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Acute viral respiratory infections can increase the risk of progression of a pre-existing condition, including a cardiovascular pathology. Life-threatening complications of Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) necessitate research into the cardiovascular effects of COVID-19 crucial for developing adequate treatment strategy for infected patients, especially those of advanced age. This article reviews the literature on the clinical and functional characteristics of patients with COVID-19, including those with poor outcomes. The article looks at the pathophysiological processes occurring in the cardiovascular system in the setting of SARS-CoV-2 infection, risk factors and death predictors. It also discusses continuation of therapy with angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers in patients with COVID-19.

Keywords: coronavirus, cardiovascular diseases, infection, severe acute respiratory syndrome, coronavirus infection 2019, angiotensin-converting enzyme, SARS-CoV-2, COVID-19

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Author contribution: Larina VN conceived and planned the study, analyzed the literature, interpreted the literature data, and revised the manuscript; Golovko MG and Larin VG planned the study, analyzed the literature, interpreted the literature data, and wrote the draft of the manuscript.

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ВЛИЯНИЕ КОРОНАВИРУСНОЙ ИНФЕКЦИИ (COVID-19) НА СЕРДЦЕНО-СОСУДИСТУЮ СИСТЕМУ

В. Н. Ларина М. Г. Головко, В. Г. Ларин


Ключевые слова: коронавирус, сердечно-сосудистые заболевания, инфекция, тяжелый острый респираторный синдром, коронавирусная болезнь 2019, ангиотензин-превращающий фермент, SARS-CoV-2, COVID-19

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Coronaviruses (CoVs) are a family of positive-sense single-stranded RNA viruses, organized into two subfamilies and, as of January 2020, numbering 40 different species. CoVs infect animals and humans and are capable of rapid mutation and genetic recombination. CoVs owe their name to their appearance: their spike-like projections resemble the sun's corona. The spikes allow the virus to penetrate the cell membrane by mimicking the molecules recognized by the transmembrane receptors. Once the receptor has bound to the "impostor" molecule, the virus pushes it into the cell and the viral RNA gets inside.

Acute respiratory infections, such as influenza, respiratory syncytial virus infection and bacterial pneumonia, are widely acknowledged as triggers for cardiovascular diseases (CVD); in turn, pre-existing CVD are associated with other comorbid conditions and can increase the risk of inflammation or aggravate its progression.

The outbreak of the novel coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has rapidly escalated into a pandemic; the majority of the infected patients are reported to have CVD [1].

The prospective cohort observational ARIC (atherosclerosis risk in communities) study has demonstrated that there is a higher risk of coronary heart disease (CHD) and ischemic stroke within up to 90 days after a past infection. The study looked at 1,312 CHD and 712 ischemic stroke cases with a past history of infections within one or two years preceding the cardiovascular event. The mean age of the participants was 75 years; 57.4% of the patients with CHD were male, whereas
54.1% of the patients with stroke were female. Of 1,312 individuals with CHD, 119 (9.1%) had a history of an inpatient infection, whereas 366 (27.9%) had a history of an outpatient infection. The most common were urinary tract infections (29%), pneumonia or respiratory infections (27%), skin/subcutaneous infections (11%), and blood infections (8%). Inpatient infections remained a stronger trigger of cardiovascular events throughout the entire follow-up period (day 14 OR = 12.83, day 30 OR = 8.39, day 42 OR = 6.24, day 90 OR = 4.48) than outpatient infections ($p < 0.05$). Therefore, hospitalized patients with an inpatient infection should be closely monitored in order to take timely measures to prevent CHD or stroke [2].

In light of this, pathophysiological processes triggered by coronavirus infections in the cardiovascular system certainly pose a great interest. Since our knowledge of the mechanisms underlying COVID-19 effects is scarce yet, the analysis of data collected during the outbreaks of viral pneumonia and MERS (Middle East respiratory syndrome coronavirus, MERS-CoV), as well as seasonal influenza, will provide a better understanding of how coronaviruses exert their effects on the cardiovascular system. This has important implications for the development of comprehensive strategies for the timely management of infected advanced-age patients with CVD.

Effects of coronavirus on human organism

Coronaviruses get their name from the crown-like spikes on their surface. These viruses are members of the Coronaviridae family clustered into 4 phylogenetic groups: α, β, γ, and δ CoVs. Only α and β CoVs can cause infection in humans. Coronaviruses have 4 main structural proteins: the spike protein (S), which ensures attachment to the host cell receptor and subsequent fusion with the cell membrane, the nucleocapsid protein (N), the membrane protein (M), and the envelope protein (E).

Human coronaviruses (HCoVs) were discovered in 1965 when the first HCoV was isolated from the culture of human embryo tracheal tissue. By 2003, two types of HCoVs had been identified: HCoV-229E and HCoV-OC43.

Today, 7 different CoV strains are known to infect humans, including HCoV-229E, HCoV-NL63, HCoV-OC43, and HCoV-HKU1 that normally cause self-resolving symptoms. The virus can also cause severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS) and fatal acute respiratory syndrome resulting from SARS-CoV-2 infection.

Endemicity of coronavirus

Four types of HCoVs, including HCoV-229E (α-CoV), HCoV-NL63 (α-CoV), HCoV-OC43 (β-CoV), and HCoV-HKU1 (β-CoV), are endemic to humans and normally cause a mild respiratory infection with self-resolving symptoms, accounting for 15 to 30% of acute respiratory infections (ARI). As a rule, mild symptoms develop in young patients; in older patients, especially those with cardiovascular or bronchopulmonary pathology, the infection can provoke hospitalization and require emergency care [3].

Severe acute respiratory syndrome caused by coronavirus

The first SARS-CoV outbreak occurred in Guangdong province, South China, in 2002 [4]. Shortly after SARS-CoV was isolated and identified, similar SARS-like CoVs were detected in Himalayan palm civets and raccoon dogs; their nucleotide sequence was 99.8% homologous to that of SARS-CoV isolated from humans [5].

SARS-CoV is a representative of the β-CoV group; it binds to a zinc peptidase angiotensin-converting enzyme 2 (ACE2), which is a surface molecule exploited by the virus to enter the host cell. ACE is present in various organs and tissues; it is an integral plasma membrane protein found in endothelial, specialized epithelial, neuroepithelial, nerve terminal, and reproductive system cells. The role of ACE is not limited to the regulation of cardiovascular system functions. It also participates in hematopoiesis and the metabolism of some bioactive peptides [6].

ACE2 is found in arterial and venous endothelial cells, smooth muscle cells of the arterial wall, the epithelium of the respiratory tract and the fetal intestine, and immune system cells. It is hypothesized that the inhibition of ACE2 expression in patients infected with SARS-CoV is implicated in the pathological changes to pulmonary tissue, causing severe pneumonia and acute respiratory failure.

Research studies on wild animals proved that SARS-CoV could have originated in bats: Chinese horseshoe bats were found to harbor a SARS-like CoV with high nucleotide homology (87–92%) to SARS-CoV isolated from humans. Palm civets and raccoon dogs are putative intermediate hosts for SARS-CoV amplification preceding its transmission to other animals through contact at a market. SARS-CoV is transmitted from human to human by respiratory droplets during close contact (airborne spread).

There is a proposition about the possibility of the fecal-oral transmission route for SARS-CoV-2 predicated on the fact that patients stricken by SARS or MERS during the past outbreaks often had gastrointestinal symptoms, such as diarrhea and abdominal pain, and SARS-CoV RNA was detected in the feces of 14.6% patients with SARS or MERS [7]. Some patients presented with fever, diarrhea, abdominal pain, and SARS-CoV RNA was detected in the feces of 14.6% patients with SARS or MERS during the past outbreaks [8]. In vitro studies have demonstrated that MERS-CoV can infect and replicate in the intestinal epithelium of humans using dipetidyl peptidase 4 as a receptor. In vivo studies have revealed that inflammation and epithelial degeneration in the small intestine can precede pneumonia in MERS, confirming that pulmonary MERS-CoV-induced infection can be secondary to the enteric infection [9].

According to some authors, the incubation period for SARS-CoV-2 varies between 2 and 11 days, being 5.2 days on average (95% CI 4.1–7.0) [10]; other researchers report that the incubation period lasts up to 14 days [11].

SARS-CoV can be excreted into the environment and transmitted by hand contact between patients and healthcare workers; this means that surfaces must be sanitized and the nose, mouth and eyes must be protected against the virus [12].

The ability of an infected individual to spread the virus to other people is inferred from the R0 (basic reproduction number) value. For SARS-CoV, R0 is about 3, i.e. a person infected with SARS-CoV will potentially infect 3 other people in the susceptible population. The average R0 value for seasonal flu (swine flu, H1N1) is about 1.3 [13] (Table 1).

So far, no effective vaccine or medication against SARS-CoV has been developed. The clinical management of patients with SARS includes supportive symptomatic treatment and prescription of broad-spectrum antimicrobials against secondary bacterial infection. Advanced age (upwards of 60 years), multimorbidity (diabetes mellitus, CVD, cancer, chronic obstructive pulmonary disease), and elevated lactate dehydrogenase are predictors of death in patients with SARS-CoV infection. Some authors point to insignificant morbidity and mortality rates in children and adolescents in the past SARS outbreaks [14].
At the same time, preliminary data on 4,226 confirmed COVID-19 cases in the USA suggest high mortality in patients aged ≥ 85 years (10–27%). In patients aged 65–84 years, the case mortality rate ranged from 3 to 11%; in patients aged 55–64 years, it was 1–3%, whereas in patients aged 20–54 years, less than 1%. No deaths were reported in patients aged ≤ 19 years. There were young patients among the hospitalized individuals. Deaths registered in the group of patients aged 20–64 years amounted to 20% of overall case mortality; in this age group, hospitalized patients aged 20–44 years made 20% [15].

Currently, there is a paucity of published studies investigating risk factors and death predictors in patients with COVID-19. Starting on December 25, 2019 through January 26, 2020, a study was conducted in 201 patients with COVID-19 aged 43 to 60 years (the median age was 51), of whom 63.7% were men, revealing that 32.8% of the participants had a pre-existing condition. The median hospital stay was 13 (10–16) days; 33% of the patients required mechanical ventilation; the median interval between admission to hospital and progression to SARS was 2 (1–4) days. Blood tests revealed that the majority of the patients had elevated lactate dehydrogenase (98%), elevated C-reactive protein (85.6%), elevated interleukin 6 (48.8%), and elevated D-dimer (23.3%). Age upwards of 65 years, neutrophilia, organ dysfunction, and coagulation dysfunction were associated with progression to acute respiratory distress syndrome (ARDS) and death. In the patients who developed ARDS, administration of methylprednisolone was associated with lower mortality (46%), as compared to methylprednisolone-free treatment (61.8%) (OR 0.38) [16].

Clinical presentations of COVID-19

The most prevalent COVID-19 symptoms include fever, cough, labored breathing (shortness of breath or rapid breathing). Myalgia, anorexia, nausea, malaise, sore throat, nasal congestion, and headache are less common. The symptoms can set in as early as 2 days after contact with an infected person or by day 14 following such contact. The viral load does not differ between symptomatic and asymptomatic patients, suggesting a possibility of transmission from an asymptomatic individual or a patient with mild symptoms to another person. The highest number of viral copies is detected in nasal swabs, as compared to throat swabs. The diagnosis of COVID-19 is confirmed by PCR. Mucosal specimens for PCR are collected either from the upper or the lower respiratory tracts. A case of COVID-19 is assumed to be confirmed if the laboratory test for the presence of SARS-CoV-2 RNA returns a positive result even in the absence of clinical symptoms. Point-of-care serological tests are expected to be available in the nearest future.

Potential mechanisms underlying the effects of coronavirus infection on the cardiovascular system

The danger of ARI lies surging mortality from chronic diseases during an epidemic, especially among patients with cardiovascular pathology. According to a systematic review which included 42 publications and 39 clinical studies and was published in 2009 patients were at heightened risk of myocardial infarction (OR 4.95; 95% CI 4.4–5.5) and stroke (OR 3.2; 95% CI 2.8–3.6) in the first few days following the onset of ARI; over time, this risk was gradually decreasing [17].

The hypothesis that influenza can provoke acute cardiovascular events and death was proposed in the 1930s. It was then that a link was noticed between the seasonal activity of flu viruses and higher mortality from all causes, including bronchopulmonary pathology, pulmonary TB, diabetes mellitus, organic heart disease, and hemorrhagic stroke [18].

In 2004, another study reported a vast array of life-threatening clinical manifestations of coronavirus infection, including death from myocardial infarction (2 of 5 deaths); this unveiled the need for prompt treatment of CVD patients during a potential epidemic of a respiratory infection [19].

The lessons learnt from past epidemics caused by coronaviruses inspired a hypothesis that viral infections can provoke acute coronary syndrome, arrhythmias, heart failure, and thromboembolic complications resulting from the pronounced systemic inflammatory response combined with localized vascular inflammation.

In this respect, COVID-19 should not be an exception. It seems to affect the clinical course of pre-existing CVD and trigger development of life-threatening complications [20].

The severity of clinical manifestations, long- and short-term cardiovascular effects of COVID-19, and the effects of specific treatment are yet to be researched. It should be noted that during flu epidemics the majority of patients die of cardiovascular complications and not of virus-induced pneumonia itself. Considering the extreme inflammatory load caused by COVID-19 and the available clinical data on other coronavirus-related infections, one can expect to see serious cardiovascular complications in patients with COVID-19; their prevalence and severity will probably be lower in outpatients than in hospitalized individuals.

In a recently published study [21], 73% of 41 inpatients with laboratory-confirmed COVID-19 were men and 32% had pre-existing conditions, such as diabetes mellitus (20%), arterial hypertension (AH) (15%), and other CVD (15%). The median age of the patients was 49 (41–58) years. Among the most common symptoms of COVID-19 were fever (98%), cough (76%), sputum production (28%), malaise (44%), headache (8%), hemoptysis (5%), and diarrhea (3%). Lymphopenia was observed in 63% of the patients, shortness of breath, in 55%. The median time from the onset of symptoms to developing shortness of breath was 8 (5–13) days. Some of the complications included ARDS (29%) and acute cardiac injury (12%).

Another study conducted in 1,099 in- and outpatients with laboratory-confirmed COVID-19 (median age of 47 years; 42% women) found that the most common pre-existing conditions were AH (14.9%), diabetes mellitus (7.4%) and CAD (2.5%), ARDS (3.4%) and shock (1.1%) were the most severe complications [22].

In most patients with myocarditis, viral infection and the immune response to it are the root causes of inflammation. The leading mechanisms underlying myocardial injury in the acute phase of myocarditis are invasion of cardiomyocytes by viral particles that have tropism for myocardial tissue, the direct

Table 1. Characteristics of coronaviruses

<table>
<thead>
<tr>
<th>Coronavirus species</th>
<th>Receptor</th>
<th>Incubation period (days)</th>
<th>R0</th>
<th>Co-existing CVD, %</th>
<th>Mean case fatality rate, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>SARS-CoV</td>
<td>ACE2</td>
<td>2–11</td>
<td>3</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>SARS-CoV-2</td>
<td>ACE2</td>
<td>2–14</td>
<td>2–3</td>
<td>от 3.4 до 40**</td>
<td>0.7–8***</td>
</tr>
</tbody>
</table>

Note: * — overall rates; ** — in hospitalized patients; *** — depending on time, geographical area and medical help available.
Patients with elevated troponin were of advanced age, had one in 5 patients had elevated high-sensitivity troponin levels. Had CAD; 5.3%, cerebrovascular disease; 4.1%, heart failure; (13.7%) of the patients eventually died [27]. Of them, 10.6% results of observation of 416 inpatients with COVID-19; 57 steroids [26].

Conductivity disorders, and the positive effect of prescribed immune manifestations, the combination of arrhythmia and the infection (≤ 1 year). Additional criteria include systemic arrhythmias and the infection, and the time elapsed from link between the onset or exacerbation of symptoms/findings: the acute onset of the disease, the established nonspecific. The diagnosis relies on a triad of medical history have high tropism for cardiomyocytes [25].

Over 50% of myocarditis cases are associated with a viral infection. Parvovirus B19, Coxsackie A and B enteroviruses, ECHO-viruses, rubella virus, adenoviruses, human herpesvirus 6, Epstein-Barr virus, influenza virus, cytomegalovirus, etc. have high tropism for cardiomyocytes [25].

Clinical presentations of myocarditis are diverse and nonspecific. The diagnosis relies on a triad of medical history findings: the acute onset of the disease, the established link between the onset or exacerbation of symptoms/arrhythmias and the infection, and the time elapsed from the infection (≤ 1 year). Additional criteria include systemic immune manifestations, the combination of arrhythmia and conductivity disorders, and the positive effect of prescribed steroids [26].

In a recently published paper, researchers describe the results of observation of 416 inpatients with COVID-19; 57 (13.7%) of the patients eventually died [27]. Of them, 10.6% had CAD; 5.3%, cerebrovascular disease; 4.1%, heart failure; one in 5 patients had elevated high-sensitivity troponin levels. Patients with elevated troponin were of advanced age, had more comorbidities, lymphopenia, higher white cell count and higher levels of atrial natriuretic peptide, C-reactive protein and procalcitonin than those with normal troponin levels. Patients with acute inflammatory heart disease developed ARDS more often than those without acute cardiac condition (58.5% vs 14.7 %, respectively; p < 0.001); the proportion of deaths in this group was also higher (51.2% vs 4.5%, respectively; p < 0.001). Multivariate analysis confirmed that acute heart failure (OR 4.26) and ARDS (OR 7.89) were predictors of poor outcome in patients with COVID-19.

Similar results are reported by other authors [24], who found that 27.8% of 187 included patients with confirmed COVID-19 had developed acute cardiovascular complications resulting in cardiac dysfunction and arrhythmias; a combination of cardiovascular complications with elevated high-sensitivity troponin was associated with high mortality.

