.7 points) states of the participants in the acute IS period before discharge from the hospital. By contrast, the physical and mental components assessed in the residual stroke period (3 years after stroke) were characterized by significantly higher scores (81.1 ± 2.1 points and 77.8 ± 1.9 points on average, respectively; $p < 0.001$). The only exception in the residual stroke period was the low vitality score (VT; $p < 0.001$) (Fig. 1).

A negative correlation was discovered between the age of the patients and their RP (physical role functioning) and vitality (VT) scores (–0.44, $p < 0.05$ and –0.5, $p < 0.01$, respectively) in the acute stroke period. The strength of this correlation increased for the residual stroke period. Advancing age was significantly correlated with PF (physical functioning; –0.5, $p < 0.01$), GH (general health; –0,5, $p < 0.01$) and VT (vitality; –0.6, $p < 0.001$). Age between 44 and 60 years was the most favorable age in terms of a better QOL prognosis; the lowest scores were observed in the age group above 75 years (Fig. 2). Our study did not establish any correlations between age and the development of depression and anxiety.

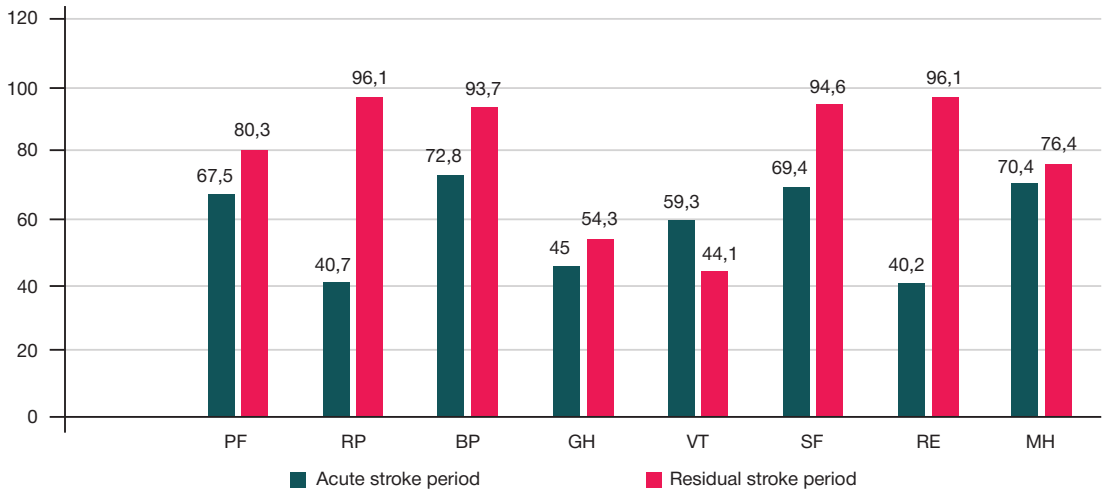


**Fig. 1.** Dynamics of QOL indicators in the acute stroke period (at the end of the early rehabilitation program) and 3 years after stroke

Women scored lower on the majority of SF-36 subscales. The differences between female and male scores were significant for RP (physical role functioning; p < 0.05) and RE (emotional role functioning; $p < 0.001$) scales in the acute stroke period and for PF (physical function; $p < 0.01$), RP (physical role functioning; $p < 0.05$), BP (bodily pain; $p < 0.05$), GH (general health, $p < 0.001$), VT (vitality; $p < 0.001$), and MH (mental health; $p < 0.01$) in the residual stroke period (Fig. 3). The analysis of the psychoemotional state revealed a more severe depression in women (13.4 ± 1.1 points) than in men (9.6 ± 1.1 points; $p < 0.05$).

Our study established negative correlations between QOL indicators in the residual stroke period and the severity of stroke on the NIHSS scale assessed on admission to hospital, PF (physical functioning; –0.6, $p < 0.001$), GH (general health; –0.4, $p < 0.05$), VT (vitality; –0.5, $p < 0.01$), SF (social functioning; –0.5, $p < 0.01$), and MH (mental health; –0.5, $p < 0.01$).

Patients with a right MCA stroke had the lowest scores for PF (physical functioning; $p < 0.05$) in the acute stroke period. In the residual stroke period, significant differences were detected in PF (physical functioning; $p < 0.05$), RP (physical role functioning; $p < 0.05$) and RE (emotional role functioning; $p < 0.05$) scores between patients with a right MCA stroke and those with a VB stroke, who scored highest on most subscales. GH (general health) and VT (vitality) scores were the lowest in patients with a left MCA stroke, differing significantly ($p < 0.05$) from GH and VT scores of patients with a VB stroke (Fig. 4). Patients with a hemispheric infarction due to internal carotid artery occlusion suffered from a more severe depression (14.2 ± 1.5 and 13.7 ± 1.3 points for left and right hemispheric lesions, respectively) than those with a VB stroke (8.3 ± 1.1 points; $p < 0.01$). Carotid and vertebrobasilar strokes also differed in terms of neurological deficit severity on admission (6.1 ± 0.3 and 4.7 ± 0.3 points, respectively; p < 0.01) and at the time of discharge (2.3 ± 0.2 and 1.5 ± 0.2 points, respectively; $p < 0.01$).

The introduction of AP into the early rehabilitation program resulted in higher scores on most SF-36 subscales both in the acute stroke period (RP, $p < 0.05$; GH, $p < 0.05$; VT, $p < 0.05$; SF, $p < 0.05$; RE, $p < 0.05$; MH, $p < 0.05$) and in the residual stroke period (PF, $p < 0.05$; GH, $p < 0.05$; VT, $p < 0.01$; MH, $p < 0.05$) (Fig. 5). AP also contributed to decreasing reactive anxiety by an average of 8.5 points (22%; $p < 0.001$) and depression by an average of 4.9 points (30%; $p < 0.001$) by the time of discharge. No significant differences in these parameters were observed in the group of patients from the no AP group.

Three years after the cerebrovascular accident, QOL was higher in patients who continued rehabilitation at RH. The differences were statistically significant for PF (physical functioning; $p < 0.05$), BP (bodily pain; $p < 0.05$), GH (general health; $p < 0.05$), VT (vitality; $p < 0.05$), and MH (mental health; $p < 0.001$) (Fig. 6).