Although pathophysiological mechanisms underlying myocardial injury in patients with COVID-19 are heavily understudied, there is evidence that the SARS-CoV genome is found in the myocardium of 35% of patients with SARS. These findings increase the probability of direct damage to cardiomyocytes by the virus, SARS-CoV-2 might utilize the same mechanism of action as SARS-CoV as these two species are genetically close, although not identical. The close correlation between elevated high-sensitivity troponin and C-reactive protein levels implies the inflammatory origin of myocardial injury in the progressive disease phase. As viral particles spread along the respiratory tract and invade host cells, they may trigger a cytokine storm ensuing from the imbalance in Th1 and Th2 production and a cascade of immune reactions that lead to myocardial injury. In the setting of infection, secretion of cytokines can cause a reduction in coronary blood flow and oxygen supply, atherosclerotic plaques destabilization and formation of microclots (Fig. 1).

Myocarditis is often manifested as arrhythmias, progressive heart failure and sudden cardiac arrest that can occur at any stage of the disease.
Among the first symptoms of myocarditis are malaise, fatigue, myalgia, and sometimes low-grade temperature caused by the inflammatory response to the virus but not by myocardial injury itself. Other manifestations of myocarditis include sudden cardiac arrest due to ventricular tachycardia or ventricular fibrillation following damage to the conducting system of the heart, thromboembolism, syncope, cardiogenic shock, and acute heart failure. The first clinical symptoms can set in at the onset of API or a few days after its onset.

There are a number of obstacles complicating the diagnosis of viral myocarditis. The primary diagnostic criterion for myocarditis is the established link between cardiac symptoms and a past infection and the signs of inflammation. The diagnosis can be facilitated by a full medical examination of the patient, which includes clinical, laboratory and instrumental tests, and by endomyocardial biopsy performed to rule out the inflammatory nature of myocardial injury [28].

Unfortunately, currently there is no substantial evidence about the efficacy of existing antivirals and vaccines against COVID-19. Patients with pre-existing CVD are at high risk of complications, a more severe course of the disease and poor outcomes; therefore, CVD patients with COVID-19 should be stratified depending on their primary condition (CVD) and its severity in order to decide on the treatment strategy. Electrocardiography and cardiac biomarker tests (NT-proBNP) can be employed to control the condition of the patient and the course of treatment.

There is a lot of controversy over whether patients infected with a coronavirus should continue angiotensin-converting enzyme inhibitors (ACE inhibitors) and angiotensin II receptor blockers (ARBs). The fears are predicated on the fact that the protease domain of ACE2 is a potential target for SARS-CoV-2 and SARS-CoV-2 and that increased expression of ACE2 can aggravate damage to the lungs in patients with COVID-19 (Fig. 2) (adapted from [29]).

ACE2 exists in 2 forms: as a structural transmembrane protein with an extracellular domain, which serves as a target for the S protein of SARS-CoV-2, and as a soluble circulating form. Invasion of host cells (alveolar type II cells in the first place) by SARS-CoV-2 occurs through the binding of the virus to the protease domain of ACE2. ACE2 expression changes following endocytosis of the viral complex. This results in the accumulation of a potent vasoconstrictor angiotensin II and, possibly, mitigates the vasodilator effect of angiotensin (1–7). Local activation of the renin-angiotensin-aldosterone system can mediate damage to the lungs in response to viral infection, whereas increased ACE2 expression can aggravate pulmonary damage in patients with COVID-19. ACE2 converts angiotensin I to angiotensin (1–9) (its functions are being studied) and angiotensin II to angiotensin (1–7). This process is accompanied by inactivation of angiotensin II and synthesis of angiotensin (1–7); the latter stimulates vasodilation, reduces oxidative stress and fibrosis. ACE — angiotensin-converting enzyme; ACEI — ACE inhibitors; ARB — angiotensin II receptor blockers; SMC — smooth muscle cells. ACE — angiotensin-converting enzyme; ACEIs — ACE inhibitors; ARBs — angiotensin II receptor blockers; SMCs — smooth muscle cells.

Fig. 2. Interactions between SARS-CoV-2 and the renin-angiotensin-aldosterone system. Interaction between SARS-CoV-2 and the renin-angiotensin-aldosterone system, invasion of host cells (alveolar type II cells, in particular) by SARS-CoV-2 occurs through the binding of the virus to the functional domain of ACE2. ACE2 expression changes following endocytosis of the viral complex. This results in the accumulation of a potent vasoconstrictor angiotensin II and, possibly, mitigates the vasodilator effect of angiotensin (1–7). Local activation of the renin-angiotensin-aldosterone system can mediate damage to the lungs in response to viral infection, whereas increased ACE2 expression can aggravate pulmonary damage in patients with COVID-19. ACE2 converts angiotensin I to angiotensin (1–9) (its functions are being studied) and angiotensin II to angiotensin (1–7). This process is accompanied by inactivation of angiotensin II and synthesis of angiotensin (1–7); the latter stimulates vasodilation, reduces oxidative stress and fibrosis. ACE — angiotensin-converting enzyme; ACEI — ACE inhibitors; ARB — angiotensin II receptor blockers; SMC — smooth muscle cells. ACE — angiotensin-converting enzyme; ACEIs — ACE inhibitors; ARBs — angiotensin II receptor blockers; SMCs — smooth muscle cells.

Also, there are no experimental or clinical data demonstrating positive or negative effects of ACEIs/ARBs or other RAAS antagonists in patients with COVID-19, as well as in patients.
Table 2. A non-exhaustive list of clinical trials of drugs for the prevention and treatment of COVID-19

<table>
<thead>
<tr>
<th>Drug</th>
<th>Start date</th>
<th>Estimated completion date</th>
<th>Trial ID at ClinicalTrials.gov</th>
</tr>
</thead>
<tbody>
<tr>
<td>Remdisivir</td>
<td>February 21, 2020</td>
<td>April 1, 2023</td>
<td>NCT04280705</td>
</tr>
<tr>
<td>Human recombinant ACE2</td>
<td>February 2020</td>
<td>April 2020</td>
<td>NCT04286786</td>
</tr>
<tr>
<td>Remdisivir</td>
<td>March 2020</td>
<td>May 2020</td>
<td>NCT04292899</td>
</tr>
<tr>
<td>Injections and infusions of the LV-SMENP-DS and antigen-specific cytotoxic T cell vaccines</td>
<td>February 24, 2020</td>
<td>December 31, 2024</td>
<td>NCT04276896</td>
</tr>
<tr>
<td>Fingolimod</td>
<td>February 22, 2020</td>
<td>July 1, 2020</td>
<td>NCT04280588</td>
</tr>
<tr>
<td>Human mesenchymal stem cells</td>
<td>February 24, 2020</td>
<td>February 1, 2021</td>
<td>NCT04293682</td>
</tr>
<tr>
<td>Carrimycin</td>
<td>February 23, 2020</td>
<td>February 28, 2021</td>
<td>NCT04286503</td>
</tr>
<tr>
<td>Methylprednisolone</td>
<td>February 14, 2020</td>
<td>May 30, 2020</td>
<td>NCT04273321</td>
</tr>
<tr>
<td>Washed microbiota transplantation</td>
<td>February 2, 2020</td>
<td>April 16, 2020</td>
<td>NCT04251767</td>
</tr>
<tr>
<td>Losartan</td>
<td>March 16, 2020</td>
<td>April 1, 2021</td>
<td>NCT04312009</td>
</tr>
<tr>
<td>2019-nCoV vaccine (mRNA-1273)</td>
<td>March 3, 2020</td>
<td>June 1, 2021</td>
<td>NCT04283461</td>
</tr>
<tr>
<td>Lopinavir / ritonavir tablets combined with xylanine injection</td>
<td>March 14, 2020</td>
<td>April 14, 2021</td>
<td>NCT04295551</td>
</tr>
</tbody>
</table>

with COVID-19 and a history of CVD. If a patient with CVD is infected with COVID-19, the treatment strategy should account for their overall symptoms and hemodynamics.

RAAS activation plays a crucial role in the pathogenesis of many CVD. Long-term effects of increased renin and angiotensin II production and the activity of the sympathetic nervous system include left ventricular hypertrophy, dyslipidemia, arrhythmias, hypercoagulation, endothelial dysfunction, insulin resistance, and metabolic syndrome. ACEIs and ARBs that have been studied and successfully used in the clinical setting for many years are first-choice drugs for treating chronic heart failure, AH, renal disorders, and diabetes mellitus [35, 36].

The ACE2-dependent entry of SARS-CoV-2 into the host cell can be blocked by caspase-14, the inhibitor of serine protease TMRPSS2 used by SARS-CoV-2 for S protein priming. Caspase-14 is a promising candidate for further trials [37].

Among the antivirals for treating flu, oseltamivir does not have any effect on SARS-CoV-2 although preliminary studies have demonstrated some positive effects of favipiravir. Table 2 features a few drugs which are now in clinical trials or being considered as candidates for clinical trials in patients with COVID-19.

CONCLUSIONS

The data collected so far suggest a high prevalence of comorbidities in middle-aged and old patients with COVID-19. The most common cardiovascular conditions are AH (about 15%), diabetes mellitus (7.4–20%) and CAD (about 2.5%). Patients with COVID-19 and a pre-existing cardiovascular pathology are at high risk of developing ARDS, shock and death. Acute cardiac failure and ARDS are regarded as predictors of poor outcomes in patients with COVID-19.

It is crucial to study different aspects of screening, diagnostics, clinical manifestations, prevention and management of patients with COVID-19. As the infection spreads and more data are accumulated, risk factors for cardiovascular complications should be thoroughly investigated.

Perhaps, creating a registry of patients with COVID-19 and systemic reporting of clinical symptoms and cardiovascular and other complications will help to elucidate the characteristics of the infected patients, develop approaches to the treatment and prevention and design a risk model for predicting complications.

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ON THE UNPREDICTABILITY OF OUTCOMES OF IMMUNOTHERAPY AND PREVENTIVE IMMUNIZATION AGAINST COVID-19

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This article analyzes the possibility of employing immunotherapy and preventive immunization to fight COVID-19. The authors think that treatment and prevention of the infection with anti-SARS-CoV-2 antibodies can have unpredictable outcomes. Although these antibodies can neutralize virus antigens (S-proteins), they also have the ability to enhance virus entry into the host cell. The article emphasizes the importance of solid evidence of efficacy and safety for candidate anti-COVID-19 therapies and protective measures.

Keywords: coronavirus, COVID-19, SARS-CoV-2, antibodies, preventive immunization, immunotherapy

Author contribution: the authors equally contributed to the manuscript

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Finding effective treatment and developing protective measures against the novel coronavirus infection COVID-19 is a critical challenge facing medical science. Today, many candidate approaches to managing this infection are under scrutiny. Obviously, the effective treatment is expected to directly block the virus and prevent it from replicating or entering the cell.

Drugs designed to inhibit HIV (ritonavir, lopinavir) and Ebola virus (remdesivir) are now being evaluated for their potential to inhibit coronavirus replication [1, 2]. However, so far there is no solid evidence of their efficacy against COVID-19; this is true for both approved drugs and those still in clinical trials [3]. The possible anticoronaviral effect of quinolines and the unclear underlying mechanism of action need further investigation.

Immunotherapy and preventive immunization might hold promise for countering COVID-19. Indeed, vaccines and passive immunization have been successful in fighting various infections, including viral infections. However, because of the features demonstrated by the causative agent of COVID-1 - SARS-CoV-2, extreme caution should be exercised when using active or passive immunization approaches.

This article is an attempt to point to the unpredictability of outcomes of using anti-SARS-CoV-2 antibodies for treating and preventing COVID-19.

Interaction with viral S-proteins

Spike-glycoproteins (S-proteins) responsible for latching onto receptors of the host cell have long been identified as the primary surface target for neutralizing antibodies. Using cell and animal models of severe acute respiratory syndrome (SARS-CoV) and Middle-East respiratory syndrome (MERS-CoV), researchers have demonstrated that antibodies can bind to and neutralize S-proteins [4, 5]. It is reported that anti-S-IgG for neutralizing MERS-CoV promote survival of viral clones that carry mutations in the S-protein encoding genes; as a result, the antibodies can no longer recognize the S-protein and neutralize the virus [6].

Antibody-dependent enhancement of virus entry

Unfortunately, the emergence of clones unrecognizable to antibodies is not the only drawback of passive immunization/immunotherapy. Therapies with anticoronaviral antibodies can be devastating due to the phenomenon of antibody-dependent enhancement of virus entry. Briefly, some IgG variants can accelerate penetration of the virus into the cell because their Fab fragments can bind to the S protein of SARS-CoV, whereas other IgG domains, like Fc or unidentified sites, bind to a number of host cell receptors, including angiotensin-converting enzyme 2, dipeptidyl peptidase-4 and the FcY-receptor (see Figure). This phenomenon has been demonstrated in the models of some coronavirus-related infections, including SARS and MERS [7, 8]. Considering the similarity of pathogenesis between SARS, MERS and COVID-19, there is a high probability that SARS-CoV-2 will also provoke IgG-dependent enhancement of virus entry. Some authors believe that IgG-enhancement of virus entry is not limited to epithelial cells and can also occur...
Fig. A schematic representation of antibody-dependent enhancement of virus entry

in immune cells via immunoglobulin FcγII receptors (CD32) [9]. IgG-dependent damage to immune cells might underlie the pathogenesis of uncontrolled immune system activation and cytokine storm in patients with SARS.

It is believed that antibodies do not always enhance virus entry, depending on the antibody binding site on the S protein, the IgG subclass, IgG concentrations and expression of cell receptors. This unpredictability means that convalescent serum and synthetic anti-S antibodies should not be used in COVID-19 patients without thorough thought. The same applies to preventive immunization against COVID-19. It cannot be ruled out that vaccination will stimulate production of polyclonal antibody variants responsible for antibody-dependent virus entry.

As COVID-19 is continuing its global rampage, a worrying trend is being born: scientists are engaged in a race to develop diagnostic, therapeutic and preventive tools for the novel infection at all costs. A similar situation unfolded in the USSR shortly after HIV was discovered. In an attempt to get ahead of their foreign counterparts, some medical teams decided to treat AIDS patients with immunostimulants. The formal yet erroneous logic behind the decision dictated that a patient who developed immunodeficiency should be treated by stimulating the immune system. Dozens of patients fell victim to the ambitions of their doctors because immunostimulation provoked the irreversible progression of the disease. We hope that the story will not repeat itself with COVID-19 and that treatments for this infection will be evidence-based.

CONCLUSIONS

1. On the one hand, antibodies against coronaviral S-proteins can neutralize the virion; on the other hand, they are also capable of enhancing virus entry into the host cell. 2. Although COVID-12 is an epidemiological emergency, its treatment and prevention should be based on solid evidence of safety and efficacy.

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ROLE OF ACE2/TMPRSS2 GENES REGULATION BY INTESTINAL microRNA ISOFORMS IN THE COVID-19 PATHOGENESIS

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Coronavirus SARS-CoV-2, the cause of the COVID-19 pandemic, enters the cell by binding the cell surface proteins: angiotensin-converting enzyme 2 (ACE2) and transmembrane serine protease 2 (TMPRSS2). The expression of these proteins varies significantly in individual organs and tissues of the human body. One of the proteins’ expression regulation mechanisms is based on the activity of the microRNA (miRNA) molecules, small non-coding RNAs, the most important function of which is the post-transcriptional negative regulation of gene expression. The study was aimed to investigate the mechanisms of the interactions between miRNA isoforms and ACE2/TMPRSS2 genes in the colon tissues known for the high level of expression of the described enzymes. The search for interactions was performed using the correlation analysis applied to the publicly available paired mRNA/miRNA sequencing data of colon tissues. Among the others, such miRNAs as mir-30c and mir-200c were identified known for their involvement in the coronavirus infection and acute respiratory distress syndrome pathogenesis. Thus, new potential mechanisms for the ACE2 and TMPRSS2 enzymes regulation were ascertained, as well as their possible functional activity in a cell infected with coronavirus.

Keywords: COVID-19, SARS-CoV-2, ACE2, TMPRSS2, miRNA, isomiR, acute respiratory distress syndrome, coronavirus

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Author contribution: Nersisyan SA, Shkurnikov MYu, Osipyants AI, Vechorko VI — study concept; Nersisyan SA, Shkurnikov MYu — bioinformatics analysis; Nersisyan SA, Shkurnikov MYu, Osipyants AI, Vechorko VI — interpretation of results; Nersisyan SA — manuscript writing.

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ROLE РЕГУЛЯЦИИ ГЕНОВ АФП2/TMPRSS2 ИЗОФОРМАМИ микроRNК КИШЕЧНИКА В ПАТОГЕНЕЗЕ COVID-19

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Коронавирус SARS-CoV-2, вызвавший пандемию COVID-19, проникает в клетку, связываясь с поверхностными белками: аниотенсин-превращающий фермент 2 (АПФ2) и серинмембранзная протеаза 2 (TMPRSS2). Экспрессия данных белков значительно различается в отдельных органах и тканях организма человека. Одним из механизмов регуляции их экспрессии является активность молекул микроRNК — коротких некодирующих РНК, важнейшей функцией которых является посттранскрипционная негативная регуляция экспрессии генов. Целью работы было выявить механизмы взаимодействия изоформ микроRNК и генов АПФ2 / TMPRSS2 в тканях толстого кишечника, известных высоким уровнем экспрессии указанных ферментов. Поиск взаимодействий функциональных взаимодействий генов было осуществлен средствами корреляционного анализа на публично доступной выборке данных парного мРНК / микроRNК-секвенирования тканей кишечника. В числе находок оказались такие микроRNК как miR-30c и miR-200c, известные своей ролью в патогенезе коронавирусной инфекции и острого респираторного дистресс-синдрома. Таким образом, были установлены новые потенциальные механизмы регуляции ферментов АПФ2 и TMPRSS2 и их возможная функциональная активность в клетке, инфицированной коронавирусом.

Ключевые слова: COVID-19, SARS-CoV-2, ACE2, TMPRSS2, микроRNК, изоформа микроRNК, острый респираторный дистресс-синдром, коронавирус

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The rapid and progressive spread of the COVID-19 infection caused by the SARS-CoV-2 coronavirus deeply affected the health of hundreds of thousands of people, which became a serious challenge to healthcare systems and global economic stability. The characteristics of SARS-CoV-2, especially distinguishing the disease from influenza, are the higher infection rate combined with the increased risk of severe course and mortality, mainly due to the acute respiratory
distress syndrome (ARDS) [1]. The mechanism of the cells infection is actively studied in many laboratories. In particular, it is known that the SARS-CoV-2 viral envelope expresses the spike protein (S protein) containing the receptor binding domain with high affinity for the extracellular domain of angiotensin-converting enzyme 2 (ACE2). The further S protein cleavage by the transmembrane serine protease 2 (TMPRSS2) aimed to produce the S1 and S2 subunits is a crucial stage for membrane fusion and virus internalization by endocytosis with ACE2 in pulmonary epithelium. It is assumed that the greater virulence of SARS-CoV-2 compared to other coronaviruses can be explained by the S1 protein’s significantly higher affinity for ACE2. This mechanism of the SARS-CoV-2 entering the cell leads to the loss of ACE2 on the cell surface, thereby contributing to chronic lung function impairment and severe tissue fibrosis [2].

MicroRNAs (miRNAs) are the small non-coding single stranded RNAs containing an average of 22 nucleotides. One of the most important intracellular functions of miRNA is the negative regulation of gene expression due to complementary miRNA binding with the target mRNA, leading to mRNA degradation or translational inhibition [3]. MicroRNAs are formed from the longer hairpin molecules of pre-miRNA as a result of the hairpin cleaving Drosha and Dicer enzymes’ activity [4]. The cleaving site inaccuracy leads to the emergence of various miRNA isoforms that differ in several nucleotides at the ends of the molecule. It is reported that many miRNAs of canonical types are expressed much weaker than some alternative isoforms [5]. It is of key importance that different isoforms of the same miRNA may have completely different target genes. This is because the most important role in binding to the target mRNA is played by the miRNA region between the 19th nucleotide of the hairpin (seed region) [6].

It is reported that the functional impairment of miRNAs and their isoforms is associated with a large number of pathological conditions, including cancer, neurological and cardiovascular diseases [7]. A large number of papers is devoted to the study of the role of miRNAs in the pathogenesis of viral infections: some of them are aimed to study the therapeutic potential of the direct miRNA interaction with the virus [8], and the others are aimed to investigate the potential interactions of miRNAs and proteins playing a key role in the viral vital processes [9]. However, the ACE2 and TMPRSS2 expression regulation by miRNA in subjects with COVID-19 remains poorly understood. The study was aimed to reveal the mechanisms of the interactions between miRNA isoforms and ACE2/TMPRSS2 genes in the colon tissues known for the high level of expression of the described enzymes.

METHODS

To search for miRNA isoforms interacting with ACE2 and TMPRSS2 enzymes, we performed the integrated analysis of the paired mRNA and miRNA expression in the normal colon tissues’ sample (the enzyme is most intensively expressed in the colon tissues). The tissue selection was also due to the fact that the gut models were often used for in vitro studies of viruses [10, 11]. The available for public access samples from The Cancer Genome Atlas Colon Adenocarcinoma (TCGA-COAD) collection were used. The sample analysis was carried out by the next-generation mRNA and miRNA sequencing [12]. The data were expression matrices of thousands of mRNA and miRNA isoforms in eight samples, the unit of expression was the binary logarithm of the corresponding transcript number of reads normalized to the upper quartile of the overall distribution (FPKM-UQ). To search for potential regulatory interactions between miRNA isoforms and TMPRSS2 the Spearman correlation coefficients between the expressions of 25% of the most highly expressed isoforms with the expressions of the corresponding mRNA were calculated, with subsequent filtering in accordance with the p-value (significance level 0.05).

RESULTS

The ACE2 and TMPRSS2 expression at the mRNA level turned out to be very high: TMPRSS2 was in the list of the most highly expressed genes (1%), and the ACE2 expression was between the 95th and 94th percentiles, which was fully consistent with published data [13] (see Figure). Correlation analysis allowed us to detect the miR-21 miRNA demonstrating a significant negative correlation with the ACE2 gene expression, as well as the following miRNA families regulating TMPRSS2: let-7a/let-7d, miR-30a, miR-30c, miR-127, miR-194, miR-200c, miR-361 and miR-423. The let-7a miRNA was represented by the hsa-let-7a-5p isoform, which differed from the canonical type by adenine added at the 5’ end of the molecule. The miR-194 was represented by the hsa-miR-194-3p isoform, which lacked the 5’ first nucleotide. The absence of the corresponding miRNA canonical forms in the list indicates the importance of taking into account the profiles of all miRNA isoforms, not just canonical isoforms.

DISCUSSION

Some of the discovered miRNAs have already been detected during the virological studies. Thus, it was shown that the miR-93c expression in the lungs of the mouse changed significantly upon infection with SARS-CoV virus [14], which made it possible to put forward a hypothesis about the involvement of that miRNA in the development of a disease caused by the virus. The miR-200c miRNA is also of great interest. In 2017, a paper was published reporting that miR-200c miRNA played a key role in the virus-induced ARDS pathogenesis [15]. The researchers found out that the H5N1 avian influenza virus promoted the miR-200c expression, the target of which was the ACE2 receptor. Moreover, the viral proteins were detected responsible for promoting the miRNA expression. The discovery of the interaction possibility between the described miRNA and
the TMPRSS2 enzyme emphasizes the need for studying the role of miR-200c in the COVID-19 pathogenesis.

CONCLUSION

The results obtained indicate the presence of numerous regulatory interactions between miRNA isofoms and ACE2/TMPRSS2 enzymes. Such information is extremely important due to the key role of enzymes in the mechanism of cell infection with SARS-CoV-2 coronavirus. Further research is needed for refining and experimental validation of the findings. In particular, it is possible to discover new treatment options based on the ACE2 and TMPRSS2 expression regulation via microRNAs.

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The emergence and rapid spread of the novel coronavirus SARS-CoV-2 has sparked the pandemic of COVID-19 [1]. Over 2 billion confirmed cases and more than 150,000 deaths were reported within less than 3 months after the infection found its way out of China [2]. In the absence of specific treatments and vaccines, quarantine and lockdown are seen as the only available containment measure [3]. Since the virus is capable of presymptomatic transmission, molecular diagnostic techniques based on the amplification of nucleic acids have become the primary tool for monitoring its spread [4]. China, Singapore, South Korea, and Germany succeeded in implementing the broad testing strategy using reverse-transcription polymerase chain reaction (RT-PCR) kits and thus were able to timely identify individuals infected with SARS-CoV-2; these countries seem to have gained control of the epidemic and saved medical resources for treating critically ill patients. By contrast, Italy, Sweden, and USA, who were late to adopt the broad testing strategy or initially denied its efficacy, are now facing a truly grave situation.

Molecular methods based on the amplification of nucleic acids boast high sensitivity and high specificity; they can detect viral RNA in both severely ill patients and asymptomatic individuals and thus significantly contribute to stemming the spread of the virus [5, 6]. Immediately after the first complete SARS-CoV-2 genome sequences were obtained, Chinese researchers and WHO released primer and probe sequences for PCR-based virus detection; in the months that followed, thousands of SARS-CoV-2 whole genomes were sequenced and new RT-PCR assays were developed. In this article, we talk about the main approaches to designing PCR assays and some of their specific characteristics.

In diagnostic tests, proper sample collection techniques and adequate, informative samples are essential to valid and reliable results. Knowledge of viral tropism is critical to choosing the type of the specimen to be collected. The efficacy of RT-PCR for SARS-CoV-2 detection depends on the specimen source and the applied sampling technique. It is reported that bronchoalveolar lavage fluids have the highest diagnostic value.
in terms of SARS-CoV-2 detection, followed by sputum, nasal swabs, fibrobronchoscope brush biopsy, pharyngeal swabs, feces, and blood (1%) [7]. Still, nasal and oropharyngeal swabs remain the most available and informative specimen type used in screening tests for SARS-CoV-2 [8, 9]. When properly performed, swabbing allows obtaining good quality samples and is safe for the medical staff [10].

Real-time PCR with fluorescent hybridization probes (real-time PCR) is the primary molecular genetic technique for SARS-CoV-2 detection [6, 11, 12]. It is widely available, highly sensitive and specific. PCR assay kits are instrumental in implementing mass screening aimed at detecting infected individuals and quantifying viral loads in each patient.

Approaches RT-PCR assay design

So far, the oligonucleotides and real-time RT-PCR kits for SARS-CoV-2 detection have been described in a few dozens of publications by international authors. In those studies, several different approaches can be identified to designing real-time RT-PCR assays for SARS-CoV-2 detection. Singleplex PCR assays, in which oligonucleotides are selected to target only one specific gene, are the simplest and the most available. Multiplex assays are more advanced and allow targeting a number of different genes simultaneously. Primers and probes for multiplex assays can have different specificity or enable discrimination between SARS-CoV-2 and the related coronaviruses or other respiratory infections. For SARS-CoV-2 detection, primers and probes are usually selected to target the nucleocapsid genes N1 and N2, the RNA-dependent RNA polymerase gene (RdRP) and the E protein gene of the viral envelope. For example, CDC (Centers for Disease Control and Prevention) recommends that identification of COVID-19 patients should start with a screening test for the E protein gene, whose nucleotide sequence does not differ from that of SARS, and then proceed to differentiating SARS from SARS-CoV-2 using oligonucleotides for the RdRP target [13]. According to the WHO protocol, the collected samples should be screened for N and Orf1b. However, the proposed oligonucleotides do not help in discriminating between SARS-CoV-2 and SARS; therefore, sequencing is advised to finalize the identification procedure [14]. Table 1 features publicly accessible primer and probe sequences for SARS-CoV-2 detection recommended by WHO and CDC.

**Characteristics of existing RT-PCR assays for SARS-CoV-2 detection**

Due to high demand, over 10 different commercial kits for SARS-CoV-2 detection have been launched on the Russian market; some of them have already received a medical device registration certificate (Table 2). Singleplex kits have higher sensitivity (up to 500 GE/ml) than their multiplex counterparts (1,000 to 10,000 GE/ml), whereas multiplex kits targeting several SARS-CoV-2 genes are more specific and help to avoid false-negative results associated with the variability of the virus resulting from mutations at the oligonucleotide binding site. Of note, results generated by multiplex kits are sometimes difficult to interpret due to the insufficient optimization of the oligonucleotide sequence. Nevertheless, all Russian manufacturers claim the sensitivity of their kits to be 1,000 GE/ml. Importantly, an internal control should be included in the kit, regardless of the number of specific targets. The internal control can be endogenous (human DNA) or exogenous (e.g., an RNA phage). It is used to control all stages of the protocol, from nucleic acid extraction to amplification.

Although the internal control is necessary, not every assay has it. For example, it is not found in the kits based on isothermal amplification, including loop-mediated isothermal amplification (LAMP). Such assays are advantageously fast (they take no longer than 40 min), do not require sophisticated instrumentation and can be used as point-of-care tests outside the lab, as no thermocycler is needed. LAMP-based assays boast a sensitivity of up to 1–3 RNA copies per reaction [15]. However, the actual sensitivity of currently available commercial LAMP assays is lower than claimed (Table 2).

**Whole-genome sequencing**

Among all techniques for molecular genetic analysis, sequencing still has the highest informative value. In the current pandemic caused by SARS-CoV-2, the number of complete

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### Table 1. Oligonucleotides recommended for COVID-19 diagnostics by WHO and CDC

<table>
<thead>
<tr>
<th>Target</th>
<th>Oligonucleotides</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WHO</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORF1b-nsp14</td>
<td>HKU-ORF1b-nsp14F TGGGGYTTTAACRGGTAACCTG HKU-ORF1b-nsp14R AACRCGCTTTAACAGAAAGCAGCTC HKU-ORF1b-nsp14F PAM-TAGTGTGATGCWATCATGACTAG-BHQ1</td>
<td>SARS coronavirus BetaCoV/bat</td>
</tr>
<tr>
<td>N gene</td>
<td>HKU-NF TAATCAGGAAAGCAGCATGATT HKU-NR CAAAGATTGCAATCTGATG HKU-NP GCAATTGTGACATTGCGG-BHQ1</td>
<td>SARS coronavirus</td>
</tr>
<tr>
<td>envelope protein</td>
<td>E_Sarbeco_F ACAGTTGACTGTTAATATTGTAATATGGC E_Sarbeco_R ATATTGGACTGTTAATATTGTAATATGGC E_Sarbeco_P1 FAM-ACAGTTGACATTGCGG-BHQ1</td>
<td>SARS coronavirus BetaCoV/bat</td>
</tr>
<tr>
<td>N gene</td>
<td>N_Sarbeco_F CACATTGGCACCAGGCAACATC N_Sarbeco_R GAGGAGCAAGGAGGCTGTT N_Sarbeco_P FAM-ACTTCCTGAGAAGCACAACATTGCGCA-BHQ1</td>
<td>SARS coronavirus BetaCoV/bat</td>
</tr>
<tr>
<td>RdRP gene</td>
<td>RdRp_SARSrs-F GTGARATGATGCTATGTTTGGCCG RdRp_SARSrs-R CARATGATTTAASGACATACTTAGCATCA RdRp_SARSrs-P1 FAM-CCAGTTGCGACTCCTACMGTTATGC-BHQ1</td>
<td>SARS-CoV-2</td>
</tr>
<tr>
<td></td>
<td>RdRp_SARSs-P2 FAM-CAGGTTGGAACCTCCTACGAGAGATGC-BHQ1</td>
<td></td>
</tr>
</tbody>
</table>

---
Table 2. Kits for SARS-CoV-2 RNA detection

<table>
<thead>
<tr>
<th>Certificate ID and registration date (Federal Service for Surveillance in Healthcare)</th>
<th>Name</th>
<th>Manufacturer</th>
<th>Claimed sensitivity (GE/ml)</th>
<th>Amplification time</th>
</tr>
</thead>
<tbody>
<tr>
<td>2020/10068 dated 17.04.2020</td>
<td>Real-time isothermal amplification kit for SARS-CoV-2 RNA detection in biological samples</td>
<td>Evotech Mirai Genomics LLC</td>
<td>10 000</td>
<td>25 min</td>
</tr>
<tr>
<td>2020/10064 dated 16.04.2020</td>
<td>SBF-DX-SARS-CoV-2 Real-time PCR kit for SARS-CoV-2 RNA detection in biological samples (fluorescent hybridization)</td>
<td>SystemaBioTech LLC</td>
<td>1000</td>
<td>1 h 40 min</td>
</tr>
<tr>
<td>2020/9957 dated 02.04.2020</td>
<td>Isotherm SARS-CoV-2 RNA-screen Real-time loop-mediated isothermal amplification kit for SARS-CoV-2 RNA detection in biological samples</td>
<td>Generium JSC</td>
<td>1000</td>
<td>25 min</td>
</tr>
<tr>
<td>2020/9948 dated 01.04.2020</td>
<td>SARS-CoV-2/SARS-CoV Real-time RT-PCR kit for SARS-CoV-2 and SARS-CoV RNA detection</td>
<td>DNA-Technology TS LLC</td>
<td>1000</td>
<td>50 min</td>
</tr>
<tr>
<td>2020/10032 dated 14.04.2020</td>
<td>Real-time PCR kit for SARS-CoV-2 RNA detection in biological samples (fluorescent hybridization)</td>
<td>MediapiTech LLC</td>
<td>1000</td>
<td>1 h 20 min</td>
</tr>
<tr>
<td>2020/9904 dated 27.03.2020</td>
<td>Polyvir SARS-CoV-2 RT-PCR kit for SARS-CoV-2 RNA detection</td>
<td>Litech LLC</td>
<td>1000</td>
<td>1 h 30 min</td>
</tr>
<tr>
<td>2020/9785 dated 27.03.2020</td>
<td>AmpiliTest SARS-CoV-2 PCR kit for SARS-CoV-2 RNA detection</td>
<td>Center for Strategic Planning, Ministry of Healthcare of the Russian Federation</td>
<td>1000</td>
<td>1 h 20 min</td>
</tr>
<tr>
<td>2020/9896 dated 27.03.2020</td>
<td>Real-Best RNA SARS-CoV-2 RT-PCR kit for SARS-CoV-2 RNA detection</td>
<td>Vector-Best JSC</td>
<td>1000</td>
<td>1 h 20 min</td>
</tr>
<tr>
<td>41956 2014/1987 dated 25.03.2020</td>
<td>AmpilSense® CoV-Bat-FL PCR kit for MERS-CoV and SARS-CoV-CoV/CoV-2 RNA detection in biological samples (fluorescent hybridization); technical specifications 9398-224-01897593-2013</td>
<td>Central Research Institute of Epidemiology, Federal Service for Surveillance in Healthcare</td>
<td>1000</td>
<td>1 h 20 min</td>
</tr>
<tr>
<td>2020/9845 dated 20.03.2020</td>
<td>Real-time isothermal amplification kit for SARS-CoV-2 RNA detection in biological samples</td>
<td>SmartLifeCare LLC</td>
<td>10 000</td>
<td>25 min</td>
</tr>
<tr>
<td>41390 2020/9700 dated 14.02.2020</td>
<td>Vector-OneStepPCR-Cov-RG Real-time PCR kit for SARS/COVID-19 RNA detection (fluorescent hybridization)</td>
<td>Vector, State Research Center of Virology and Biotechnology, Federal Service for Surveillance in Healthcare</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>41240 2020/9677 dated 11.02.2020</td>
<td>Vector-real-time PCR-2019-nCoV-RG Real-time PCR kit for 2019-nCoV RNA detection (fluorescent hybridization)</td>
<td>Vector, State Research Center of Virology and Biotechnology, Federal Service for Surveillance in Healthcare</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Genomic sequences of the virus obtained within very short time is record-breaking. Whole-genome sequencing has never been so close to adoption in the clinical setting as it is now. There are a few approaches to whole-genome sequencing of SARS-CoV-2. The classic approach consists in the extraction of nucleic acids from nasopharyngeal and/or oropharyngeal swabs, subsequent depletion of the host’s ribosomal RNA for library preparation and sequencing itself carried out according to the protocols supplied by the manufacturer. However, this approach requires a fair amount of viral RNA and good read depth. With low viral loads, the virus can be replicated using cell cultures. For that, serial passages are performed in Vero V, Vero E6, LLC-MK2, and some other cell lines. This approach has been successfully used in some laboratories, including the Reference center for coronavirus infection (GISAID ID: EPI_ISL_421275).