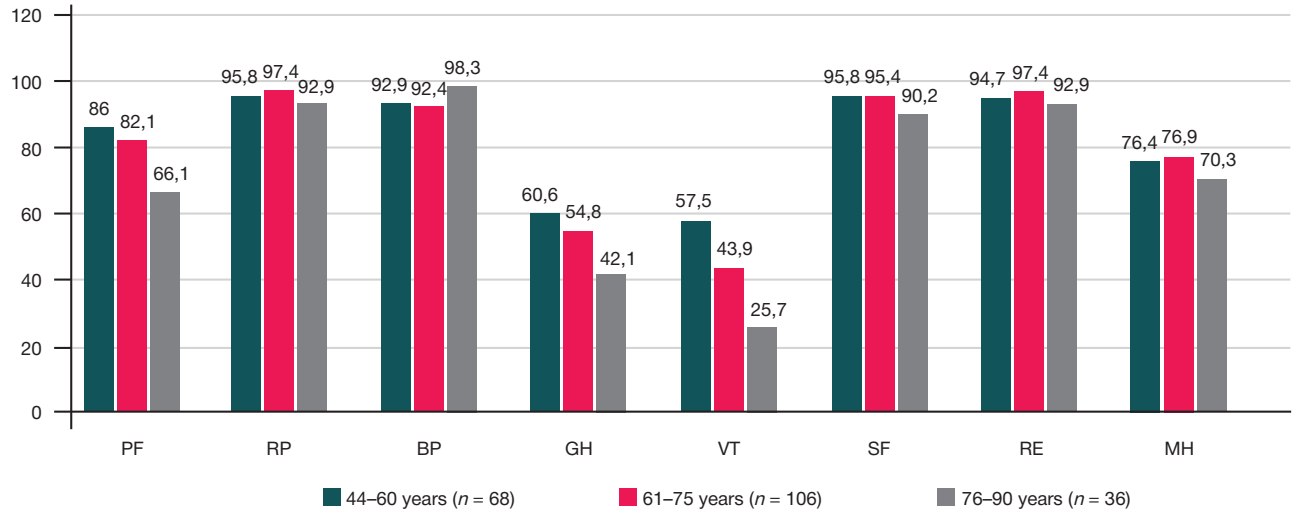


**Fig. 2.** QOL indicators in the residual stroke period in patients of different age

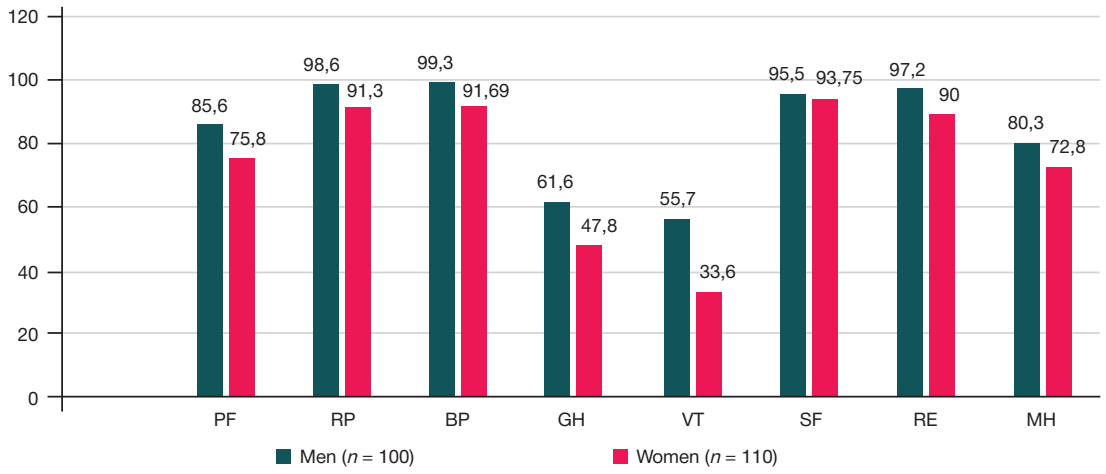


**Fig. 3.** QOL indicators in the residual stroke period in male and female patients

## DISCUSSION

Our study revealed that some QOL parameters declined with advancing age. The lowest QOL scores were observed in the age group above 75 years, which is consistent with other reports [23]. The highest scores on SF-36 scales were demonstrated by middle-aged patients (44–60 years). At the same time according to another publication, it was this age group (45–64 years) where the QOL decline was the most dramatic, perhaps due to the suddenness of stroke, its profound impact on all aspects of life and the unpreparedness of the patients to deal with stress [11]. Another study did not report any associations between QOL deterioration and age [10].

The pronounced decline in all QOL indicators observed 3 years after stroke in female patients was previously reported by other researchers. For example, a study showed that men had higher physical component summary scores on days 10 and 180 after stroke than women [10]. Some sex-related differences were observed by other researchers in the acute stroke period and during early poststroke rehabilitation [9]. Perhaps, the underlying reason is a more severe depression in women than in men.

Low general health (GH) and vitality (VT) scores in patients with a left hemispheric stroke resulting from carotid artery occlusion might be due to right-hemisphere motor deficit. For example, patients with a VB stroke had a stronger (by 20%) motivation to work towards recovery than those with a hemispheric stroke [15]. Motivation is often the key to effective rehabilitation, which was also confirmed by our study: the highest scores on the majority of the applied subscales were observed in patients with VB strokes. Low PF (physical function), RP (physical role functioning) and RE (emotional role functioning) scores observed in patients with a right MCA stroke suggest a more profound impact of the right hemispheric lesion on the psychoemotional state; this observation was previously reported by other authors [14–16] and is confirmed by our study. The highest QOL scores demonstrated by patients with a vertebrobasilar stroke might be associated with less severe neurological deficit in the acutest stroke period and at the end of the acute stroke period before discharge.

Our study confirmed the effectiveness of complex non-pharmacological rehabilitation programs [18, 19], which was illustrated by high scores on most SF-36 subscales in the acute and residual stroke periods following the inclusion of AP into the early rehabilitation regimen resulting in the improved physiological and emotional state of our patients. The high effectiveness of poststroke rehabilitation at RH is consistent with the literature reports on the advantages of sanitarium vs. outpatient rehabilitation [20, 21, 24].

## CONCLUSION

Our study has identified a few predictors of poor quality of life after stroke in the residual stroke period, including age over 60 years, female sex and strokes in the MCA territory. A relatively better outcome can be expected for younger patients under 60

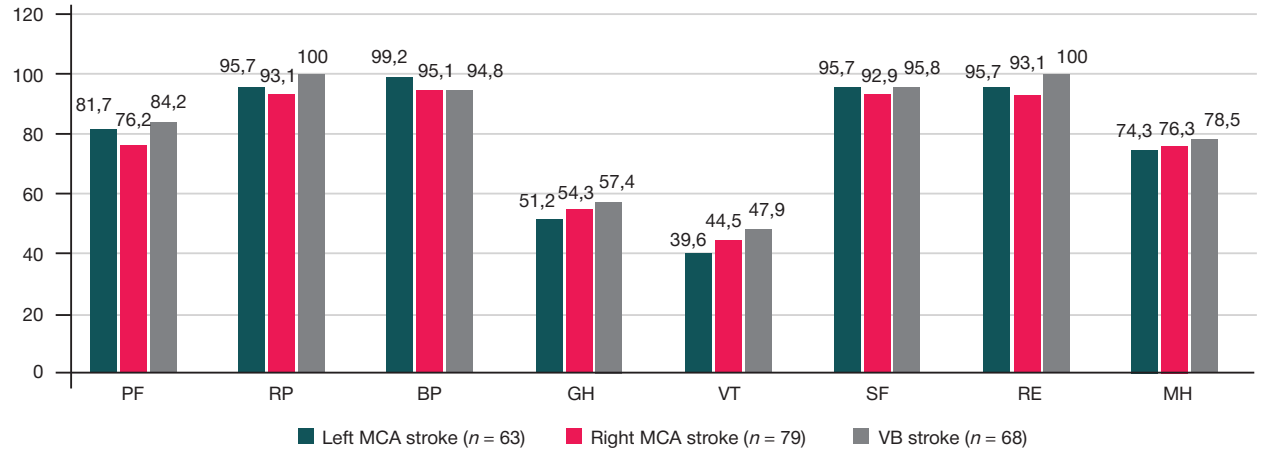


**Fig. 4.** QOL indicators in the residual stroke period for patients with different lesion locations

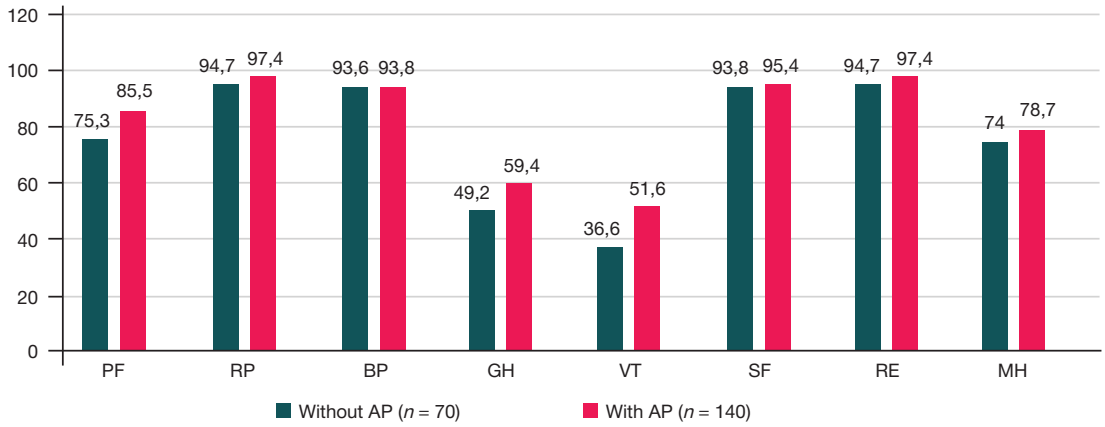


**Fig. 5.** QOL indicators in the acute stroke period in patients undergoing different types of rehabilitation

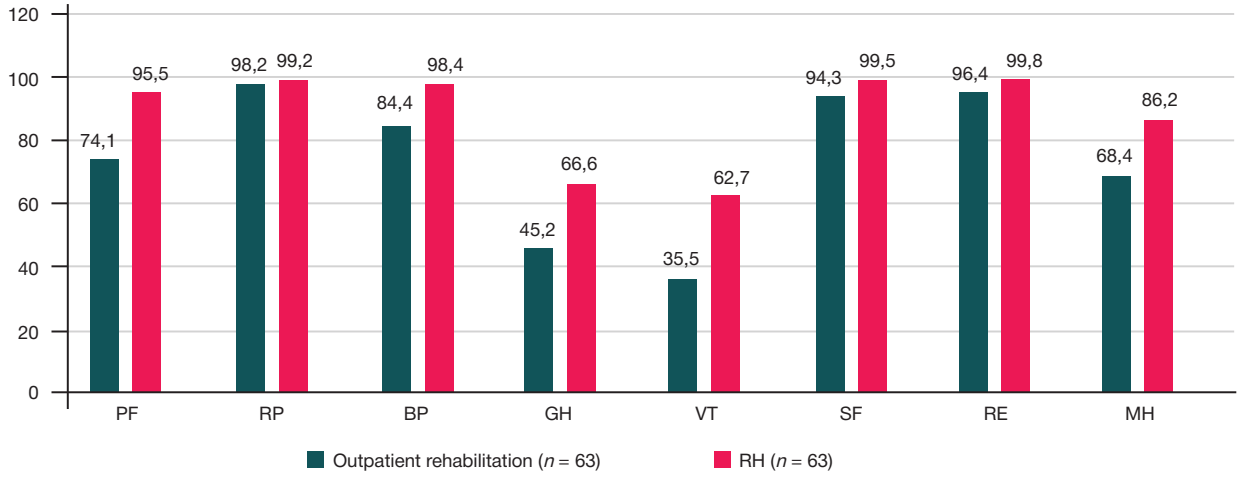


**Fig. 6.** QOL indicators 3 years after stroke depending on the type of rehabilitation after discharge from the hospital

years, men, patients with VB strokes and those who undergo rehabilitation complemented with acupuncture in the acute stroke period and continue rehabilitation in specialized inpatient facilities.

Understanding factors that affect QOL in stroke patients can help to predict the success of rehabilitation programs and improve their effectiveness. Discrepancies between our findings and data produced by other studies necessitates further research.

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# COVID-19 PATIENTS' SATISFACTION WITH QUALITY OF MEDICAL CARE PROVIDED IN THE FORM OF TELEMEDICINE CONSULTATIONS

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The paper reports the results of survey carried out in order to assess patients' opinion on the remote medical care provided by the telemedicine center of the Department of health of Moscow during the pandemic. Survey of 216 COVID-19 patients who received outpatient care made it possible to assess their health condition and determine the factors contributing to satisfaction with care provided. Patients' health condition was evaluated based on the course of underlying disease and comorbidities, which were revealed in 24.3% of COVID-19 patients. The following three groups were formed: patients with favorable (37.5%), satisfactory (36.7%) and unfavorable (25.8%) health condition. The majority of patients (76.4%) were satisfied with telemedicine consultations; men (79.8%), individuals with favorable condition (83.1%) and patients under 50 (81.9%) demonstrated significantly higher level of satisfaction. The following arguments in favour of telemedicine consultations were specified by patients: appointment of the specialist's consultation, promptness of treatment appointment, provision of medical recommendations, including recommendations on a healthy lifestyle, as well as promptness of house call and ambulance call. The main dissatisfaction reasons were as follows: lack of appropriate equipment, difficulties when setting up the equipment, complexity of the instructions for connecting to telemedicine consultations, poor quality of video/audio conferencing; these indicated the patients' inadequate technical resources. Telemedicine consultations may be considered an effective method of outpatient care provision for COVID-19 patients, especially since 64.7% of patients plan to use telemedicine consultations in the future, and 72.3% of patients are ready to recommend them to others.

**Keywords:** telemedicine consultations, morbidity, COVID-19, pandemic, satisfaction

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**Compliance with ethical standards:** the study was approved by the Ethics Committee of the City Policlinic № 2 of the Department of health of Moscow (protocol № 9 dated September 30, 2020). The survey was carried out by consent of the patient under full anonymity; the patient was informed that his/her personal data would never be used anywhere else.

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# УДОВЛЕТВОРЕННОСТЬ ПАЦИЕНТОВ С COVID-19 КАЧЕСТВОМ МЕДИЦИНСКОЙ ПОМОЩИ, ОКАЗАННОЙ В ФОРМЕ ДИСТАНЦИОННЫХ ТЕЛЕМЕДИЦИНСКИХ КОНСУЛЬТАЦИЙ

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В статье представлены результаты исследования мнения пациентов об оказании дистанционной консультативной медицинской помощи в телемедицинском центре ДЗМ в условиях пандемии. Обследование 216 пациентов с COVID-19, находившихся на амбулаторном лечении, дало возможность оценить их здоровье и определить факторы, влияющие на удовлетворенность предоставляемыми услугами. Здоровье пациентов оценивали на основании изучения течения основного заболевания и наличия сопутствующих заболеваний, которые были выявлены у 24,3% заболевших COVID-19. Были сформированы три группы — с благоприятной (37,5%), удовлетворительной (36,7%) и неблагоприятной (25,8%) характеристиками здоровья. Большинство пациентов (76,4%) остались удовлетворены телемедицинскими консультациями, при этом достоверно чаще — мужчины (79,8%), лица, имеющие благоприятное здоровье (83,1%), и пациенты моложе 50 лет (81,9%). В качестве аргументов использования телемедицинских консультаций пациенты указывали получение консультаций специалистов, оперативность назначения лечения, получение медицинских рекомендаций, в том числе по здоровому образу жизни, а также быстроту оформления вызова врача на дом и скорой медицинской помощи. Основными причинами неудовлетворенности были отсутствие у пациентов соответствующего оборудования, трудности с настройкой оборудования, сложность инструкции по подключению к телемедицинским консультациям, неудовлетворительное качество аудио- и видеосвязи, что свидетельствует о недостаточной технической оснащенности пациентов. Телемедицинские консультации могут быть рассмотрены в качестве эффективного метода при амбулаторном лечении пациентов с COVID-19, тем более, что 64,7% пациентов планируют в будущем их использование, а 72,3% готовы рекомендовать их другим.

**Ключевые слова:** телемедицинские консультации, заболеваемость, COVID-19, пандемия, удовлетворенность

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**Соблюдение этических стандартов:** исследование одобрено этическим комитетом ГБУЗ «Городская поликлиника № 2 Департамента здравоохранения г. Москвы» (протокол № 9 от 30 сентября 2020 г.). Анкетирование проводили при условии согласия пациента с соблюдением анонимности, информируя пациента о том, что его личные данные нигде не будут использованы.

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The epidemic situation of 2020 posed new challenges both for global health care and for domestic health care system. The most important epidemiological aspect during the pandemic is limiting the spread of infection by reducing contacts, including the patient's visits to a polyclinic or home visits by doctors [1].

During the pandemic, the growing people's need in timely and quality medical care resulted in need for implementation of novel information and communication technologies when providing the outpatient care. Remote technologies make it possible not only to expand access to medical services, but also to reduce the risk of the novel coronavirus infection COVID-19 spread, allowing the patients not to violate the self-isolation regime and not to expose others to the risk of infection. According to the Moscow Operational Headquarters for preventing the spread of coronavirus infection data of March 29, 2020, on average, 62% of patients infected with SARS-CoV-2 have mild or asymptomatic disease. Despite the fact that the disease is more often mild, such patients require medical supervision, however, if they need medical care, this allows physicians to follow-up this group of patients on an outpatient basis.

Remote technologies, which include tele-health [2], make it possible to provide the patient with high-quality and affordable medical care, regardless of the location of the physician, ensuring epidemiological safety of both healthcare specialists and the patient. Telemedicine development trends demonstrate the relevance of studying the opinions of patients about the implementation of telemedicine consultations, which is necessary for the development of proposals for improvement of those [3].

In this paper, the experience of remote medical care (telemedicine consultations) provision is reported, and the assessment of patients' satisfaction with quality of such consultations is presented.