Whole-genome amplification is an alternative to cell cultures. So far, a few panels have been designed for sequencing the entire genome of SARS-CoV-2. Among them is the Ion AmpliSeq SARS-CoV-2 Research Panel (Thermo Fisher Scientific; USA). It consists of two primer pools for the amplification of 125–275 bp-long fragments [16].

Another panel was developed by Paragon Genomics Inc (USA). It is a multiplex PCR research panel with two primer pools and an average amplicon size of 99 bp [17]. The panel can potentially detect 1.15 viral copies at 95% probability. Using two overlapping pools of primers will ensure full coverage of the entire viral genome, with a calculated detection limit of 0.29 copies at 95% probability. So far, there is no data on the actual SARS-CoV-2 detection limit for the AmpliSeq SARS-CoV-2 Research Panel.

A new protocol for sample preparation and bioinformatic analysis was proposed by the ARTIC network [18]. It was developed for Oxford Nanopore sequencing platform and generates results within 8 h.

CONCLUSIONS

Methods based on the molecular genetic analysis of nucleic acids are instrumental in the surveillance and monitoring of SARS-CoV-2 spread and help to contain the COVID-19 pandemic. Their primary advantage over thermometry or evaluation of symptoms is the ability to detect asymptomatic carriers or infected presymptomatic individuals. In spite of a plethora of designs, classic RT-PCR is still the preferred detection technique. Refinement of isothermal amplification tools will make molecular analytical techniques more accessible in the future and improve their efficacy in monitoring and controlling biological threats. Sequencing is now accumulating more data about changes occurring in the viral genome and using it for RT-PCR primer optimization, vaccine development, study of the evolution of the virus, and
reconstruction of epidemiological processes that drive the epidemic. Sequencing platforms make it possible to analyze
reconstruction of epidemiological processes that drive the epidemic. Sequencing platforms make it possible to analyze


References


Литература

CHARACTERIZATION OF THE GENOTYPE AND THE PHENOTYPE OF NONTOXIGENIC STRAINS OF CORYNEBACTERIUM DIPHTHERIAE SUBSP. LAUSANNENSE ISOLATED IN RUSSIAN RESIDENTS

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In 2018, a few sequencing studies were published revealing the existence of two monophyletic clusters within the C. diphtheriae species, meaning that this species can be divided into two subspecies: C. diphtheriae subsp. diphtheriae and C. diphtheriae subsp. lausannense. The objective of our study was to describe the genotype and the phenotype of 2 nontoxigenic C. diphtheriae strains isolated in Russia in 2017–2018, which were classified by us as C. diphtheriae subsp. lausannense based on the aggregated data yielded by a variety of techniques, including microbiological and molecular genetic techniques, as well as a bioinformatic search for subspecies-specific genes in the publicly available genomes of C. diphtheriae. The isolated strains had morphological and biochemical characteristics of C. diphtheriae. The strains were assigned to the MLST type ST199 included in the clonal complex associated with subsp. lausannense. PCR revealed that both analyzed strains of C. diphtheriae subsp. lausannense carried the ptsI gene encoding phosphoenolpyruvate-protein phosphotransferase and did not carry the narG gene encoding the synthesis of nitrate reductase subunits, whereas the strains of C. diphtheriae subsp. diphtheriae had the narG gene and did not have the ptsI. We experimentally proved the ability of lausannense strains to ferment N-acetylgalactosamine. Our findings expand the knowledge of the biological diversity of C. diphtheriae and indicate the need for estimating the spread of these microorganisms in Russia, as well as their pathogenic potential.

Keywords: diphtheria, nontoxigenic Corynebacterium diphtheriae, Corynebacterium diphtheriae subsp. lausannense, multifocus sequence typing, phylogenetic analysis

Author contribution: Borisova OYu carried out molecular genetic tests, analyzed the literature and the obtained data, contributed to manuscript preparation; Chaplin AV performed phylogenetic analysis, analyzed the experimental data and contributed to manuscript preparation; Gadua NT, Pimenova AS carried out microbiological tests and contributed to manuscript preparation; Alexeeva IN, Rakitsky GF examined the patients on admission and contributed to manuscript preparation; Afanasiev SS conducted molecular genetic tests and contributed to manuscript preparation; Donskih EE analyzed the literature and the experimental data and contributed to manuscript preparation; Kafarskaya LI analyzed the experimental data and contributed to manuscript preparation.

Compliance with ethical standards: the study was approved by the Ethics Committee of G. N. Gabrichevsky Research Institute for Epidemiology and Microbiology. Informed consent was obtained from all participants.

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ОБЩЕСТВЕННАЯ ПАТОЛОГИЯ ЛЕКАРСТВЕННЫХ ЗАБОЛЕВАНИЙ

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Ключевые слова: дифтерия, нетоксигенные Corynebacterium diphtheriae, Corynebacterium diphtheriae subsp. lausannense, мультифокусный секвенирование, филогенетический анализ

Вклад авторов: О. Ю. Борисова — молекулярно-генетические исследования, анализ данных, анализ литературы, подготовка рукописи; А. В. Чаплин — филогенетический анализ, анализ данных, подготовка рукописи; Н. Т. Гадуа и А. С. Пименова — молекулярные биологические исследования, подготовка рукописи; И. Н. Алексеева и Г. Ф. Ракицкий — обследование пациентов и первичная идентификация, подготовка рукописи; С. С. Афанасьев — молекулярно-генетические исследования, подготовка рукописи; Е. Е. Донских — анализ данных, анализ литературы, подготовка рукописи; Л. И. Кафарская — анализ данных, подготовка рукописи.

Соблюдение этических стандартов: исследование одобрено этическим комитетом Московского научно-исследовательского института гигиены и микробиологии имени Г. Н. Габричевского. Все пациенты подписали добровольное информированное согласие на участие в исследовании.

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ORIGINAL RESEARCH I MICROBIOLOGY

BULLETIN OF RSMU | 2, 2020 | VESTNIKRGMU.RU
Diphtheria is caused by toxigenic strains of Corynebacterium diphtheriae harboring integrated bacteriophage DNA containing the toxin gene. The infection spreads from person to person via airborne droplets and develops into classic pharyngeal or nasal diphtheria.

Over the past century, mass immunization programs have dramatically cut down the incidence of diphtheria [1]. In Russia, the incidence rate of the disease has stabilized due to good vaccination coverage (> 95%) [2]. In 2017, no incident cases of diphtheria and only 2 asymptomatic carriers were reported in Russia. In 2018, 4 incident cases of the disease and 3 carriers were reported, whereas in the first 9 months of 2019, there were 3 new cases of diphtheria and 2 carriers [3]. In the past few years, there have been no reports of the secondary cases or lethal infection. Most clinical forms of diphtheria are mild localized forms.

Today, diphtheria is a rare disease; therefore, it can pose a diagnostic difficulty to the clinician. This, as well as the existence of latent carriers, who act as a reservoir for the infection, and the fact that the epidemic process unfolds in the vaccinated population, still renders diphtheria a clinically important problem [3].

Recently, infections caused by nontoxigenic C. diphtheriae strains have been on the rise. They manifest atypically as pharyngitis, respiratory tract infections, endocarditis, osteomyelitis, septic arthritis or skin infections [4–8].

Historically, C. diphtheriae were classified into 4 biotypes based on their biochemical phenotypes: gravis, mitis, intermedius and belfanti [9, 10]. Representatives of the same biovar, though, can be genetically distant [11, 12]. This is why genomics does not support the use of biovars as a reliable classification tool for C. diphtheriae [13]. Besides, there is no correlation between the biovar and pathogenicity [14]. This is why the analysis of C. diphtheriae strains was carried out using the following the guidelines 4.2.3065-13 for laboratory diagnostics of diphtheria. The isolates were plated onto telluride blood agar (2% fishmeal hydrolysate agar base, State Research Center for Applied Microbiology & Biotechnology; Obolensk, Russia) supplemented with 7% bovine blood (Letran; Russia) and potassium tellurite (State Research Center for Applied Microbiology & Biotechnology; Obolensk, Russia) and kept in a temperature-controlled chamber at 37 °C for 24–48 hours. Grown colonies of C. diphtheriae were evaluated for their morphological, toxigenic and biochemical properties.

To evaluate the ability of the analyzed strains to ferment N-acetylglucoasamine, a phenol red broth was ex tempore supplemented with N-acetylglucoasamine (Sigma-Adrich; USA). Then, a loop full of overnight C. diphtheriae cultures grown on serum agar was added to 3 ml of the solution. The cultures were incubated at 37 °C for 24–48 h. Fermentation was evaluated based on the change in the color of the solution. Two toxigenic and two nontoxigenic gravis strains, as well as two toxigenic and two nontoxigenic mitis strains, were used as controls.

The sample of the analyzed published genomic sequences comprised 204 C. diphtheriae genomes representing diphtheriae and lausannense subspecies deposited in the NCBI Refseq database, 3 genomes of C. diphtheriae subsp. lausannense from the NCBI Genbank and one genome of Corynebacterium ulcerans BR-AD22, which served as an outgroup for phylogenetic reconstruction. In total, 208 genomes were included in the analyzed dataset.

Coding sequences retrieved from the genome annotations in the corresponding databases were clustered into ortholog groups using OrthoMCL [16] with standard settings (inflation index of 1.5; protein sequence similarity threshold of 50%; e-value of 10–5). For phylogeny reconstruction, we used the groups of orthologs that were made up of the genes present in every genome in the amount of 1 copy. Nucleotide sequences were aligned in MUSCLE software [17] and then concatenated. Phylogeny reconstruction was performed following the Maximum Likelihood algorithm implemented in FastTree software [18] using the GTR+CAT model. MLST types of the published sequences were predicted based on the
data retrieved from PubMLST. Clonal clusters were formed in Phyloviz 2 using the goeBURST algorithm at the SLV level [19].

Total DNA was isolated from overnight C. diphtheriae cultures grown on fishmeal hydrolysate agar (State Research Center for Applied Microbiology & Biotechnology; Obolensk, Russia) supplemented with 10% bovine serum (Leitran; Moscow) using a standard boiling extraction method with subsequent centrifugation.

Detection of tox gene fragments in nontoxigenic C. diphtheriae strains was performed in accordance with the protocol described in [20]. The PCR reaction mix contained 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1 µM of forward and reverse primers, 200 mM of each dNTP, and 1 unit of Taq polymerase (Thermo Fisher Scientific; USA). DNA of the control toxigenic C. diphtheriae strain (gravis biovar, accession number 665) was used as a positive amplification control.

MLST types of C. diphtheriae strains were determined following the international protocol [14]; fragments of 7 housekeeping genes were Sanger-sequenced, including atpA, dnaE, dnaK, fusA, leuA, odhA, and ppoB. Sequencing was carried out by Evrogen JSC (Moscow). Allele identification was done using the PubMLST database.

To identify dbfR fragments in the sequences of C. diphtheriae strains, PCR was carried out with one pair of primers for the entire region of the dbfR gene: GGGACTACAACGCAACAAAGAA and TCATCTAATTTCGCCGCCTTTA as described in [20, 21]. The following primers were used for subspecies-specific PCR: _F: ACCTTCCGAACCTGCCATCC and _R: GAGTTGTCATAACGCCACTG.

RESULTS

The C. diphtheriae strains B-8759 and B-8760 had been isolated from the pharynx of two patients (26 and 77 years) admitted to 2 different units of a psychiatric hospital; the isolated cultures were grown on fishmeal hydrolysate agar (State Research Center for Applied Microbiology & Biotechnology; Obolensk, Russia) supplemented with 10% bovine serum (Leitran; Moscow) using a standard boiling extraction method with subsequent centrifugation. Toxigenicity of the grown colonies was determined from their morphological, biochemical properties of the culture, like saccharolytic and nitrate reductase activity. The cultures exhibited cysteinase activity and formed a brown halo following inoculation into the Pisu medium; the cultures fermented glucose, maltose, fructose and galactose, did not ferment saccharose and starch, and exhibited no urease or nitrate reductase activity (Table 1). The tests allowed us to provisionally assign the analyzed C. diphtheriae strains to the belfanti biotype typically seen in lausannense subspecies.

In the next step, we analyzed the previously published genomes of C. diphtheriae, which was necessary to verify that the studied species can be distinctly divided into subspecies and to conduct a search for species-specific protein-encoding genes.

The constructed phylogenetic tree (Fig. 1) confirmed the results previously obtained on a smaller sample indicating that representatives of C. diphtheriae constituted two clades corresponding to the subspecies _diphtheriae_ and _lausannense_. The tree also showed that the representatives of these subspecies belonged to non-overlapping groups of sequence types. The goeBURST clustering analysis of MLST types described in PubMLST (Fig. 2) revealed that all representatives of the _lausannense_ subspecies whose genomes had been previously sequenced belonged to the sequence types ST106, ST360, or ST409, and to one previously undescribed type that differed from ST359 in just one allele. All these sequence types formed one clonal complex.

It could be hypothesized that other sequence types (such as ST35, ST37, ST69, or ST81) constituting the same clonal complex also belong to the _lausannense_ subspecies. An additional argument in favor of our hypothesis is that almost all isolates representing the sequence types from this clonal complex have been described in the PubMLST database as representing the belfanti biotype typical to the _lausannense_ subspecies.

The analysis of ortholog groups revealed the existence of loci specific to C. diphtheriae subspecies. For example, all strains of the _lausannense_ subspecies had a region (presumably, an operon) harboring genes of the phosphotransferase system, for which N-acetylglucosamine is a hypothesized substrate. The following primers were selected for the gene coding

### Table 1. Characteristics of the analyzed C. diphtheriae cultures

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>C. diphtheriae strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Strain 665 (control)</td>
</tr>
<tr>
<td>Glucose fermentation</td>
<td>+</td>
</tr>
<tr>
<td>Saccharose fermentation</td>
<td>–</td>
</tr>
<tr>
<td>Maltose fermentation</td>
<td>+</td>
</tr>
<tr>
<td>Fructose fermentation</td>
<td>+</td>
</tr>
<tr>
<td>Galactose fermentation</td>
<td>+</td>
</tr>
<tr>
<td>Starch fermentation</td>
<td>+</td>
</tr>
<tr>
<td>Urease</td>
<td>–</td>
</tr>
<tr>
<td>Nitrate reductase</td>
<td>+</td>
</tr>
<tr>
<td>Cysteinase test</td>
<td>+</td>
</tr>
<tr>
<td>Toxigenicity (the Feldman method)</td>
<td>+</td>
</tr>
<tr>
<td>Presence of the tox gene</td>
<td>+</td>
</tr>
</tbody>
</table>
for phosphoenolpyruvate-protein phosphotransferase: ptsI_F: ACTTTCCGAACCTGCCATCC and ptsI_R: GTGTAICTCTTGCTGTC (the expected product length was 489 bp). At the same time, a locus encoding the synthesis of nitrate reductase subunits was detected only in the genomes of the *C. diphtheriae* subspecies. The following primers were selected for the gene encoding its α-subunit (the gene was present in the sequences of 201 out of 202 strains representing this subspecies in the analyzed sample): narG_F: CTGACCACTGGGGCGAGG and narG_R: GAGTTGTCATAACGCCACTG (the expected product length was 691 bp).

PCR with primers for the amplification of *ptsI* and *narG* fragments (Fig. 3) showed that the samples containing DNA of В-8759 and В-8760 strains carried the *ptsI* gene and did not carry the *narG* gene, whereas “classic” *C. diphtheriae* strains had the *narG* gene and did not have *ptsI*. There were no samples that carried either both of these genomic loci or none of them.

These findings and the results of biochemical identification allowed us to conclude that the analyzed *C. diphtheriae* strains belonged to *C. diphtheriae* subsp. *lausannense*. The conclusion was corroborated by the fact that the isolated strains represented the sequence type ST199 included in the clonal complex presumably typical to the representatives of this subspecies (Fig. 2). Another piece of evidence confirming our conclusion was the sequence of the *dtxR* gene that coincided with the sequences found in the genomes of *lausannense* subspecies.

Considering that strains of *C. diphtheriae* subsp. *lausannense* carried the gene coding for phosphoenolpyruvate-protein phosphotransferase, which is part of the phosphotransferase system for N-acetylglucosamine, we conducted a few experiments to investigate the phenotypic manifestations of this gene. The experiments showed that unlike *C. diphtheriae* subsp. *diphtheriae*, both analyzed strains, which we classified as *C. diphtheriae* subsp. *lausannense*, fermented N-acetylglucosamine (Fig. 4).

The analyzed strains, which we classified as *C. diphtheriae* subsp. *lausannense*, were deposited in the State collection of pathogenic microorganisms (SCPМ-Obolensk).
Fig. 2. The clonal complex reconstructed from PubMLST data. The complex comprises lausannense strains with publicly available sequenced genomes.

Fig. 3. Gel electrophoresis of PCR products with the following primers: \textit{pstI} \textit{F} — \textit{pstI} \textit{R} and \textit{narG} \textit{F} — \textit{narG} \textit{R}. \textit{M} is a GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific; USA) (example).

Fig. 4. Saccharolytic activity of \textit{C. diphtheriae} subsp. lausannense strains: the ability to ferment N-acetylglucosamine. The crimson color of the medium means the test results are positive. 1, 2 are isolated strains of \textit{C. diphtheriae} subsp. lausannense; 3 is a strain of gravis \textit{C. diphtheriae} subsp. diphtheriae; 4 is a strain of mitis \textit{C. diphtheriae} subsp. diphtheriae; 5 — negative control.
DISCUSSION

We were able to identify the two analyzed C. diphtheriae strains isolated from the samples of Russian residents as nontoxigenic representatives of subsyb. lausannense. Our findings along with the reports of foreign researchers [14, 22] suggest that these strains are ubiquitous. They belong to the sequence type ST199, which is part of the lineage-2 cluster typical to the lausannense subspecies, and carry the sequence of the dtxR gene characteristic of lausannense representatives. The analysis of ortholog groups established the existence of loci specific to the subspecies of C. diphtheriae: the region containing the genes of the N-acetylglucosamine-phosphotransferase system (specific to the lausannense subspecies) and the region encoding the synthesis of nitrate reductase subunits (specific to the diphtheriae subspecies). Our findings are consistent with the results of earlier genomic studies of the lausannense subspecies [15] and the studies of the biochemical properties of the belfanti biotype [9, 10]. Primers designed for these genes and the subsequent PCR allowed us to classify the two analyzed strains as C. diphtheriae subsp. lausannense.

CONCLUSIONS

We have identified the 2 analyzed strains collected on the territory of Russia as nontoxigenic strains of C. diphtheriae subsp. lausannense. Our findings expand the knowledge of the biological diversity of C. diphtheriae and indicate the need for estimating the spread of these microorganisms.

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SYNTHESIS OF $^{13}$C- AND $^{14}$C-LABELLED LINOLEIC ACIDS FOR USE IN DIAGNOSTIC BREATH TESTS FOR HEPATOBILIARY SYSTEM DISORDERS

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At present, there is a need for a simple, noninvasive, highly specific and sensitive diagnostic test for hepatobiliary system disorders. Compounds labeled with carbon isotopes are widely used in various diagnostic breath tests; they are safe and can reliably detect a metabolic disorder or enzyme deficiency. The aim of this study was to synthesize $^{13}$C- and $^{14}$C-labeled linoleic acids suitable for use in hepatobiliary breath tests in terms of purity. In the synthesis of $^{13}$C-labeled linoleic acid, the chemical yield for 1-bromo-8,11-heptadecadien was 86.4% and the chemical yield for barium carbonate-$^{13}$C was 96.0%. In the synthesis of $^{14}$C-labeled linoleic acid, the chemical yield for 1-bromo-8,11-heptadecadien was 87.39%; for barium carbonate-$^{14}$C it was 97.1%. The specific radioactivity of $^{13}$C-labeled linoleic acids was 45.36 ± 0.02 mCi/g. The radiochemical yield of the reaction was 96.0%. The proposed method is suitable for batch production.

Keywords: breath test, linoleic acid, $^{13}$C, $^{14}$C, hepatobiliary system, liver disease

Author contribution: Tynio YY conceived and supervised the study; Morozova GV synthesized the final product by carboxylation of the Grignard reagent with $^{13}$C and $^{14}$C dicarboxylic acids, did preparative calculations; Biryukova YuK conducted NMR-analysis of the final product; Sivokhin DA analyzed the literature and wrote the manuscript; Pozdniakova NV analyzed the literature; Zykov MV measured the radioactivity of carbon atoms in the reagents, intermediate and final products; Bogdanova ES analyzed the NMR spectra of the final products; Smirnova MS determined the melting point of the final products; Shevelev AB provided reagents and instrumentation and revised the manuscript.

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SYNTHESIS OF $^{13}$C- AND $^{14}$C-LABELLED LINOLEIC ACIDS FOR USE IN DIAGNOSTIC BREATH TESTS FOR HEPATOBILIARY SYSTEM DISORDERS

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В настоящее время для диагностики заболеваний печени и билиарных органов требуется разработка простого неинвазивного теста с высокой чувствительностью и специфичностью. Соединения, меченные изотопами углерода в исходных веществах, полупродуктах синтеза и конечных соединениях; Е. С. Богданова — расшифровка ЯМР-спектра конечных продуктов синтеза; М. С. Смирнова — определение температуры плавления конечных соединений; А. Б. Шевелёв — материально-техническое снабжение и редактирование перевода рукописи.

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The prevalence of chronic liver diseases that progress to cirrhosis, including hepatitis B and C, alcoholic liver disease, toxic hepatitis, primary sclerosing cholangitis, etc., is on the rise [1].

The gold standard for evaluating the liver is a liver biopsy. However, being an invasive procedure, it is associated with the risk of complications and, therefore, cannot be used as a routine test [2]. Adoption of highly reliable, simple and safe noninvasive diagnostic tests into clinical practice [3] will allow clinicians to monitor the efficacy of treatment and estimate the functional reserve of the liver [2, 4]. Such tests have a strong advantage over elastography, as well as APRI [5] and FORNS [6] scores calculated from a patient’s laboratory data.

Linoleic acid is a long-chain water-insoluble compound. Bile secreted by the gall bladder catalyzes hydrolisis of linoleic acid in the small intestine; the reaction results in the formation of mixed micelles. The lack of bile salts in the bile caused by a hepatobiliary disorder slows absorption of a labeled fatty acid, which can be inferred from the isotopic composition of exhaled carbon dioxide [20].

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The literature reports an 11-step method for stereospecific synthesis of [1-13C] isomers of monounsaturated fatty acids using olefin inversion [21]. Advantageously, this method allows obtaining monounsaturated fatty acids with a C=C double bond at different positions. However, it is very labor-intensive, involves preparative separation of stereoisomers upon epoxidation and requires an expensive and toxic source of 13C, such as cyanide. These factors impede adoption of the method in large-scale manufacturing.

So far, there are no published reports describing chemical synthesis of 13C- and 14C-labeled derivatives of most common dietary unsaturated fatty acids (linoleic and linolemic) from the most common source (CO2) Synthesis by biological methods involving protists and fungi, like Thraustochytrium and Mortierella alpina, has a low radiochemical yield under 60% [22, 23]. The residual isotope is disposed of as waste. Given that 13C is a long-lived radionuclide, this method of synthesis is a hazard to the environment. Another drawback of the method is distribution of labeled atoms along the entire carbon chain of the acyl group, which limits application of synthesized 13C- and 14C-labeled fatty acids in breath tests. The highest sensitivity, repeatability and safety of a liver function breath test can be achieved by using fatty acids in which 100% of labeled atoms are at position 1 (the carboxyl group). Biogenic synthesis often results in a mix of fatty acids differing in their composition. For instance, Thraustochytrium-based synthesis produces 10 different fatty acids with a yield range of 0.72 to 21.82%. Besides, 1.9% of the isotope is included in the structure of unidentified fatty acids [23].

This study aimed to develop a method of synthesis of linoleic acids labeled with 13C- and 14C-atoms at position 1 of the acyl group using CO2 as an isotope source for use in diagnostic tests for hepatobiliary disorders.

**Methods**

**Equipment**

Purity of intermediate and final reaction products was controlled by means of thin-layer chromatography using precoated silica gel Kieselgel 60 F254 TLC plates (Merck; Germany) and Sorbfil plates PTLC-AF-V-U. The ethyl alcohol : n-hexane (1 : 1) eluent mixture was used as a mobile phase. The separated analytes were visualized by exposing the plates to iodine vapors. The structure of the final product was confirmed by means of a DPM 7001 liquid scintillation counter (RadiEk Scientific and Technical Center; Russia) equipped with 2 photomultiplier tubes. Microcalorimetric analysis of mixtures was carried out using a Setaram C80 Calvet calorimeter (SETARAM Instrumentation; France). Potentiometric pH measurements of aqueous solutions were taken with a Sartorius PB-11 basic meter (Sartorius; Germany).

**Materials**

The following reagents were used in the experiment:

- 1-bromo-8,11-heptadecadienyl CH(CH2)7CH=CH=CH(CH2)16Br (AppliChem; USA); molar mass 315.332 g/mol, melting point 3.4 °C, boiling point 112 °C [24];
- the source of a stable carbon isotope: anhydrous barium carbonate-13C (JSC Isotope; Russia); isotopic purity 99.32%; molecular weight 198.3539 g/mol; melting point 1,558 °C;
- the source of a radioactive carbon isotope: anhydrous barium carbonate-14C (Mayak Production Association, Rosatom; Russia); isotopic purity 97.8%; molar mass 199.3539 g/mol; melting point 1,566 °C; specific activity 66.92 mCi/g;
- among other reagents were high-purity dry argon and nitrogen (M-Gas; Russia); ethyl acetate, GOST 22300-76 rev.1-3 (Chimmed; Russia); n-hexane, specifications 2631-158-44493179-13 (Lenreactiv; Russia); magnesium turnings, GOST 804-93 (Interchim; Russia); crystalline iodine, reagent grade (Lenreactiv; Russia); diethyl ether, specifications 2600-001-45682126-13 (Chimmed; Russia); sulfuric acid, pure grade, GOST 4204-77 (Chimmed; Russia); hydrochloric acid, pure grade, GOST 3118-77 (Chimmed; Russia); sodium hydroxide 98% (Fluka; Switzerland; catalog number 71695); acetonitrile, specifications 6-09-3534-87 (Chimservice; Russia).
Absolute ether was prepared as described below. Briefly, diethyl ether was washed in the saturated solution of calcium chloride (50 ml of the solution per 1 L of ether) and dried for 48 h over calcium chloride precalcined at +120 °C for 24 h (130 g of calcinated calcium chloride per 1 L of ether). The reagent was filtered through a fluted paper filter into a dry flask; then, sodium metal (1 g per 1 L of the reagent) was added to the flask. The flask was cooled with a holed stopper holding a calcium chloride drying tube. Absolute ether was used to carboxylate the Grignard reagent once hydrogen was no longer released after sodium addition.

RESULTS

Linoleic acid labeled with $^{13}$C and $^{14}$C at position 1 was synthesized in two steps: 1) preparation of the Grignard reagent; 2) carboxylation of the Grignard reagent with $^{13}$C and $^{14}$C dioxides.

Preparation of the Grignard reagent

The apparatus for synthesizing the Grignard reagent was set up as shown in Fig. 1 (adapted from [25]).

A 250 ml three-necked flask was clamped on a stand; a reflux condenser with a calcium chloride drying tube and a pressure-equalizing dropping funnel were fitted into the side necks of the flask. An electric stirrer was introduced into the middle neck through an oil seal. Magnesium turnings (3.0 g) and an iodine crystal were put inside the flask. High purity grade argon was blown through the apparatus for 20 min. Then, 160 ml of absolute ether was added into the flask through the dropping funnel, the stirrer was switched on, and 3.78 g (12 mmol) of the 1-bromo-8,11-heptadecadien solution in diethyl ether (80 ml) was added under weak argon flow. To initiate the reaction, the flask was warmed over a water bath until the ether started to boil. Once the reaction started, the water bath was switched off and stirring continued until the magnesium was completely consumed.

Carboxylation of the Grignard reagent with $^{13}$C and $^{14}$C dioxides

Isotopically labeled acids were obtained through carboxylation of the Grignard reagent using a high vacuum manifold with ports for connecting a reaction flask, a CO$_2$ source, a mercury column manometer, and tubes for nitrogen inlet/outlet to the line. A cone-shaped three-necked reaction flask resistant to freezing was equipped with a magnetic stirrer that allowed carrying out reactions in vacuum at low temperatures (Fig. 2; adapted from [25]).

The source of $^{13}$C and $^{14}$C dioxides was represented by 5.4 mmol of isotopically labeled barium carbonate (a weighted amount of 1.071 g for the $^{13}$C isotope and a weighted amount of 1.076 g for the $^{14}$C isotope) placed in a round-bottom flask. The flask was connected to a pressure-equalizing dropping funnel filled with concentrated sulfuric acid. This part of the apparatus was connected to the vacuum manifold via a desiccant-containing tube.

First, the apparatus was evacuated to 0.1 mmHg using an oil pump and filled with dry nitrogen. Then, the Grignard reagent solution prepared from 6 mmol of 1-bromo-8,11-heptadecadien (half of the total synthesized amount) was taken up into a prewashed dispenser pipette filled with nitrogen and quickly injected into the flask. The free side neck of the flask was closed with a stopper. The flask was cooled in liquid nitrogen, and the apparatus was evacuated to 0.1 mmHg. Then, the solution in the reaction vessel was thawed to −77 °C in a mixture of dry ice and acetone, frozen in liquid nitrogen, and the apparatus was again evacuated to remove nitrogen.

Carboxylation of the Grignard reagent was performed at −20 °C under continuous stirring. To initiate liberation of labeled CO$_2$, concentrated sulfuric acid was slowly added to barium carbonate through the dropping funnel, making sure that the pressure did not exceed 500 mmHg. To finish off liberation of labeled CO$_2$, the reaction flask was carefully heated until barium carbonate was completely dissolved. After the Grignard reagent was depleted, manometer readings indicated that CO$_2$ pressure in the apparatus was no longer decreasing. For both $^{13}$CO$_2$ and $^{14}$CO$_2$, the reaction was completed in 15 minutes.

The reaction flask with the Grignard reagent was cooled in liquid nitrogen to collect the remaining labeled CO$_2$; the stopcock connecting the apparatus to the source of labeled CO$_2$, was closed and the reaction mass was stirred for 15 min at −20 °C until labeled CO$_2$ was fully absorbed. Then, the apparatus was filled with nitrogen and connected to the air inlet tube. The obtained complex was decomposed by diluted hydrochloric acid. The acidified mixture was extracted in ether. The resulting ether extract was treated with 100 mM NaOH, followed by extraction with ether. A 200 ml volume of ether was used to collect the ether-soluble part of the extract.

The resulting ether extract was treated with 100 mM NaOH, followed by extraction with ether. A 200 ml volume of ether was used to collect the ether-soluble part of the extract. The aqueous part was then re-extracted with ether.

Fig. 1. The apparatus for preparing the Grignard reagent in argon atmosphere

Fig. 2. The apparatus for carboxylation of the Grignard reagent. 1 — source of carbon dioxide; 2 — drierite-containing tube; 3 — high-vacuum manifold (tube diameter of 13 mm); 4 — mercury column manometer; 5 — flask; 6 — 3-phase magnetic stirrer (110 V); 7 — cooling bath; 8 — nitrogen inlet; 9 — glass joint, 29/12; 10 — stopcock, bore size 3 mm; 11 — stopcock, bore size 2 mm; 12 — ground-glass joint, 18/8; 13 — ground-glass joint, 14/35; 14 — ground-glass joint, 14/20.
and the obtained alkaline solution was acidified to achieve pH = 7.0. The released acid was filtered. The precipitate was collected, washed in water and recrystallized from acetonitrile at –20 °C (for linoleic acid, t_freez was assumed to be –11 °C).

The total mass of the synthesized 13C-linoleic acid equaled 1,459 mg (5.184 mmol). The final chemical yield of 1-bromo-8,11-heptadecadien was 86.4%; the 13CO2 yield was 96.0%. For the final product, the empirically measured freezing point was –11.0 °C.

The total mass of the synthesized 14C-linoleic acid was 1,480 mg (5.243 mmol). The yield of 1-bromo-8,11-heptadecadien was 87.39%; the 14CO2 yield was 97.1%. The specific activity of 14C-linoleic acid was measured using the scintillation counter and equaled 45.36 ± 0.02 mCi/g. Thus, the total radiation and chemical yield was 96.0%. The final reaction product had a freezing point at –10.7 °C.

The synthesized 13C- and 14C-linoleic acids were analyzed by means of thin layer chromatography using Kieselgel 60 F254 plates; the ethyl acetate : n-hexane (1 : 1) eluent mixture was used as a mobile phase (see Methods); the obtained linoleic acids contained admixtures. 13C-linoleic acid made up 98.2% of the dry product weight after elution.

The 1H NMR spectrum of 14C-labeled linoleic acid was compared to the reference spectrum of 13C/C18H32C/O/c1-2-3-4-5-6-7-8-9-10-11-12-13-14-15-16-17-18(19)20/H6-7, 9, 10H, 2-5, 8, 11-17H, 1H, (H, 19, 20)/b7-6-, 10-9 described in the literature [26]; the synthesized compound matched the structure of the linoleic acid (Fig. 3).

**DISCUSSION**

The proposed method of synthesis of 13C- and 14C-labeled linoleic acids has a few strengths, including shorter reaction time, reduced loss of labeled CO2 and increase in the total chemical and radiation yield. Importantly, the isotopically labeled atoms are not distributed along the entire carbon chain of acyl but instead occur only at position 1.

Shorter reaction time due to the optimization of reagents ratios and measurements of the CO2 pressure in the system taken to check the completion of Grignard reagent carboxylation ensured operational simplicity and cost-effectiveness of the method. The method significantly increases the radiation and chemical yield of the product in comparison with other known techniques [21–23]. The labeled isotope is almost entirely included in the final reaction product, meaning that the amount of radioactive waste is near zero. This makes the proposed method appealing in terms of cost reduction, given that disposal of long-lived radioactive waste is difficult and expensive.

The amount and purity of the synthesized 13C- and 14C-linoleic acids make them suitable for use in preclinical trials of acute, subchronic, chronic and other types of toxicity [27]. Once the safety of the compounds has been confirmed, they can be recommended for clinical trials of hepatobiliary function breath tests.

**CONCLUSIONS**

This paper describes a method of synthesis of 13C- and 14C-linoleic acids with carbon isotopes occurring at position 1. The method is advantageously simple and consists of 2 steps instead of 11 reported in other works. For 13C, the radiochemical yield of the method is very close to quantitative (96%), which almost rules out the issues associated with radioactive waste disposal. The method requires no sophisticated analytical and preparative instrumentation, which may facilitate its adoption to batch production.

![Fig. 3. The 1H-NMR spectrum of 14C-labeled linoleic acid](image-url)
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TRANSABDOMINAL ULTRASOUND AS A SCREENING STAGE FOR THE DIAGNOSIS OF TUBERCULOUS PERITONITIS

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In recent years, the incidence of tuberculous peritonitis increased. Peritoneal tuberculosis is difficult to diagnose, and often the diagnosis is verified with significant delay. In clinical practice, a quick and affordable diagnostic radiology method, ultrasonography (USG), is proposed for patients with suspected tuberculous peritonitis. The study was aimed to describe the sonographic semiotics of tuberculous peritonitis, to create the integrated scale for the individual peritoneal tuberculosis sonographic symptoms significance assessment, and to determine the role of ultrasound imaging in the diagnosis verification. Retrospective study of the invasive and ultrasound investigation results of 37 patients with confirmed tuberculous peritonitis was carried out in 2009–2019. Similar data obtained by investigation of 28 patients with the disorders which often mimic the tuberculous peritonitis (peritoneal carcinomatosis and sarcoidosis, non-specific ascites) were used as a comparison group. Direct and indirect signs of peritoneal lesion in patients with tuberculosis were identified. On the basis of that, an integral scale for the individual sonographic symptoms significance assessment was created. Each sonographic symptom received a 0–3 score. Assessment of those sonographic signs visualization allowed us to evaluate the probability of the disorder’s tuberculous etiology. The following data were obtained: score under 4 corresponded to low probability, score 5–8 corresponded to medium probability, and score over 9 corresponded to high probability of tuberculous peritonitis based on the visualization of all described sonographic symptoms. The proposed integrated scale for the sonographic signs assessment allows the clinician to verify the tuberculous peritonitis diagnosis based on the ultrasound imaging data or to select the further tactics of diagnosis.