Patients' satisfaction is one of the medical care quality indicators recognized both in the Russian Federation and other countries. Patient's satisfaction with the quality of services results from comparison of patient's expectations and the actual experience associated the use of the service. Thus, the level of satisfaction and subjective assessment of the service quality directly correlates with expectations and requirement not necessarily stated in administrative regulations and other regulations governing the provision of certain services [4, 5].

Due to growing abundance of digital and telemedicine technologies in the system of medical care provision, it is important to study the key indicator characterizing the quality of the health care system, i. e. the patient's satisfaction and the factors contributing to patient's satisfaction [6–8].

Monitoring of patients' opinions is required to ensure flexibility in improving new technologies in accordance with the patients' basic needs when integrating telemedicine technologies into the health care system. Since satisfaction with medical care consists of many factors, which are most often subjective [9], surveys were recognized as the most informative method for studying the patients' opinion about the work of the health care system [10]. The development of telemedicine technologies contributes to availability of outpatient care, regardless of the patient's condition and location [11].

The study was aimed to assess the patients' satisfaction with quality of medical care provided in the form of telemedicine consultations.

## METHODS

The study was carried out in the Telemedicine Centre of the Department of health of Moscow (TMC DHM) organized in the Consultative and Diagnostic Polyclinic no. 121. Inclusion criteria: documentary evidence of COVID-19 diagnosis, outpatient treatment.

In order to assess the patients' perception in relation to satisfaction with medical care provided and quality of remote consultation, the 216 patients' survey was carried out using the specially developed "Questionnaire for Assessment of COVID-19 Patients' Satisfaction with Quality of Care Provided by the Telemedicine Center". The answers were ranked using the 5-point scale. When compiling the questionnaire, we used verification questions, which made it possible to exclude incorrectly completed forms (see Appendix). The survey was carried out from April 30 to May 10, 2020 by the Telemedicine Center administrator, who interviewed the patients with confirmed COVID-19 diagnosis, who had received telemedicine consultations, by phone and filled the form.

Morbidity was studied using information about the symptoms of the underlying disease and chronic diseases in patients with COVID-19 copied from the "Medical record of a patient receiving medical care on an outpatient basis". These data was entered in a sample card, which also contained patient's full name, date of birth, place of residence, date of consultation and results of remote health monitoring.

Statistical analysis performed with the IBM SPSS Statistics software for Windows, version 20.0 (IBM Corp.; USA), included the following: calculation of extensive and intensive indicators, calculation of mean and the error of the mean, assessment of significance, comparison of mean values and indicators. The differences are considered significant when $P \geq 0.95$.

## RESULTS

The data obtained indicate that almost every 3rd patient was aged 30–40 (32.7%), every 4th was aged 40–50 (23.1%), and every 5th was aged 50–60 (19.4%). The smallest groups included patients aged 20–29 (15.6%) and 60+ (9.2%).

The average age of surveyed patients was 40.3 ± 0.72 in men, and in women the average age was significantly higher (44.2 ± 0.97, $P < 0.01$). It was shown that among men 40.4% of patients were aged 30–40, and the proportion of women was 26.6% ($P \leq 0.05$). Among women, the patients aged 40–50 predominated (27.3%), and the proportion of men in this age group was 15.7% ($P \leq 0.05$). The smallest number of both men and women were aged 20–29 (20.2% and 12.5% respectively, $P \geq 0.05$) and 60+ (10.1% and 10.2% respectively, $P \geq 0.05$).

Significant gender differences were observed in all age groups except two: under 30 and 60+.

**Table 1.** Distribution of male and female COVID-19 patients based on the symptoms reported (% of the total number)

| Number of symptoms in patients with COVID-19 | Men | Women | Forecasting probability, *P* |
|---|---|---|---|
| 0–3 symptoms | 44.9 | 29.9 | ≥ 0.99 |
| 4–7 symptoms | 30.3 | 43.3 | ≥ 0.95 |
| 8–12 symptoms | 24.7 | 26.8 | ≤ 0.95 |
| Total | 100.0 | 100.0 | |

**Table 2.** Ranked distribution of disorders by classes in surveyed COVID-19 patients (% of the total number)

| Rank | Men | | Women | |
|---|---|---|---|---|
| | Classes of disorders | % | Classes of disorders | % |
| 1 | Circulatory system diseases | 14.8 | Circulatory system diseases | 19.1 |
| 2 | Digestive diseases | 11.8 | Digestive diseases | 13.1 |
| 3 | Musculoskeletal system diseases | 10.9 | Musculoskeletal system diseases | 11.3 |
| 4 | Neoplasms | 10.7 | Neoplasms | 9.5 |
| 5. | Urogenital disorders | 10.6 | Urogenital disorders | 8.7 |
| 6 | Respiratory disorders | 10.2 | Respiratory disorders | 7.8 |
| 7 | Disorders of eye and adnexa | 6.8 | Disorders of eye and adnexa | 7.6 |
| | Other | 24.2 | Other | 22.9 |
| | Total | 100 | Total | 100 |

Analysis of the health condition performed based on the medical records included the assessment of both underlying disease course and comorbidities. The data obtained showed that in most surveyed patients who received outpatient care the mild course of COVID-19 was observed, and patients with severe course of the disease were hospitalized to appropriate hospitals.

However, the patients with mild course of the disease may have various symptoms. The study of the prevalence rate of the disorder showed that most COVID-19 patients who received outpatient treatment complained of elevated body temperature (84.9 cases per 100 surveyed patients), dry or minimally productive cough (74.3 cases per 100 surveyed patients), shortness of breath (48.5 cases per 100 surveyed patients), weakness (78.6 cases per 100 surveyed patients), fatigue (69.2 cases per 100 surveyed patients), loss of smell (37.5 cases per 100 surveyed patients), conjunctivitis (29.7 cases per 100 surveyed patients), and sleep disturbances (61.8 cases per 100 surveyed patients). Headache (14.3 cases per 100 surveyed patients), nausea and vomiting (9.6 cases per 100 surveyed patients), and diarrhea (7.3 cases per 100 surveyed patients) were the least prevalent symptoms.

It was noted that one patient experienced an average of 5.39 ± 0.09 symptoms. The patients were divided into three groups based on the number of symptoms. The first group included patients with favorable health conditions who reported no complaints or up to three complaints. The proportion of such patients was 37.5%. The third group included patients with unfavorable health conditions who reported 8 or more complaints; the proportion of such patients was 25.7%. Other patients comprised the second group; their proportion was 36.8%.

The data obtained showed that patients with more favorable course of the disease predominated among men, and patients who reported 4–7 symptoms predominated among women (Table 1). At the same time, the proportion of patients who reported 8 or more symptoms was 24.7% in men and 26.8% in women.

The study of comorbidities in patients with COVID-19 was performed due to several reasons. First, comorbidities may worsen the course of COVID-19 and lead to disability. Second, comorbid patients require extra medical supervision and extra medication.

The in-depth study of the supervised COVID-19 patients' health conditions indicated that 24.3% of the infected patients were comorbid. Comorbidities were detected in 14.7% of men, and among women the proportion of comorbid patients was significantly (by 2.3 times, $P < 0.01$) higher (33.9%). The comorbidity rate was 350.2%, among them in patients under 40 it was 312.9%, and among patients 40+ it was 387.8%. The morbidity rate analysis in men and women showed that the morbidity rate was significantly (by 1.4 times, $P < 0.99$) in men compared to women (290.8% and 409.7% respectively).

In the structure of comorbidity the first seven places belonged to circulatory system diseases (17.3%), urogenital disorders (12.1%), musculoskeletal system diseases (11.2%), digestive diseases (10.5%), neoplasms (9.5%), respiratory disorders (8.7%), disorders of eye and adnexa (7.4%). The proportion of those was 76.7% of the total number of detected comorbidities.

It was found that the majority of the disorders, revealed in surveyed male and female patients (75.8% and 77.1% respectively), was comprised by the listed classes of disorders. However, the ranked distribution of morbidity was different (Table 2).

Despite the fact that the first place in the structure of comorbidities in COVID-19 patients belonged to circulatory system diseases, the morbidity rate for this class of disorders was 1.8 times higher in women than in men (78.1% and 43.1% respectively). The morbidity rate was higher in women compared to men for almost all classes of disorders; the most significant differences in the prevalence rate were observed for urogenital disorders (by 1.7 times — 53.8% and 30.9% respectively), disorders of eye and adnexa (by 1.6 times — 31.9% and 19.8% respectively), and musculoskeletal system diseases (by 1.4 times — 46.1% and 31.8% respectively).