Keywords: ultrasonography, peritoneal tuberculosis, peritonitis, carcinomatosis, sarcoidosis

Author contribution: Plotkin DV, Reshetnikov MN, Nikanorov AV, Sinitsyn MV — study concept and design, overall management; Kirillova OV, Shtykhno AO, Loshkareva EO — sample collection; Korotkova ES, Plotkin DV — statistical analysis; Plotkin DV, Reshetnikov MN, Kirillova OV, Nikanorov AV — data analysis; Plotkin DV, Reshetnikov MN — manuscript writing.

Compliance with ethical standards: The study was approved by the Ethics Committee of the Moscow Research and Clinical Center for TB Control (protocol №12 dated December 9, 2019). The informed consent was submitted by all study participants.

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ТРАНСАБДОМИНАЛЬНОЕ УЛЬТРАЗВУКОВОЕ СКАНИРОВАНИЕ КАК СКРИНИНГОВЫЙ ЭТАП ДИАГНОСТИКИ ТУБЕРКУЛЕЗНОГО ПЕРИТОНИТА

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За последние годы отмечен рост числа случаев туберкулезного перитонита. Туберкулезная брюшина — сложный объект для диагностики и нередко верификация диагноза происходит со значительными задержками. В клинической практике предложен непродолжительный и доступный метод лучевой диагностики при подозрении на туберкулезный перитонит — ультразвуковое исследование (УЗИ). Целью работы было описать эксо-симплотику туберкулезного перитонита с созданием интегральной шкалы оценки значимости отдельных ультразвуковых симптомов туберкулеза брюшины и определить роль УЗИ-сканирования в верификации диагноза. Произведен ретроспективный анализ инвазивной и УЗ-диагностики 37 пациентов с подтвержденным туберкулезным перитонитом в период с 2009 по 2019 г. В качестве группы сравнения использовали такие же данные исследований у 28 больных с заболеваниями, часто имитирующими туберкулезный перитонит (канцероматозом и серьезным брюшино, неспецифическим асцитом). Выделены прямые и косвенные признаки поражения брюшины при туберкулезе, на основании этого создана интегральная шкала оценки значимости отдельных эксо-симптомов. С этой целью каждому эксо-симптому присваивали от 0 до 3 баллов. При оценке визуализации описанных ультразвуковых признаков возможно прогнозировать вероятность туберкулезной этиологии заболевания. Получены следующие статистические данные: низкая вероятность наличия ультразвуковых признаков — до 4 баллов, средняя — от 5 до 8 баллов, высокая 9 и более баллов.

Ключевые слова: ультразвуковое исследование, туберкулез, брюшина, перитонит, канцероматоз, саркоидоз

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After the 45-year neglection, tuberculous peritonitis showed up again in European and Russian clinics. That was due to HIV pandemic, people’s migration from endemic regions, emergence of extensively drug-resistant mycobacteria strains and drug-induced immunosuppression. Peritoneal tuberculosis (primary peritonitis) is a chronic inflammatory process with nonspecific clinical manifestations, which often causes significant difficulties and a delay in diagnosis [1–3]. According to the vast majority of scientists, the most accurate method of peritoneal tuberculosis verification is the diagnostic laparoscopic biopsy of the abdominal cavity serous membrane affected areas [4–5]. The growth of Mycobacterium tuberculosis (MTB) from exudates demonstrates the positive result 4–6 weeks after inoculation only in 10% of cases, and PCR analysis of effusion is informative in one-third of cases and also requires invasive intervention [5–6]. In clinical practice, short-term and affordable diagnostic radiology methods, ultrasonography and computed tomography (CT), are the first choice for patients with suspected tuberculous peritonitis [1, 7, 8], however, in most cases, they do not allow one to identify the inflammatory process in the peritoneum accurately.

Ultrasonography is the most harmless, efficient and cost-effective imaging method which can help clinicians to make decisions on the diagnosis and timely treatment of tuberculous peritonitis. Today, transabdominal ultrasound plays an important role in assessment of inflammatory, benign, and malignant diseases of the peritoneum, both in outpatient and inpatient settings. Medical literature describes different sonographic signs allowing one to suspect tuberculous peritonitis, as well as methods allowing one to distinguish between tuberculous peritonitis and peritoneal carcinomatosis or nonspecific ascites [7–11]. The accuracy of these methods depends on the qualification and experience of the doctor, as well as the class of equipment used. Over the past 10 years, most studies report the individual observations of the TB-associated peritoneal changes’ visualization. However, there is no analysis of the prevalence of symptoms and their combinations [9, 11].

The study was aimed to describe the sonographic semiology of tuberculous peritonitis, to create the integrated scale for assessment of the significance of individual sonographic symptoms of peritoneal tuberculosis, and to determine the role of ultrasonography in the diagnosis verification.

**METHODS**

In 2009–2019, in the TB surgery department of the Hospital № 2 of the Moscow Research and Clinical Center for TB Control, Moscow, a retrospective study of the ultrasonography results of 37 patients with tuberculous peritonitis was carried out. The main clinical manifestation was a large volume of effusion in the abdominal cavity (exudative, adhesive, caseous and mixed forms of tuberculous peritonitis). Inclusion criteria: peritoneal tuberculosis diagnosed in all 37 patients via histological (100%) and bacterioscopic (81.1%) examination of peritoneal biopsies obtained using laparoscopic invasive methods. Among hospitalized patients, males predominated (22 men, 59.5%; 15 women, 40.5%) aged 20–65 (median age 37.2). Twenty three patients (62.7%) were HIV-positive, 34 patients (91.6%) had pulmonary tuberculosis, mostly the infiltrative and disseminated forms. Exclusion criteria: no histological confirmation of peritoneal tuberculosis.

For the comparison group, the patients were selected with the diseases verified using laparoscopy, histological and laboratory analysis data, which most often mimic tuberculous peritonitis, both in clinical picture and ultrasonography. The control group included 28 patients with non-specific ascites of various origin (21 patients; 75.0%), peritoneal sarcoidosis (1 patient; 3.5%) and peritoneal carcinomatosis (6 patients; 21.5%). The patients were aged 29–54 (median age 36.1). Pulmonary tuberculosis was diagnosed in all patients. 10 patients (35.7%) of the control group were HIV-positive. Exclusion criteria for the control group: tuberculosis granulomas and acid resistant mycobacteria in the peritoneal biopsy; mycobacterial DNA positive effusion PCR-test.

The patients were examined using polypositional radiography, thoracic computed tomography, abdominal ultrasonography, diagnostic video-assisted laparoscopy, laboratory and morphological techniques. Diagnostic studies were expanded using histological, cytological, and molecular genetic analysis of intraoperative material (biopsies and exudate). Microbiological studies included Ziehl-Neelsen (ZN) microscopy for acid-fast bacilli examination, inoculation of solid and liquid media using the automated systems.

Abdominal ultrasonography was performed using the Toshiba Apilo 500 expert-class ultrasound system (Toshiba; Japan) and the LOGIC ER7 portable imaging system (General

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**Fig. 1. Tuberculous peritonitis. A. Sonogram: 1 — paretic bowel loops, 2 — exudate. B. Sonogram: 1 — intra-peritoneal caseous abscess, 2 — tubercle, 3 — parietal peritoneum striated pattern. C. Laparoscopy. D. Sonogram: heterogeneous fibrinous effusion and paretic loops of the small intestine.**
Fig. 2. Tuberculous peritonitis. A. Laparoscopy: fibrin overlays forming septa. B. Sonogram: 1 — intestinal loops, 2 — exudate, 3 — fibrin strands forming septa. C. Sonogram: tubercle (7 mm). D. Laparoscopy: tubercles on the parietal peritoneum

Electric; Republic of Korea) working in the grayscale, real-time mode. Abdominal cavity examination was carried out using the 2.5–5.0 MHz convex probe for assessment of presence and prevalence of free fluid, as well as the spleen and liver state. High-frequency linear probe (10–15 MHz) was used for evaluation of the intestinal loops, mesentery, greater and lesser omentum, lymphatic apparatus state.

Elective surgery (laparoscopic-assisted biopsy or laparotomy) was performed in patients with ascites of unknown origin or in order to clarify the nature of the pathological process detected by ultrasound imaging and computed tomography. Emergency surgery was performed in patients with clinical picture not allowing one to exclude peritonitis.

The criterion for the diagnosis verification was the detection of tuberculous granulomas in the peritoneum biopsy specimens, acid-fast bacilli during bacterioscopic examination and a positive result of PCR testing of effusion, as well as a combination of these signs.

Table 1. Direct and indirect signs of tuberculous peritonitis (the significance level is 0.95)

<table>
<thead>
<tr>
<th>Sonographic sign</th>
<th>Tuberculous peritonitis ( n = 37 )</th>
<th>Ascites and carcinomatosis ( n = 28 )</th>
<th>Sign character</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free fluid in all abdominal cavity parts</td>
<td>51.4% ± 16.1</td>
<td>82.1% ± 14.1</td>
<td>Direct signs</td>
</tr>
<tr>
<td>Free fluid in pelvis and between intestinal loops</td>
<td>21.6% ± 13.2</td>
<td>3.6% ± 6.8</td>
<td></td>
</tr>
<tr>
<td>Encysted fluid</td>
<td>27.0% ± 14.3</td>
<td>14.3% ± 12.9</td>
<td></td>
</tr>
<tr>
<td>Heterogeneous effusion</td>
<td>75.7% ± 13.8</td>
<td>3.6% ± 6.8</td>
<td></td>
</tr>
<tr>
<td>Striated pattern and heterogeneity of the parietal peritoneum and omentum leaflets</td>
<td>37.8% ± 15.6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Intra-peritoneal caseous abscesses</td>
<td>2.7% ± 1.6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Peritoneal tubercles</td>
<td>24.3% ± 13.8</td>
<td>10.7% ± 11.5</td>
<td></td>
</tr>
<tr>
<td>Bowel wall thickening</td>
<td>18.9% ± 12.6</td>
<td>21.4% ± 15.2</td>
<td></td>
</tr>
<tr>
<td>Ileus</td>
<td>16.2% ± 11.9</td>
<td>10.7% ± 11.5</td>
<td></td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>13.5% ± 11.0</td>
<td>71.4% ± 16.7</td>
<td></td>
</tr>
<tr>
<td>Enlarged and heterogeneous mesenteric lymph nodes</td>
<td>51.4% ± 16.1</td>
<td>14.3% ± 12.9</td>
<td></td>
</tr>
<tr>
<td>Enlarged liver and vascular pattern depletion</td>
<td>27.0% ± 14.3</td>
<td>82.1% ± 14.2</td>
<td></td>
</tr>
<tr>
<td>Expansion of the portal vein and diffuse changes of the liver</td>
<td>8.1% ± 8.8</td>
<td>85.7% ± 12.9</td>
<td></td>
</tr>
</tbody>
</table>

To evaluate the sonographic symptoms of tuberculous peritonitis, we identified the following direct signs that were related to the inflammatory process in the serous leaflets and the associated exudation: presence of free fluid and its location in the abdominal cavity, exudate homogeneity, fibrin septa or incomplete septa in the abdominal cavity, striated pattern and heterogeneity of peritoneum and omentum, tubercles in serous leaflets. Indirect signs of the peritoneum tuberculous lesions include the intestinal loops changes, spleen enlargement and heterogeneity, and the visualization of the enlarged mesenteric lymph nodes groups. Indirect signs do not reflect the inflammatory process in the peritoneal membranes, but may be the result of long-term ascites (thickening of the bowel walls),
as well as the effect or specific lymphadenitis and splenitis in patients with abdominal tuberculosis.

When performing the abdominal ultrasound, various volumes of free fluid were detected in all patients with tuberculous peritonitis. The effusion in all parts of the abdominal cavity was visualized in 19 patients (51.4%). In eight patients (21.6%) free fluid was localized mainly in the pelvis and between the loops of the small intestine, and in 10 patients (27.0%) the encysted free fluid was revealed together with the formation of incomplete or complete septa from fibrin layers. The effusion heterogeneity due to freely floating layers of fibrin and small fibrin sequestra with a diameter of up to 6–8 mm was noted in the vast majority of patients (75.7%). Striated pattern and heterogeneity of certain areas of parietal peritoneum leaflets (primarily in the ileocecal region) were observed in 14 patients (37.8%), moreover, in one patient, the caseous foci were visualized inside the anterior abdominal wall (Fig. 1). Heterogeneity and striated pattern of the greater omentum were found in 9 patients (24.3%). Tubercles characteristic of tuberculous peritonitis were visualized in 9 patients (24.3%). The tubercle size varied between 5–9 mm, tubercles were described as hyperechoic avascular foci with uneven contour rising above the parietal peritoneum (Fig. 1, 2).

The other features allowing one to suspect tuberculosis as the cause of peritoneal changes included the sonographic signs of the visceral peritoneum, spleen and mesenteric lymphatic apparatus involvement in the pathological process. Bowel wall changes were registered in 7 patients (18.9%). Most commonly, there was a local thickening of the intestinal wall exceeding 3 mm with its length within 45–60 mm. In 6 patients (16.2%), the expansion of the intestinal lumen of more than 33–35 mm and the weakening or complete absence of peristalsis were revealed, which was considered a paralysis. Enlarged mesenteric lymph nodes (more than 10–16 mm in diameter) with a heterogeneous structure were detected in 19 patients (51.4%). Splenomegaly with spleen heterogeneity was observed in 5 patients (13.5%). Comparison of the control group patients’ ultrasonography results is presented in Table 1.

To create an integrated scale for assessment of the tuberculous peritonitis probability using ultrasonography, all variants of signs (sonographic symptoms) combinations were considered. For that, each sonographic symptom received a 0–3

![Fig. 3. Granulomatous peritonitis and carcinomatosis. A. Sonogram: peritoneal carcinomatosis associated with sigmoid colon cancer. 1 — disseminated tumors (17 and 8 mm), 2 — ascites. B. Sonogram: encysted ascites associated with ovarian cancer. C. Laparoscopy: parietal peritoneum carcinomatosis. D. Laparoscopy: peritoneal sarcoidosis](image-url)
score. The 1st three signs (free fluid distribution) determined the 1st complete event space, probability (effusion in the abdominal cavity). Tubercles of various sizes could be considered the 2nd complete event space (based on the tubercle size). The other signs (heterogenous effusion with fibrin fragments, striated pattern and heterogeneity of parietal peritoneum and omentum leaflets, intra-peritoneal caseous abscesses) were independent. Next, calculations were made for all features combinations using Python 3.6 language and Bayes’ theorem, allowing one to determine the probability based on the data provided. Assumptions about the approximation of binomial distributions to normal and the conclusion on the inclusion of patients in the groups (low, medium, high probability) were made using the stats and numpy libraries (Table 2.).

DISCUSSION

Thus, it can be noted that the visual signs of tuberculous peritonitis, benign peritoneal granulomatosis (sarcoidosis), non-specific ascites, and peritoneal carcinomatosis are very similar and in most cases confront the researcher with a choice of diagnosis.

Our experience of ultrasound scanning in patients with tuberculous peritonitis, and the literature data [7–10] demonstrate that the main sonographic symptom is the presence of heterogeneous free of encysted fluid in the abdominal cavity (75–80%). A characteristic feature that allows one to distinguish tuberculous peritonitis from non-specific ascites is the more frequent (about 75%) visualization of fibrin sequestrations freely floating in the exudate, which in sometimes form complete or incomplete septa causing the encysted effusion (Fig. 2). Nevertheless, similar sonographic features also occur in 50% of patients with peritoneal carcinomatosis [13]. Conversely, in patients with carcinomatosis, there is a high probability of disseminated tumors detection with a diameter of 5–18 mm (Fig. 3). Accoding to our data, similar hypechoic foci on the peritoneum can also be detected in patients with benign granulomatosis, for example, with sarcoidosis (Fig. 3), which also does not give any reason to consider this feature specific. Tubercles in the peritoneum usually have a smaller diameter, and the maximum tubercle size is up to 9 mm (median value 4–6 mm) [11, 13–16]. A more reliable sonographic symptom of tuberculous peritonitis is the heterogeneity (striated pattern) of the parietal peritoneum and omentum areas, however, this symptom can be determined in just over one third of all cases. The most common finding is the extended hypechoic thickening of the serous parietal leaflet (4–8 mm), which is associated with chronic inflammation. According to a number of authors, this variant of the thickened peritoneum is probably associated with tuberculosis [8, 17–20] and is less common in patients with peritoneal carcinomatosis, where the thickening of the serous membrane is most often small and nodular [20]. Various authors are equally likely to describe the involvement of the greater omentum and its changes as a characteristic sign of tuberculous omentitis or carcinomatosis, therefore we consider this symptom to be specific for both disorders, given that it is rarely possible to visualize the omental changes (less than 25% of observations) [21].

Indirect signs of peritoneal damage, such as lymphadenopathy with adenomegaly, occur in half of patients with tuberculous peritonitis, which reflects the pathogenetic character of lymphogenous dissemination in the peritoneal leaflets. The mesenteric lymphatic apparatus changes are most often destructive, and the lymph nodes are visualized as grouped anechoic rounded foci with a diameter exceeding 10 mm. According to the literature, the prevalence of joint

lesions of intra-abdominal lymph nodes and peritoneum is 10–54% [22–24].

Thus, it is clear that tuberculous peritonitis does not have any reference or unique sonographic symptoms, which makes it extremely difficult to diagnose it using ultrasound imaging. At the same time, various combinations of some direct and indirect signs increase the probability of the ultrasound image correct interpretation in patients with peritoneal tuberculosis.

Based on the calculations and the probability distribution, the following data has been obtained: score under 4 corresponds to low probability, score 5–8 corresponds to medium probability, and the score over 9 corresponds to high probability of tuberculous peritonitis based on the visualization of all described sonographic symptoms. Based on the sum score obtained after the ultrasound scanning, a diagnostic algorithm can be proposed that clearly indicates further diagnostic and treatment strategies for each combination of sonographic symptoms, and for patients with HIV and/or pulmonary tuberculosis (Fig. 4).

CONCLUSION

The obtained integrated scale for the sonographic signs assessment is quite simple and allows the clinician to select the further tactics of diagnosis based on the ultrasound imaging results and to start the treatment in a timely manner. The proposed scheme may also complete the diagnostic search algorithm for any form of ascites or granulomatous peritonitis.

Due to the clinical symptoms non-specificity and the subacute course, the diagnosis of peritoneal tuberculosis remains a difficult task. Transabdominal ultrasound, which is affordable and commonly used in medical practice, can become a first-line screening test for verification of tuberculous ascites, and peritoneal tuberculosis is probably associated with tuberculosis [9, 17–20] and is less common in patients with peritoneal carcinomatosis, where the thickening of the serous membrane is most often small and nodular [20]. Various authors are equally likely to describe the involvement of the greater omentum and its changes as a characteristic sign of tuberculous omentitis or carcinomatosis, therefore we consider this symptom to be specific for both disorders, given that it is rarely possible to visualize the omental changes (less than 25% of observations) [21].

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Литература


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OPTIMIZATION OF A SINGLE-EMBRYO TRANSFER IN PATIENTS WITH GOOD OVARIAN RESERVE

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Due to refinements of assisted reproductive technology, the number of multiple pregnancies has increased substantially. Time-lapse microscopy (TLM) is a tool for selecting quality embryos for transfer. This study aimed to assess the outcomes of single-embryo transfer of autologous oocytes performed on day 5 of embryo incubation in a TLM-equipped system in patients with good ovarian reserve. The study was carried out in 208 infertile women with good ovarian reserve (over 8 oocytes retrieved). Single-embryo transfer following incubation in a TLM-equipped incubator was performed in 95 patients, who formed the main group; the control group consisted of 113 patients undergoing single-embryo transfer following a traditional culture and embryo selection procedure. We assessed the quality of transferred embryos, the rates of clinical pregnancy and pregnancy loss. Two subgroups were identified in each group of the participants: the sSET subgroup (nonelective single-embryo transfer), which included 45 patients from the main group and 67 controls, and the SeSET subgroup (elective single-embryo transfer), which consisted of 50 main group patients and 46 controls. The groups did not differ in terms of age, infertility factors and infertility duration. The quality of transferred embryos was excellent or good in all main group patients (100%); in the control group, the quality of transferred embryos was excellent or good in 93.8% of cases (p = 0.037). Clinical pregnancies were achieved in 64.2% of women in the main group and in 60.2% of controls (p = 0.65). Delivery rates were 54% and 51.1% in the sSET and SeSET subgroups of the main group, respectively (p = 0.940). For the control group, delivery rates were 54.4% and 34.3% in the SeSET and sSET subgroups, respectively (p = 0.066, Fisher exact test). Elective single-embryo transfer (SeSET) and the use of TLM increased the chance of pregnancy 2.17-fold (p = 0.01).

Keywords: assisted reproductive technology, single-embryo transfer, elective blastocyst transfer, time-lapse microscopy

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Author contribution: all authors equally contributed to the study and manuscript preparation.

Compliance with ethical standards: the study was approved by the Ethics Committee of Samara State Medical University (Protocol 194 dated September 12, 2018). Informed consent was obtained from all study participants.

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ОПТИМИЗАЦИЯ ПЕРЕНОСА ОДНОГО ЭМБРИОНА У ПАЦИЕНТОК С ХОРОШИМ ОВАРИАЛЬНЫМ РЕЗЕРВОМ

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Совершенствование вспомогательных репродуктивных технологий привело к росту числа случаев многоплодной беременности. Одним из инструментов выбора качественного эмбриона на перенос — использование time-lapse микроскопии (TLM). Целью работы было оценить исходы переноса одного эмбриона на пятье сутки культивирования у пациенток с хорошим овариальным резервом в программе ЭКО с использованием TLM. Исследовали 208 женщин с бесплодием, с хорошим овариальным резервом (при пункции фолликулов получено более восьми ооцитов): у 95 пациенток провели перенос одного эмбриона с использованием системы TLM (группа исследования); у 113 пациенток — с использованием традиционного культивирования и выбора эмбриона для переноса (группа контроля). Проведена оценка качества перенесенных эмбрионов, частоты наступления клинической беременности, частоты рождения и случаев потери беременности. В каждой группе выделены две подгруппы: с неэлективным переносом одного эмбриона (подгруппа sSET: 45 пациенток в группе исследования, 67 — в контрольной) и с элективным (подгруппа SeSET: 50 пациентов в группе исследования, 46 — в контрольной). Группы не различались по среднему возрасту, фактору бесплодия, длительности бесплодия. В группе исследования в 100% случаев перенесены эмбрионы хорошего и отличного качества, в группе контроля — в 93,8% (p = 0,037). Частота наступления клинической беременности составила 64,2% в основной группе и 60,2% — в контрольной (p = 0,65). В группе исследования частота родов составила 54% в подгруппе SeSET и 51,1% — в подгруппе sSET (p = 0,940). В группе контроля частота родов составила 54,4% а в подгруппе sSET — 34,3% (p = 0,069 по методу Фишера). Проведение элективного переноса эмбриона (SeSET) или использование TLM повышало вероятность родов в 2,17 раза (p = 0,01).

Ключевые слова: вспомогательные репродуктивные технологии, перенос одного эмбриона, элективный перенос бластоцисты, time-lapse микроскопия

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Соблюдение этических стандартов: исследование одобрено этическим комитетом СамГМУ (протокол № 194 от 12 сентября 2018 г.). Всем пациентам дана добровольная информированная согласия на проведение вспомогательных репродуктивных технологий.

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Due to advancements in assisted reproductive technology (ART), implantation rates have significantly improved in the past 15–20 years, resulting in an increased incidence of multiple pregnancies. A multiple pregnancy is a recognized high-risk factor for obstetric and neonatal complications [1–3]. Therefore, transfer of a single embryo, as opposed to multiple embryos, is a top-priority task in ART-based infertility treatment [4, 5].

Selecting an embryo with the best developmental potential is crucial to ART success. Selection allows reducing time to pregnancy and simplifies embryo grading for cryopreservation, which, in turn, ensures that high-quality embryos are transferred first [6, 7].

Since the advent of in vitro fertilization (IVF), morphological evaluation has been the primary method proposed by embryologists to assess the development of human embryos and identify those with the highest implantation potential. Later, grading systems were proposed to estimate the viability of embryos, but their practical application was impeded by the rapid pace of embryo development in the preimplantation phase. In other words, it is possible that an embryo evaluated at 8:00 am will look very different in only a few hours [8]. So, it is very difficult to offer a correct interpretation of the morphological data without analyzing the dynamics of embryo development at a number of different time points.

Introduction of time-lapse microscopy (TLM) into IVF laboratories heralded a new age in embryology. TLM is a modern technique of embryo selection for subsequent implantation. It is used for continuous evaluation of embryo morphology in a series of images taken every few minutes [9–11].

Published reports on TLM results are conflicting. A retrospective study has demonstrated that incubation of human embryos in the EmbryoScope system can improve live birth rates whereas traditional culture techniques can negatively affect the development of embryos [12]. Other retrospective and prospective studies point to the advantages of this promising technology [13–16], as well as to the absence of differences in outcomes in comparison with the conventional technique for morphological evaluation [17, 18].

The aim of our study was to assess the outcomes of single-embryo transfer following embryo incubation in a TLM-equipped incubator in patients with good ovarian reserve undergoing IVF.

METHODS

The study was conducted in 208 infertile women receiving a single-embryo transfer as part of their IVF treatment at the IDK Medical Company (Samara) in 2013–2015.

We analyzed 208 patients’ clinical and embryo protocols using SPSS21 Statistics (License 20130626-3; IBM Company; USA) and Microsoft Excel (Microsoft; USA).

The following inclusion criteria were applied: participation in the IVF program, fresh autologous IVF cycles with 8 or more oocytes retrieved per cycle, embryo transfer on day 5 of incubation, and endometrial thickness of at least 8 mm on the day of transfer.

Exclusion criteria: participation in the ICSI program, donor oocyte cycles (with ≤ 8 oocytes), frozen-thawed embryo transfer, transfer on day 3 of incubation, multiple (2) embryo transfer, endometrial thickness of < 8 mm on the day of transfer.

There was no age limit applied. The lowest age was 20 years, whereas the highest, 42 years.

The patients were divided into two groups. The main group comprised 96 patients with good ovarian reserve undergoing a single-embryo transfer following embryo incubation in a TLM-equipped system.

The control group consisted of 113 patients with good ovarian reserve undergoing a single-embryo transfer following conventional embryo incubation and selection. The average age of the participants, infertility factors, the duration of infertility, and the number of the current IVF program did not differ between the groups. The average age was 31.40 ± 0.38 and 30.65 ± 0.37 years in the main and control groups, respectively (p > 0.05).

In the main group, embryo cultures were monitored using a Primo Vision time-lapse system (Vitrolife; Sweden).

In both groups, embryo quality was assessed using the alphanumeric blastocyst grading system proposed by Gardner and Schoolcraft in 1999 [19]. Grades AA, AB, and BA represented excellent quality blastocysts; grade BB indicated good quality; grades AC, CA, CB, BC, and CB were considered to be satisfactory quality blastocysts.

Two subgroups were identified in each group based on the type of embryo transfer: a subgroup of nonelective single-embryo transfer on the 5th day of culture (the 5SET subgroup, which included 45 patients from the main group and 67 women from the control group) and a subgroup with elective single-embryo transfer on the 5th day of culture (the 5eSET subgroup consisting of 50 patients from the main subgroup and 46 controls). A transfer was classified as elective if there were 2 or more excellent quality embryos to choose from.

In the main group, embryos were selected for transfer based on their morphokinetic parameters. The following developmental events were assessed: time of the first cleavage division; an interval between the first and second divisions; time of the third cleavage; and time of blastocyte formation. If these parameters fell within the reference range of the Primo Vision system and the embryo was of excellent or good quality, it was selected for transfer (a reference-positive embryo). The reference-negative subgroup comprised 52 patients. If one or more parameters of embryo development did not fall within the system’s reference range, the standard morphological assessment technique for embryo selection was applied (a reference-negative embryo).

The reference-negative subgroup included 43 patients.

In both groups, the embryos were cultured in a Continuous Single Culture medium (Irvine Scientific; USA). Embryo quality was assessed on day 5 of incubation, 116–118 h after fertilization.

RESULTS

We assessed the quality of transferred embryos and calculated the rates of successful pregnancies, delivery and pregnancy loss. Patients of late reproductive age (≥ 35 years) made up 21.05% of women in the main group and 23.89% of women in the control group (p > 0.05). Because the study included only females with good ovarian reserve and a single-embryo transfer, our sample was dominated by patients of early reproductive age.

The average number of retrieved oocytes was 11.87 ± 0.32 and 12.49 ± 0.40 in the main and control groups, respectively (p > 0.05).

It is known that the quality of transferred embryos significantly affects the chance of pregnancy in patients undergoing IVF treatment. It is reported that transfer of excellent or good quality embryos results in much higher pregnancy rates than observed for satisfactory quality embryos [20]. In the main group, transferred embryos were of either good (16) or excellent (79) quality in 100% of cases. In the control group, good (18) or excellent (88) quality embryos were transferred in 93.8% of cases (p = 0.037) (Fig. 1). Satisfactory quality embryos were transferred to 7 patients in the control group (6.2%).
The analysis of embryo quality did not reveal any significant differences between the reference-positive and reference-negative subgroups. In the reference-positive subgroup, 87.5% of embryo transfers were performed with excellent quality embryos, whereas in the reference-negative subgroup, the proportion of such cycles was 78.95% (p = 0.44).

Thus, the clinical pregnancy rate did not differ between the groups and was 64.2% in the main group and 60.2% in the control group (p = 0.65) (Table 1).

Live births accounted for 52.6% and 42.5% of all embryo transfer outcomes in the main and control groups, respectively (p > 0.05). Early pregnancy loss (biochemical pregnancy and pregnancy loss before gestational week 12) was observed in 11.6% of cases in the main group and in 17.7% of cases in the control group, but this difference was statistically insignificant.

No statistically significant differences were noted between the reference-positive and reference-negative subgroups in terms of clinical pregnancy rates (66.7% vs 60.5%, respectively) and delivery rates (50% vs 52.6%, respectively).

According to the literature, elective embryo transfer increases the probability of a positive outcome [21]; therefore, we decided to compare the delivery rate among patients who had undergone different types of transfer.

In the SeSET subgroup (elective single-embryo transfer) of the main group, the delivery rate reached 54%; in the SET subgroup (nonelective single-embryo transfer), it was 51.1% (p = 0.940) (Fig. 2). In the control group, this parameter was significantly affected by the type of transfer; the delivery rates for the SeSET and SET subgroups were 64.3% and 34.3%, respectively (p = 0.052, Fisher exact test). The difference in the delivery rate was 20.1% (95%CI 1.5–37%), with OR = 2.28 (95%CI 1.06–4.91). Thus, the delivery rate was high in the TLM group, regardless of the type of transfer (54.0% and 51.1%), and did not differ significantly between the subgroups.

Considering this finding, we analyzed a possible correlation between the positive outcome of an IVF cycle (live birth) and the following factors: the absence/presence of TLM and the type of embryo transfer (elective or nonelective; Table 2).

The delivery rate was as high as 53.2% in the group with the combination of two factors (SeSET in both groups and SET in the main group), whereas in the control group, it was lower (34.3%) (p = 0.01; OR = 2.17 (1.19–3.97)). Thus, it could be hypothesized that there is a positive trend showing an increase in live births in patients undergoing IVF treatment aided by TLM regardless of the embryo transfer type.

DISCUSSION

The TLM technology minimizes exposure of the incubated embryo to environmental factors, which might be a contributor to a higher implantation potential. Continuous monitoring within short time intervals provides more information about the kinetics and morphology of embryos in comparison with traditional...
marginal significance (p = 0.052). No statistically significant differences were observed in the number of good quality embryos on day 3 of incubation, as well as in the rates of clinical pregnancy and implantation [23]. In our study, patients did not differ in terms of age, infertility factors and infertility duration; therefore, it could be hypothesized that the difference in the proportion of good and excellent quality embryos can be attributed to the absence of impact of environmental factors (ambient temperature, light, pH conditions) in the TLM group.

The efficacy of TLM might be determined by 2 factors: stable incubation conditions (there is no need to remove an embryo from an incubator for morphological evaluation) and the possibility of selecting an embryo for transfer using specialized software [24].

According to a recent Cochrane review that analyzed the data on 2,995 couples, there is no convincing evidence about the advantage of TLM over the conventional culture technique: no significant differences were observed in terms of clinical pregnancy rates (OR 0.95; 95%CI 0.78–1.16) and live birth rates (OR 1.12; 95%CI 0.92–1.36) [25].

By contrast, in a meta-analysis of data of 1,637 patients, TLM was shown to have an advantage over traditional incubation and morphological evaluation procedures [26]. This study reports high rates of clinical pregnancies (51.0% vs 39.9%; OR 1.54, 95%CI 1.21–1.97) and live births (44.2% vs 31.3%; OR 1.67, 95%CI 1.13–2.46) and lower rates of pregnancy loss (15.3% vs 21.3%; OR 0.66, 95%CI 0.47–0.94).

In our study, pregnancy rates were high in both groups (64.2% in the main group and 60.2% in the control group), which may suggest the absence of TLM negative effect on the incubated embryos. The use of time-lapse microscopy resulted in a reduction in the number of early pregnancy losses.

The absence of differences between the groups in terms of pregnancy rates, delivery rates and early pregnancy loss in our study might be associated with a small sample size (95 patients in the TLM group).

In another retrospective cohort study, the TLM group demonstrated an increase in clinical pregnancy rates (+15.7% per embryo transfer) [27]. However, unlike ours, that study was heterogeneous in terms of patient sample (IVF cycles with donor oocytes were also included), number of transferred embryos (1–3) and time of transfer (in the majority of cases in the TLM group transfer was performed on day 3 of incubation, which decreased the overall pregnancy rate). In the TLM group, clinical pregnancies achieved after performing transfer of retrieved oocytes on the 5th day of culture were observed in 50% of cases, whereas for our patients, the pregnancy rate (embryo transfer on day 5 of incubation) was as high as 64.2%. One of the strengths of our study is a prognostic mathematical model developed by the authors of this work. The model predicted a 15.7% increase in pregnancy rates per transfer achieved through the use of TLM. Ever better outcomes can be achieved by increasing the number of IVF cycles with TLM to ≥ 200.

Like many technological advances, TLM may not ensure immediate results in every laboratory, and some standardization might be required. Indeed, TLM does not always demonstrate an advantage in terms of embryo selection [22]. However, its growing value for continuous incubation and embryo biopsy scheduling cannot be overestimated [28, 29]. At present, there are attempts to integrate artificial intelligence into TLM in order to identify the right combination of parameters predicting the potential of the embryo for implantation and live birth [24].

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**Table 2.** Delivery rates in the absence/presence of TLM for different types of embryo transfer

<table>
<thead>
<tr>
<th>Delivery</th>
<th>Transfer type</th>
<th>χ²</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5eSET in both groups + 5SET in the main group</td>
<td>5SET in the control group</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>Abs.</td>
<td>%</td>
<td>Abs.</td>
</tr>
<tr>
<td>No</td>
<td>66</td>
<td>46.8%</td>
<td>44</td>
</tr>
<tr>
<td>Yes</td>
<td>75</td>
<td>53.2%</td>
<td>23</td>
</tr>
</tbody>
</table>

Fig. 2. Delivery rate depending on the type of the embryo transfer
CONCLUSIONS

Our study did not reveal any differences in the rates of clinical pregnancies, delivery and early pregnancy loss between the TLM group and patients with traditional embryo incubation. This might be explained by a small number of patients in the TLM group. With TLM incubation, delivery rates were high regardless of the type of embryo transfer (selective or nonselective) and there were no differences in terms of pregnancy rates and early pregnancy losses. With traditional embryo incubation and selection, the transfer type significantly affected the delivery rate: in the elective transfer subgroup, the delivery rate was higher than in the nonelective transfer subgroup (p = 0.052; Fisher exact test). Performing elective embryo transfer on day 5 of incubation (SeSET) and the use of TLM regardless of transfer type were favorable factors and increased the chance for live birth (p = 0.01).