Worth noting is the relationship between comorbidities and the number of symptoms reported (Table 3). Among patients with no comorbidities, the number of individuals who reported no more than three symptoms was significantly higher (by 2.1 times, $P \geq 0.95$), and the number of individuals who reported 8 and more symptoms was significantly lower (by 1.7 times, $P \geq 0.95$).

**Table 3.** Distribution of COVID-19 based on the number of complaints and reported comorbidities ((% of the total number)

| Number of symptoms in patients with COVID-19 | Patients with no comorbidities | Comorbid patients | Forecasting probability, *P* |
|---|---|---|---|
| 0–3 symptoms | 49.6 | 26.5 | ≥ 0.99 |
| 4–7 symptoms | 30.8 | 39.4 | ≤ 0.95 |
| 8–12 symptoms | 19.6 | 34.1 | ≥ 0.95 |
| Total | 100.0 | 100.0 | |

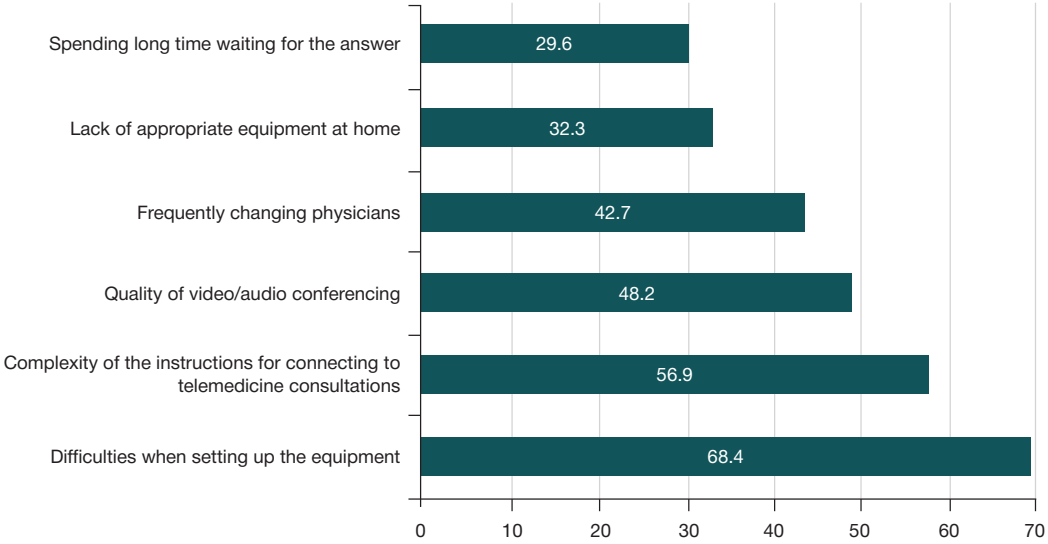


**Fig. 1.** Frequency of COVID-19 patients based on the reasons for dissatisfaction with telemedicine consultations (per 100 surveyed patients)

The data show that unfavorable course of the disease is more common in comorbid patients.

A specialized telemedicine center was created for patients with COVID-19, the main task of which was to provide online consultations for patients with a confirmed diagnosis of coronavirus infection. Since the patients' condition allowed for outpatient treatment, such patients were provided with remote telemedicine consultations from specially equipped telemedicine center.

The online consultations were 24-hour. The use of video monitoring together with audio/video conferencing makes it possible to assess the patient's condition, the use of electronic patient records and other data from UMIAS makes it possible to assess the course of the disease, and the data on comorbidities allow the physicians to adjust treatment. During the consultations the physicians provide medical recommendations and answer the patient's questions.

The data indicated that the majority of surveyed COVID-19 patients (76.4%) were satisfied with telemedicine consultations. It was found that among patients satisfied with telemedicine consultations there were significantly ($P < 0.95$) more men than women (79.8% in the group of men and 68.5% in the group of women).

It was noted that the level of satisfaction with telemedicine consultations was higher in patients with no comorbidities who reported no more that 3 symptoms of the disease compared to patients classified as patients with unfavorable health condition (83.1% vs. 62.1%, $P < 0.95$).

The main arguments in favour of remote consultations reported by patients were as follows: appointment of the specialist's consultation (73.9% of patients), promptness of care appointment (61.6% of patients), provision of recommendations for a healthy lifestyle (52.8% of patients) and medical recommendations (47.3% of patients), promptness of laboratory tests' results (49.2% of patients), continuous health monitoring (39.6% of patients), as well as promptness of house call (31.7% of patients) and ambulance call (29.3% of patients).

It was noted that men more often specified such arguments in favour of contacting the telemedicine center as promptness of care appointment and provision of medical recommendations, and women more often specified such arguments as appointment of the specialist's consultation, continuous health monitoring and promptness of house call. An average of 3–4 in favour of remote consultations per COVID-19 patient was noted: the average number of arguments in male patients was $3.1 \pm 0.21$, and in female patients it was $4.6 \pm 0.36$ ($P < 0.99$).

Analysis of reasons for dissatisfaction with remote care quality showed that the leading place belonged to technical problems, such as lack of appropriate equipment, difficulties when setting up the equipment, complexity of the instructions for connecting to telemedicine consultations, quality of video/audio conferencing, frequently changing physicians, spending long time waiting for the answer (Fig. 1).

It was found that patients of older age were more sensitive to the quality of medical care, including the remote care. Generally, it was noted that the significantly higher (by 1.7 times) proportion of patients over 40 compared to patients under 40 was dissatisfied with provided remote medical care (29.7% vs. 17.5% respectively).

**Table 4.** Frequency of COVID-19 patients based on the reasons for dissatisfaction with telemedicine consultations and age (per 100 surveyed patients)

| п/№ | Dissatisfaction reasons | Number of cases per 100 surveyed patients | | Forecasting probability, *P* |
|---|---|---|---|---|
| | | Under 50 | Over 50 | |
| 1 | Difficulties when setting up the equipment | 53.2 | 73.6 | ≥ 0.99 |
| 2 | Complexity of the instructions for connecting to telemedicine consultations | 44.5 | 61.3 | ≥ 0.95 |
| 3 | Quality of video/audio conferencing | 39.3 | 57.1 | ≥ 0.95 |
| 4 | Frequently changing physicians | 32.6 | 46.8 | ≤ 0.95 |
| 5 | Lack of appropriate equipment at home | 21.3 | 43.2 | ≥ 0.99 |
| 6 | Spending long time waiting for the answer | 30.5 | 18.7 | ≥ 0.95 |
| 7 | Lack of timeliness | 27.3 | 15.3 | ≥ 0.95 |
| 8 | Lack of information completeness | 14.6 | 19.2 | ≤ 0.95 |

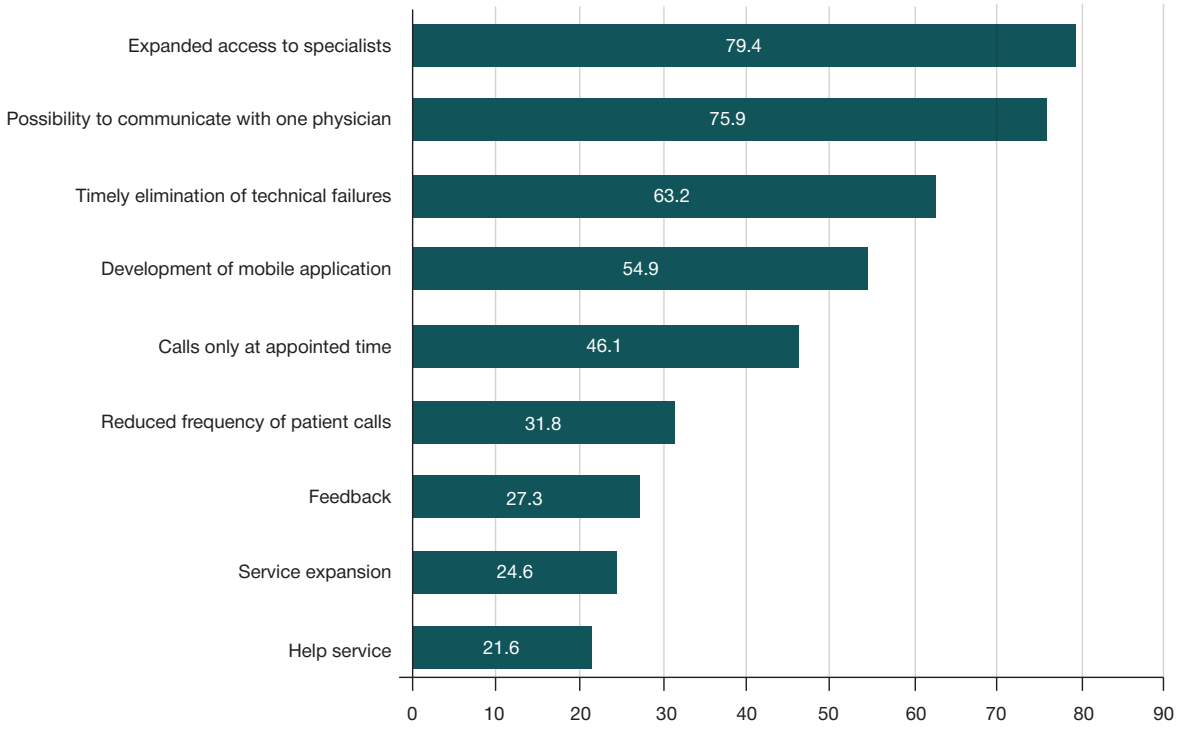


**Fig. 2.** Distribution of COVID-19 based on the proposals concerning the telemedicine consultation quality improvement (per 100 surveyed patients)

Compared to younger patients, patients aged 40+ (Table 4) more often have no equipment for remote communication, they more often experience difficulties when setting up the equipment and poor communication quality.

One of the criteria of satisfaction with medical care quality is timeliness and completeness of providing the patient with health data. It has been found that the patients of older age tend to be more patient. They less often specified spending long time waiting for the answer and lack of timeliness in provision of consultations as the reasons of dissatisfaction compared to younger patients.

## DISCUSSION

Analysis of answers showed that the answers about the lack of information completeness were mainly concerning situations not related to medical care characterized by organizational or legal orientation. The patients paid special attention to evaluation of the communication quality, since visual assessment of the patient's condition was one of the most important tasks of telemedicine consultations. At the same time, the physicians communicated with the patients via audio conferencing in every 5$^{th}$ case (19.2%), in 18.1% they used audio/video conferencing, and in 14.5% they used video conferencing.

Unfortunately, every second patient reported poor quality of video conferencing, which contributed to dissatisfaction with telemedicine consultations (48.2%). Among patients of older age, significantly more (by 1.4 times) people experienced problems with video conferencing compared to younger patients (57.1% vs. 39.3%, $P \leq 0.95$).

The study of patients' proposals concerning the improvement of telemedicine is an important aspect of the outpatient medical center medical and organizational performance (Fig. 2).

The most frequent proposals were as follows: expanded access to specialists, possibility to communicate with one physician, and timely elimination of technical failures. In addition to the patients' satisfaction with the telemedicine center activities survey results, the quality of telemedicine consultations can be judged based on the patients' desire to use this medical care type of in the future, as well as to recommend it to their friends and acquaintances. The data obtained has shown that 64.7% of surveyed COVID-19 patients plan using the telemedicine consultations in the future, and 72.3% of patients are willing to recommend it to others.

Despite the subjectivity component, the indicator of patient satisfaction with medical care is highly informative and can be used not only to assess the quality of telemedicine consultations, but also to develop measures for medical care improvement.

## CONCLUSION

1. Implementation of remote telemedicine consulting into management of patients with confirmed COVID-19 makes it possible to assess patient's health condition, to perform the dynamic monitoring of the course of the disease and ensure timely treatment adjustment. 2. The indicator of patients' satisfaction with quality of medical care received is subjective and associate with patients' health condition. Thus, the level of satisfaction with telemedicine consultations was higher in patients who reported no COVID-19 symptoms or no more than 3 symptoms compared to patients classified as the group with unfavorable health condition. 3. Among reasons for dissatisfaction with remote medical care the leading place belongs to technical problems and organizational aspects; this served as the basis for development of measures required for this medical care type improvement. 4. The main directions of the telemedicine services quality improvement are as follows: improvement of technical requirements for equipment, taking into account the elimination of causes leading to failures during telemedicine consultations; provision of patients with mobile applications for telemedicine consultations; expanded access to specialists in accordance with comorbidity revealed in patient with COVID-19; remote consultations provided by the same physician throughout the follow-up period. 5. Taking into account the fact that 64.7% of surveyed COVID-19 patients plan to use telemedicine consultations in the future, and

72.3% of patients are ready to recommend them to others, implementation of remote medical services as the new form of quality medical care provision by outpatient centers and the expansion of those using the information and communication technologies should be considered. This is one of the promising areas of modern healthcare.

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# EVALUATION OF EFFICACY OF PROVIDING HYGIENE EDUCATION TO SCHOOLCHILDREN AND STUDENTS IN THE PROCESS OF DEVELOPMENT OF THE SAFE ELECTRONIC DEVICE USE SKILLS

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The negative impact of the digital environment on the health of young people necessitates the search for new methods of hygienic education. This study aimed to test and assess the hygienic qualities of the practice designed to give students and schoolchildren the skills allowing safe use of electronic devices. The study involved 256 students, 200 senior schoolchildren, 400 teachers and 251 parent. The hygienic education practice relied on the healthy lifestyle materials published in scientific literature, as well as materials posted on the Internet resources of medical organizations professionally engaged in the area considered, as well as their groups in the social networks Odnoklassniki, VKontakte, Facebook, Instagram, etc. We observed physical development of the schoolchildren and students dynamically and polled schoolchildren, students, teachers, and parents. For statistical processing of the results, we used methods of descriptive statistics, Student's t-test, correlation, discriminant and cluster analysis, and calculated risks. As the most popular source of information about health maintenance, Internet scored as follows: among schoolchildren — 79.0%, students — 88.6%, parents — 64.9%, teachers — 50.4%. The tested hygienic education practice allowed for a reduction of the number of schoolchildren and students who did not have the skills to safely use electronic devices to 20 and 25%, respectively. The practice also taught the participants to reduce their daily smartphone use time, engage in physical activity more often, which ultimately increased the share of children whose physical development was normal ($p \leq 0.01$), and helped to increase the duration of night sleep. The tested methods of education are not costly; they can be replicated in other regions and organizations.

**Keywords:** schoolchildren, students, electronic devices, rules of use, hygiene education, assessment of the effectiveness

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**Compliance with ethical standards:** the study was approved by the Ethics Committee of the Pirogov Russian National Research Medical University (Minutes #159 of November 21, 2016), conducted in compliance with the ethical standards provided by the Declaration of Helsinki and the European Community Directives (8/609 EU). Each participant signed a voluntary informed consent form. The participating adults (parents and teachers) were polled voluntary with the help of an online service.

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# ОЦЕНКА ЭФФЕКТИВНОСТИ ГИГИЕНИЧЕСКОГО ВОСПИТАНИЯ ШКОЛЬНИКОВ И СТУДЕНТОВ ПО ФОРМИРОВАНИЮ НАВЫКОВ БЕЗОПАСНОГО ИСПОЛЬЗОВАНИЯ ЭЛЕКТРОННЫХ УСТРОЙСТВ

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Негативное влияние цифровой среды на здоровье молодежи стимулирует поиск новых приемов гигиенического воспитания. Целью работы были апробация и гигиеническая оценка эффективности практики формирования здорового образа жизни у студентов и школьников в части формирования навыков безопасного использования электронных устройств. В исследовании приняли участие 256 студентов, 200 учащихся старших классов, 400 преподавателей и 251 родитель. Гигиеническое воспитание осуществляли с использованием опубликованных в научной литературе материалов по здоровому образу жизни, а также материалов, размещенных на Интернет-ресурсах медицинских организаций, профессионально работающих в этой области и их группах в социальных сетях «Одноклассники», «ВКонтакте», «Facebook», «Инстаграм» и др. Было организовано динамическое наблюдение за физическим развитием школьников и студентов, а также анкетирование школьников, студентов, преподавателей, родителей. Для статистической обработки результатов использовали методы описательной статистики, *t*-критерий Стьюдента, корреляционный, дискриминантный и кластерный анализы, проводили расчет рисков. Наиболее популярным источником информации о сохранении здоровья респонденты отметили Интернет: среди школьников — 79,0%, студентов — 88,6%, родителей — 64,9%, преподавателей — 50,4%. Апробированная практика гигиенического воспитания позволила снизить число школьников и студентов, не имевших навыков безопасного использования электронных устройств до 20 и 25% соответственно; сократить продолжительность использования смартфона в течение дня; увеличить двигательную активность, а также долю детей с нормальным физическим развитием ($p \leq 0,01$); способствовала увеличению продолжительности ночного сна. Апробированные способы воспитания финансово не затратны и могут быть тиражированы в других регионах и организациях.

**Ключевые слова:** школьники, студенты, электронные устройства, правила использования, гигиеническое воспитание, оценка эффективности

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The target of the Healthy Lifestyle Popularization (Public Health Improvement) Priority Project is to raise the share of the Russian population living a healthy lifestyle to 60% by 2025 [1]. Project execution includes a large-scale information campaign in the media, on various Internet resources, in social networks. The campaign will focus on maintaining health. The project aims to invite healthy lifestyle (HL) experts to collect, test and replicate the health maintenance best practices relevant for various groups of population.

Popularization of HL among children, adolescents and youth has always been regarded by specialists as a complex, systemic, multi-level process, covering all spheres of life of the younger generation [2–5].

All aspects of life are digitalized nowadays; this is a global trend. Digital environment affects significantly the learning process of children, adolescents and youth, their leisure time, socialization, health status and lifestyle [6-10].

Against the background of digitalization, children, adolescents and the youth have switched to the new main source of information on health preservation, the internet, and they rely on their parents, teachers and medical workers to a lesser extent in this regard [11].

Innovative methods of HL promotion become more important. Such include flash mobs, quests covered on social media, economic incentives and the like. They are more attractive to the young people compared to the traditional methods that were used several decades ago [12–14].

However, with the digitalization as it is currently, there are practically no research reports assessing effectiveness of the internet as the hygienic education enabler for the younger generation. The Yamal Center for Public Health and Medical Prevention can be taken as an example of the internet resource containing relevant information on the healthy lifestyle. Employees of the Center run an active information and communication campaign on various internet resources and social networks: they post materials on prevention of diseases and regularly hold various creative contests promoting healthy lifestyle. Similar work promoting prevention, including prevention of non-infectious diseases, is done by the specialists of the National Medical Research Center of Therapy and Preventive Medicine and the Center for Hygienic Education of the Population under Rospotrebnadzor.

The share of people living the healthy lifestyle and observing the rules of safe use of electronic devices (ED) is low [15–16]. This fact indicates that the existing preventive measures are insufficient, and there is a need to further research and intensify education efforts aimed at giving the younger generation hygiene skills, including those allowing them to use ED safely.

This study aimed to test and assess the effectiveness of the hygiene education of students and schoolchildren relying on the information available online, including education granting skills needed to use electronic devices safely.

## METHODS

In the context of this study, 2nd and 3rd year medical students received hygiene education over the course of two years. The students were studying hygiene as part of the Federal State Educational Higher Education Standard. Another track of the study had Department of Hygiene employees and postgraduate students teaching ED safe use skills for study and leisure at Dolgoprudnenskaya Grammar School (Moscow region). The hygiene skills were taught to schoolchildren, their parents and teachers.

The study inclusion criteria were: voluntarily signed informed consent form; age of schoolchildren, students; correct completion of the questionnaire. The participating adults (parents and teachers) were polled voluntary with the help of an online service'.

Standard methods and tools enabled physical development dynamic control through the study [17].

The experimental group consisted of 128 medical students (mean age 20 years) and 100 schoolchildren (mean age 16 years); the participants attended Dolgoprudnenskaya Grammar School. The control group consisted of 128 medical students and 100 senior schoolchildren attending other schools at Dolgoprudny; they were not receiving hygiene education. The study also involved 251 parents and 400 teachers/professors working at primary, secondary and higher education organizations.

We used all educational methods (verbal, printed, visual, mixed) and such means as conversation, discussion, lecture, memos, leaflets, posters, video materials, telecommunications etc. In particular, teaching schoolchildren and students hygiene, we employed materials published to the official websites and social network pages of the Yamal Center for Public Health and Medical Prevention, National Medical Research Center of Therapy and Preventive Medicine and the Center for Hygienic Education of the Population under Rospotrebnadzor, as well as videos posted to the YouTube channel.

Factoring in the high interest the youth, their parents and teachers have in social media and websites, and considering how deeply engaged they are with the online resources, we recommended them to seek HL information online, from the specialists available for consultations in the Odnoklassniki, VKontakte, Facebook, Instagram, Telegram social networks, as well on the websites of specialized medical organizations.

In addition, the official website of the Dolgoprudny Grammar School offered HL information with practical recommendations for teachers and parents.

We tested how some HL apps by various developers work in the context of provision of hygiene education. Such apps were installed by schoolchildren and students on their personal smartphones and allowed counting/monitoring body mass index, screen time, steps, nighttime sleep.

The program of the hygiene education measures relied on the use of the following: materials covering prevention of risks associated with the technical and audiovisual properties of ED (electromagnetic radiation, air ionization, screen diagonal, screen brightness level etc.), room microclimate parameters and illumination level; workplace ergonomics, work-rest balance information; information on prevention and health-improving measures.

To assess the effectiveness of HL skills development in students and schoolchildren, we developed special questionnaires in Google Forms, which were made available to the participants [18]. The questionnaires contained questions designed to learn the peculiarities of ED use by schoolchildren and students, their level of awareness of the risks associated with uncontrolled use of ED, shape of their safe ED use skills, and subjective assessment of the respondents' health status [18].

The statistical processing of the data was enabled by Statistica 13.0 (StatSoft Inc.; USA). In our work, we used the methods of descriptive statistics, Student's t-test, correlation, discriminant and cluster analysis, risks calculation. The differences were considered significant at $p = 0.05$.

## RESULTS

We employed both the traditional and innovative teaching methods and means in providing hygiene education to

**Table.** Time schoolchildren spent using smartphones during the day and their body fat mass depending on the effectiveness of mastering the hygiene education program

| Indicator | Group of schoolchildren that responded positively to the hygiene education program | | Group of schoolchildren that did not receive hygiene education or for whom it was ineffective | | *p* |
|---|---|---|---|---|---|
| | Me | [$Q_{25}$; $Q_{75}$] | Me | [$Q_{25}$; $Q_{75}$] | |
| Time spent using a smartphone a day, minutes | 180 | [90; 300] | 720 | [480; 900] | < 0.001 |
| Body fat, kg | 12 | [9; 17] | 14 | [11; –23] | < 0.001 |

schoolchildren and students. The traditional methods and means were conversations, discussions using visual materials, leaflets and posters; the innovative set included business games, web quests, topical discussions with specialists online (on social media), information blogs, HL apps. The program was based both on the official materials and on the previously published information on the ED safe use rules that the authors developed in the context of their previous research efforts.

The questionnaires allowed identifying the time slot when using an ED is safe, i.e. when schoolchildren and students report no complaints about their health. We registered significant differences ($p \leq 0.05$) when comparing time spent using an ED by schoolchildren and students that complained and that did not complain about the state of their health. The complaining schoolchildren and students spent considerable more time using ED (given as Me [$Q_1$; $Q_3$]) in minutes — (660 [420; 960]), than the schoolchildren/students reporting no complaints — (480 [360; 750]). To determine the likelihood of complaints associated with ED use, as well as to find the maximum allowable duration of such use that would not entail health complaints, we carried out discriminant analysis that yielded the following theoretical model:

$$Y_c = -1.655 + 0.02 \times X_{time},$$

where $Y_c$ is the discriminant function characterizing the likelihood of complaints; $X_{time}$ — time spent with an ED.

The constant of discrimination that divided the participants into two groups was determined as the value of the function equidistant from the centroids. In the no-complaints group it was — 0.216, and in the complaints group it equaled 0.080. The sensitivity of the model reached 73.3%, its specificity — 62.6%.

According to this model, schoolchildren and students may spend 78 minutes with an ED without developing health conditions entailing complaints afterwards. This figure formed the background for the hygiene education program developed. Based thereon, we established the allowed ED use time, work-rest balance, breaks involving eye and body exercises.

The data obtained indicate there is a relationship between physical activity and the average time spent with a mobile ED daily, with the correlation ratio between the number of steps per day and the screen time at –0.36. Based on these findings, we recommended replacing a portion of screen time with physical activity, decreasing the former and increasing the latter. The suggested educational effectiveness assessment criteria are the counted minutes of screen time and steps made.

The participants trust internet as the source of information, including health maintenance and HL advice. The aggregated levels of trust reported by them are as follows: students — 88.6%; senior schoolchildren — 79.0%; parents — 64.9%; teachers — 50.4%. Considering this fact, we integrated materials published to the websites of specialized medical organizations and their social network pages, as well as online communication with specialists, into the hygiene education program.

Parents of schoolchildren and teachers who had completed the adapted training course and also seek information on the websites of specialized medical organizations participated in provision of hygiene education to the children.

The work in the context of this study allowed compiling a group of schoolchildren and students that responded well to the hygiene education.

The HL apps schoolchildren used on their personal smartphones enabled them to monitor their body mass index, steps taken, screen time, and nighttime sleep duration. Students participating in the hygiene education program had the number of steps taken increased significantly compared to their peers not included in the study ($p \leq 0.01$), the figures being 13.068 ± 70 vs 9033 ± 90 steps for boys and 8555 ± 50 vs 7807 ± 70 steps for girls, respectively.

According to the app registering nighttime sleep, schoolchildren participating in the study saw the duration of their sleep growing to 8 hours (482 ± 42 minutes), which is close to hygienically justified recommendations, while schoolchildren not involved in the program did not exhibit such a trend. On average, the participating schoolchildren decreased their ED use time to 3.8 hours (230 ± 30 minutes) per a school day.

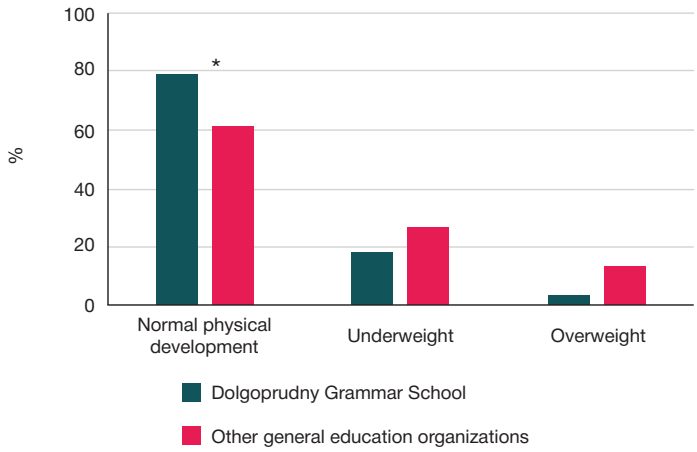


**Fig.** Senior schoolchildren from the Dolgoprudny Grammar School and other general education organizations of Dolgoprudny by level of their physical development ($p < 0.05$)

Cluster analysis based on the dynamics of the studied indicators allowed dividing the schoolchildren into groups. On average, schoolchildren and students who have successfully mastered the hygiene education program spent less time using their smartphones during the day and had better physical development indicators (see Table).

Thus, their average body fat mass was 12 kg and that of students that did not learn the hygienic education program effectively was 14 kg ($p \leq 0.001$). The results of comparison of the groups by the excess body weight were significant ($p \leq 0.004$). Schoolchildren that received no hygiene education or responded poorly to the effort were 2.44 times more likely (OR — 0.41; 95% CI: 0.23–0.73) to gain excess weight than those who mastered th program, the relative risk for the former being 2.01 (95% CI: 1.29–3.21).

Through the two years of the study, it allowed increasing the share of Dolgoprudny Grammar School's senior schoolchildren with normal physical development characteristics (to 79.0 ± 1.7%; $p \leq 0.01$). The comparison was made with other general education organizations of the city that did not offer hygiene education to their pupils. In these schools, the number of senior schoolchildren whose physical development could be considered normal was low (61.0 ± 1.5%) (see Figure).

Only 25% of the senior schoolchild ren from Dolgoprudny Grammar School had dynamometry indicators below average, while the same share among their peers from other schools was 45% ($p \leq 0.05$), which may indicate they suffer from a physical activity deficit.

Questionnaires filled by the schoolchildren and students when the hygiene education program was complete revealed smaller number of complaints about eye conditions from the participants, as well as the decrease of the share of schoolchildren and students not having the ED safe use skills to 20% and 25%, respectively.

In addition, as part of the hygiene curriculum practice, professors gave medical students general cultural competencies using business game elements, web quests, which motivated the said students to not only lead a healthy lifestyle but also to study the subject. Some students began to call themselves "rational nutrition bloggers", "health bloggers", "ED safe use bloggers" etc. They became more active in publishing HL-related information to their blogs and their social media pages, which is a good HL promotion experience for future doctors.

## DISCUSSION

The current education system is a "risk zone": the educational process intensifies, and this intensification entails mental loads, poor physical activity, lack of night sleep, work-rest balance violations [19–22]. The learning process is dynamic, highly labor intensive; there is a wide range of forms and methods of teaching used, the amount of information involved is growing, and educational technologies rely on ED, which makes children, adolescents and youth draw upon their adaptation reserves [23–29]. In such conditions, popularization of HL and ED safe use skills should be an effective preventive measure [12, 13, 15]. With the educational environment turning digital, raised awareness of the safe use of ED and the related HL principles, as well as mastered skills needed to implement them, should be the basis for safe behavior of the educational process participants (teachers/professors, medical workers, schoolchildren/students) [30].

This study has shown that all categories of respondents consider internet to be the most popular source of information on health maintenance. As for the "sources" of HL-related information, two-thirds of students said such were their professors, and more than half of schoolchildren — their parents. This should be taken into account by parents of schoolchildren and teachers when they develop the general cultural competence in youth. It also necessitates raising the HL awareness among adults and fostering their HL skills practiced both in their professional activities and everyday life [15, 30]. In view of development of the digital educational environment, it is advisable to improve the training of teachers on the safe use of ED and HL principles [2, 15].

Previous studies have shown that over a third of parents (35%) and teachers (40%) lack ED safe use skills. Among medical students, the share of those belonging to the same "risk group" was 40%, and among schoolchildren — 35% [15].

The study allowed formulating the key points that need to be added to the program of hygiene education of schoolchildren and students aimed at teaching them ED safe use skills:

– keeping the correct work-rest balance, rational organization of night sleep and physical activity;

– reduction of the ED use time to 3 hours a day, with breaks every 40 minutes — 1 hour;

– training eye strain prevention exercises and exercises to relieve general fatigue;

– use of apps to monitor screen time, physical activity and other parameters;

– participation in social media groups/pages maintained by medical organizations and containing correct information on health and healthy lifestyle;

– increasing motivation to participate in hygiene education programs by using innovative elements popular among young people, stimulating interest among medical students to blog in the field of health;

– involvement of the closest circle of schoolchildren and students (parents, teachers, professors) in promoting healthy lifestyles.

The data obtained allows recommending the tested program for the purposes of teaching hygiene to the younger generation, their parents and specialists the professional duties of which include giving youth the knowledge, skills and abilities to use ED safely and lead a healthy lifestyle.

## CONCLUSION

The study has shown the effectiveness of giving hygiene education to schoolchildren and medical students aimed at development of the electronic devices safe use skills and relying on traditional and innovative teaching methods. To increase the effectiveness of hygiene education, it is necessary to also popularize HK among teachers and parents, whom children, adolescents and youth perceive as carriers of the relevant information. HL promotion should be included in the preventive discipline curricula, for example, the hygiene course read to the medical and pediatric faculty students, since in their future professional activities they will become "sources" of information about HL for the younger generation and the patients. It is strongly recommended to teach various categories of the population the techniques of monitoring parameters related to physical activity, sleep, screen time control etc. Mobile apps installed on personal smartphones can enable such monitoring. We suggest judging the degree of mastering the hygiene education program by the participants' level of awareness about risk factors associated with uncontrolled use of ED and their ED safe use skills. The assessment may rely post-course polling of the participants. The tested methods are not costly; they can be replicated in other regions and organizations.

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