Our findings hold promise for exploring advantages of TLM in patients of different age groups with reduced ovarian reserve. Further accumulation of data is required to assess cumulative pregnancy rates following IVF with the use of TLM and to monitor the long-term results of this technology. There is no doubt that complex systems will soon be created for noninvasive evaluation of embryo quality (morphology, kinetics and metabolism) allowing automatization of embryo selection for transfer. They will reduce the probability of negative impact of environmental factors and thereby increase the rate of live births following embryo transfer.

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Borderline ovarian tumors (BOTs) are common in women in their reproductive years. In more than one-third of patients tumors are detected at the age of 15–29, the average age at initial diagnosis is 40. The study was aimed to improve methods for BOTs diagnosis in pregnancy and to determine the possibilities of organ preservation treatment. A group of 300 pregnant women with various tumor-like formations and ovarian tumors was examined. Of them, 25 patients had borderline epithelial tumors (22 patients had serous and 3 patients had mucinous tumors). Ultrasound examination together with blood serum CA-125, sFas, VEGF and IL6 level assessment were performed prior to surgery. The results obtained were compared with the results of morphological studies. Organ preservation and radical surgical treatment were carried out, and chemotherapy, if necessary. Perinatal outcomes were studied when performing the cross-comparison. It was discovered, that ultrasonography and logistic surgical treatment regression analysis made it possible to distinguish between benign ovarian tumors, BOTs and malignant ovarian tumors. The levels of VEGF above the 500 pg/ml, IL6 above the 8.1 pg/ml and CA-125 above the 300 U/ml indicated the high probability of malignant ovarian tumors in pregnant women. Only the morphological study of ovarian tissue, obtained regardless of surgical methods, ensured understanding of the ovarian tumor’s true nature during pregnancy. At the same time, in three pregnant women with ovarian tumors, the morphological examination revealed some tissue areas common both for BOTs and malignant ovarian tumors. Thus, the predominance of the tumor early stages, relatively mild course and, favorable prognosis in patients with BOTs make it possible to use gentle surgical treatment making it possible to preserve menstrual function and fertility.

Keywords: ultrasound, morphological examination, ovarian tumors in pregnant women, CD31

Author contribution: all authors contributed to the research and manuscript preparation equally, read the approved the final version of the article before publishing.

Compliance with ethical standards: the study was approved by the Ethics Committee of Pirogov Russian National Research Medical University (protocol Nr. 176 dates June 25, 2018). The informed consent was submitted by all study participants.

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BORDERLINE OVARIAN TUMORS IN PREGNANCY
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Borderline ovarian tumors (BOTs) are common in women in their reproductive years. In more than one-third of patients tumors are detected at the age of 15–29, the average age at initial diagnosis is 40. The study was aimed to improve methods for BOTs diagnosis in pregnancy and to determine the possibilities of organ preservation treatment. A group of 300 pregnant women with various tumor-like formations and ovarian tumors was examined. Of them, 25 patients had borderline epithelial tumors (22 patients had serous and 3 patients had mucinous tumors). Ultrasound examination together with blood serum CA-125, sFas, VEGF and IL6 level assessment were performed prior to surgery. The results obtained were compared with the results of morphological studies. Organ preservation and radical surgical treatment were carried out, and chemotherapy, if necessary. Perinatal outcomes were studied when performing the cross-comparison. It was discovered, that ultrasonography and logistic surgical treatment regression analysis made it possible to distinguish between benign ovarian tumors, BOTs and malignant ovarian tumors. The levels of VEGF above the 500 pg/ml, IL6 above the 8.1 pg/ml and CA-125 above the 300 U/ml indicated the high probability of malignant ovarian tumors in pregnant women. Only the morphological study of ovarian tissue, obtained regardless of surgical methods, ensured understanding of the ovarian tumor’s true nature during pregnancy. At the same time, in three pregnant women with ovarian tumors, the morphological examination revealed some tissue areas common both for BOTs and malignant ovarian tumors. Thus, the predominance of the tumor early stages, relatively mild course and, favorable prognosis in patients with BOTs make it possible to use gentle surgical treatment making it possible to preserve menstrual function and fertility.

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ПОГРАНИЧНЫЕ ОПУХОЛИ ЯИЧНИКОВ У БЕРЕМЕННЫХ
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Пограничные опухоли яичников характерны для женщин репродуктивного периода, более чем у трети больных опухоли выявляют в возрасте 15–29 лет, средний возраст при первичной постановке диагноза составляет 40 лет. Целью исследования было усовершенствовать методы диагностики пограничных опухолей яичников на фоне беременности и определить возможности выполнения органосохраняющего лечения. Обследовано 300 беременных с различными опухолеподобными образованиями (ООЯ) и опухолями яичников (ОЯ), из которых 25 имели пограничные эпителиальные опухоли: 22 — серозные, три — мукозные. До операции проводили УЗИ, определяли концентрацию в сыворотке крови СА-125, sFas, VEGF и IL6. Полученные результаты сопоставляли с морфологическими исследованиями. Проводили органосохраняющее и радикальное хирургическое лечение, при необходимости — химиотерапию. При перерыве между операциями проводили внутриабдоминальные исследования. Уровни VEGF выше 500 пг/мл, IL6 выше 8,1 пг/мл и CA-125 выше 300 ЕД/мл свидетельствуют о высокой вероятности ОЯ у беременных. И только морфологическое исследование тканей яичника, полученных независимо от хирургических способов, давало истинное представление о характере опухоли яичника у беременных. Вместе с тем у трех беременных с ОЯ при морфологическом исследовании выявлены участки ткани, характерные как для ООЯ, так и для ОЯ. Таким образом, преобладание пограничных в форме ОЯ возможно с помощью УЗИ и логрегрессионных моделей. Уровни VEGF выше 500 пг/мл, IL6 выше 8,1 пг/мл и CA-125 выше 300 ЕД/мл свидетельствуют о высокой вероятности ООЯ у беременных. И только морфологическое исследование тканей яичника, полученных независимо от хирургических способов, давало истинное представление о характере опухоли яичника у беременных. Вместе с тем у трех беременных с ООЯ при морфологическом исследовании выявлены участки ткани, характерные как для ООЯ, так и для ООЯ. Таким образом, преобладание пограничных в форме ООЯ возможно с помощью УЗИ и логрегрессионных моделей. Уровни VEGF выше 500 пг/мл, IL6 выше 8,1 пг/мл и CA-125 выше 300 ЕД/мл свидетельствуют о высокой вероятности ООЯ у беременных.

Ключевые слова: внутриабдоминальное исследование, морфологическое исследование, опухоли яичников у беременных, CD31

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examination [9–10]. In more than 70% of pregnant women, the tumors are detected during the ultrasound scan in the early stages of gestation (tumor early stages according to the FIGO system). Surgical treatment of malignant ovarian tumors and BOTs in pregnant women is normally performed in the first and second trimesters of pregnancy [5, 11–12], which leads to increased perinatal morbidity and early infant mortality.

The study was aimed to improve methods for BOTs diagnosis in pregnancy and to determine the possibilities of organ preservation treatment.

METHODS

In 2000–2017 a group of 300 pregnant women with various tumor-like formations and ovarian tumors was prospectively examined. Inclusion criteria: pregnant women with tumor-like formations/ovarian tumors diagnosed during I–III trimesters. Exclusion criteria: the woman’s refusal to participate in the study; pregnant women with cancer diagnosed before the study; patients with threatened abortion, intrauterine infection, impairments in a fetus diagnosed before the study. The results of the study were evaluated by cross-analysis. The results’ distribution in accordance with the morphological structure, tumor stage and the abnormality degree is presented in Fig. 1.

In 76 of 300 pregnant women with ovarian neoplasms, BOTs and malignant ovarian tumors were detected. Of 25 patients with BOTs, 22 patients had serous and 3 had mucinous forms. It should be noted that the study was carried out for a long time and the patients’ recruitment was random, not population-based.

Ultrasonographic examination was performed with the Voluson 530 MT (Kretztechnik; Austria) and Voluson E8 (General Electric; USA) systems, and the RIC5-9-D (4–9 MHz), C1-5-D (2–5 MHz), RAB4-8-D (2–8 MHz) probes. An ultrasound scan was carried out in 2D and 3D mode, combined with color and energy Doppler mapping, as well as with three-dimensional angiography. The color Doppler mapping was used for assessment of the following features: vascularization pattern (tumor periphery, central parts of the tumor, septa, papillary features), the curve of the blood flow velocity analysis together with resistance index (RI) and peak systolic blood flow velocity (cm/s) determination. Of 30 ultrasound signs of tumor-like formations, benign ovarian tumors, BOTs and malignant ovarian tumors, 17 signs appeared to be informative. For ultrasound diagnostics the proposed model was used allowing one to distinguish between benign ovarian tumors, BOTs and malignant ovarian tumors [13]. Our previous studies [14] demonstrated that ovarian tumors in pregnant women had ultrasound signs allowing one to differentiate between benign and malignant ovarian tumors with high accuracy. During the study it was noted that the differences in the ultrasound features of various ovarian neoplasms were significant. When studying the ultrasound signs of malignant epithelial tumors of the ovaries (ovarian cancer), four types of structure, and, which in most important, unique hemodynamic parameters were identified. At the same time, the assessment scale based on the ultrasound signs analysis was created. To evaluate the accuracy of the model, in addition to the actual percentage of correct assignments, the sensitivity (Se) and specificity (Sp) parameters were taken into account.

Molecular biology techniques were applied as follows. Concentration of CA-125 was determined using the enzyme immunoassay test system (Siemens; Germany). Enzyme immunoassay method was used to determine the sFas concentration in the blood serum using monoclonal antibodies, and the VEGF concentration using the reagent kits (R&D; USA). The concentration of IL6 was evaluated by the Sandwich Enzyme-Linked ImmunoSorbent Assay (ELISA) using the reagent kits (R&D; USA).

Different pathologists examined the hematoxylin and eosin stains. The WHO Classification of Tumors of Female Reproductive Organs (2003) was used for morphological diagnosis, since that classification was adopted in the Russian Federation at the time of the study. For immunohistochemical studies, paraffin blocks of 15 pregnant women with BOTs and 10 pregnant women with malignant ovarian tumors were selected. Analysis of the angiogenesis was performed using antibodies to the vascular endothelial growth factor, VEGF, the major signal transducer for angiogenesis (VENTANA; USA), and antibodies to CD31 endothelial marker, the type 1 platelet endothelial

![Fig. 1. Ovarian tumors/tumor-like formations distribution in accordance with the histological structure, stage (BOTs/ malignant ovarian tumors) and abnormality degree (ovarian cancer)](image-url)
cell adhesion molecule (JC70 clone; VENTANA, USA). When evaluating the expression of CD31 under the microscope with small magnification, first, the areas with the largest number of microvessels were selected. Subsequently, in two separate fields of view with the increased microvasculature density, the number of all positive microvessels was calculated (200-fold magnification). The VEGF expression level was evaluated by semi-quantitative method (comparison of staining intensity and number of positive cells) in five fields of view (400-fold magnification). When measuring the staining intensity, unstained cells were assigned score 0, cells with pale yellow staining were assigned score 1, yellow-brown stained cells were assigned score 2, and brown stained cells were assigned score 3. The number of positively stained cells varied: score 0 corresponded to less than 10% of all cells, score 1 corresponded to 10–49% of stained cells, score 2 corresponded to 50–74% of stained cells, score 3 corresponded to over than 75% of stained cells. The results of both counts were added, the score over 2 was considered positive.

In addition, histories and outcomes of pregnancy and childbirth after treatment were studied in 300 patients with ovarian neoplasms. Statistical analysis was carried out using the SPSS 15.0 software package (IBM; USA). Data were analyzed by the frequency method using the crosstabs. The differences were considered significant when \( p < 0.05 \).

RESULTS

The study demonstrated that the examined pregnant women’s clinical characteristics did not vary significantly between the groups. Thus, the age of 76 pregnant women with BOTs and malignant ovarian tumors varied in a wide range, from 18 to 45 years. More than 60% of patients were aged 30. Pain in the lower abdomen and impaired function of neighboring organs (9% of cases), increase in abdomen size (10.9% of cases) were registered, and the history of menstruation irregularities (10.9% of cases) and infertility (2.7% of cases) was revealed in pregnant women with BOTs/malignant ovarian tumors. The structure of concomitant extragenital, gynecological pathologies and previous gynecological operations before pregnancy in patients with tumor-like formations/ovarian tumors correlated mainly with age and did not depend on the tumors’ morphology.

Among the BOTs histological types the serous types prevailed (22 (88%) patients). Mucinous tumors were detected in 3 (12%) pregnant women. The 28% of patients had bilateral ovarian lesions. In most pregnant patients stage I BOTs were diagnosed (19 (76%) patients). Stage II was revealed in 5 (20%) patients, and stage III was verified only in one patient.

Ultrasonic signs in pregnant women with BOTs matched several morphological types: in 32.6% of patients, mixed tumors with the predominant solid pattern were diagnosed. About 55% of patients had tumors with the predominant cystic component, over 10% of patients had solid tumors. Doppler sonography revealed central and peripheral hypervascularization with low RI values (less than or equal to 0.4) and high values of peak systolic blood flow velocity (over 15 cm/s) obtained during the curve of the blood flow velocity analysis, as well as the mosaic vessels indicating the presence of arteriovenous shunting in the tumor vasculature.

The use of the proposed model for the differential diagnosis of ovarian tumors in pregnant women made it possible to distinguish between tumor-like formations, benign ovarian tumors, BOTs and malignant ovarian tumors (sensitivity was 100%, specificity 92.3%, with an overall accuracy of the model 92.8%). Due to the similarity of images and hemodynamic indicators during the ultrasound scan, it was impossible to distinguish BOTs from malignant ovarian tumors. At the same time, in all patients with neoplasms of the described type, blood vessels were located in the center with a branched network in the septa, solid component, and papillary components. The low-resistance blood flow was revealed.

In pregnant women with BOTs, the CA-125 concentration varied in the range from 24.4 to 361 U/ml in the I trimester, and from 24.1 to 223 U/ml in the II trimester of pregnancy. The level of sFas was 40–200 ng/ml in the I trimester, and 46–180 in the
II trimester. The VEGF concentration varied in the range from 89 to 286 pg/ml in the I trimester, and from 92 to 480 pg/ml in the II trimester of pregnancy. IL6 reached 3.6–12 in the I trimester and 8–40.9 pg/ml in the II trimester.

In patients with malignant ovarian tumors (compared to patients with BOTs) the significant increase of CA-125 and other tumor markers (sFas, VEGF, IL6) levels in blood serum was observed at any time during pregnancy. In the blood of 3 patients with adenocarcinoma of the ovary the CA-125 level was 540–1224.6 U/ml, the sFas level was 180–312.6 ng/ml, the VEGF level was 510–1028 pg/ml, and the IL6 level was 9.8–40.9 pg/ml. The same concentration of molecular factors was observed in the blood of patients with dysgerminoma, mixed germ cell tumor and immature teratoma. In these patients, the CA-125 level exceeded 361 U/ml, the sFas level was above 240 ng/ml, the VEGF level above 490 pg/ml, and the IL6 level above 8.1 pg/ml.

When studying the BOTs morphology (Fig. 2), the features making it possible to distinguish BOTs from benign and malignant ovarian tumors were detected in 22 cases. In 3 cases, the inconsistencies were found in the final histological response of patients diagnosed with serous adenocarcinoma against the background serous borderline tumor. During the second preparations review no elements of the malignant tumor were found.

The borderline serous cystadenoma was a cystic tumor with discohesive wall and the pronounced papillary features which filled the entire inner surface and in 70% of cases were present on the outer surface. BOTs were characterized by the presence of epithelial features with the formation of cell bundles and separation of cells groups simultaneously with strictly ordered branching, in which small papilla came from large, ordered branching. Cells of the mucous tumor had some features of epithelial and mesothelial differentiation. Ciliated cells similar to cells of the fallopian tube were detected in one third of tumors. Cells with abundant eosinophilic cytoplasm and rounded nuclei resembled mesothelium, they were located on the tops of papilla. Cell nuclei were located basally, oval or round, with slight atypia, delicate chromatin, and sometimes with pronounced nucleoli. Rare mitoses were detected (usually 4–10 in the fields of view). Psammoma (sand) bodies were revealed in a half of preparations.

Serous carcinomas reached large sizes (up to 20 cm in diameter), they consisted of cysts with serous or sanious contents, filled with soft loose papillary features. The outer surface was smooth with some papillary structures on it. The solid tumors usually had less pronounced pink gray papilla, they were soft or dense depending on the underlying stroma type. At the same time the foci of hemorrhage and necrosis were observed. Under the microscope the serous carcinomas had a papillary structure with solid foci, large round cells with polymorphic hyperchromatic nuclei, clumpy nuclear chromatin pattern and increased nuclear-cytoplasmic ratio, pseudostratified epithelium. Those were characterized by the loss of polarity, no cilia on the cell surface, increased mitotic activity.

The borderline mucinous cystadenoma of the ovary was usually multilocular with a diameter up to 30 cm, it contained the straw-colored liquid or mucus. Morphological examination of the described tumors’ preparations revealed areas lined with the multilayered mucinous epithelium of the intestinal type with the villous glandular and papillary features and slight atypia of cell nuclei.

Mucinous carcinoma differed from the borderline mucinous cystadenoma by the foci with a glands complex arrangement lined with cells with moderate and severe nuclei atypia, mitoses, as well as by the foci of necrosis inside the tumor.

The medical history analysis of pregnant women with BOTs and malignant ovarian tumors showed that those of them who had disseminated tumors underwent the cytoreductive surgery with abortion. The other patients underwent the cytoreductive surgery twice upon the detection of a tumor and after the cesarean section.

All patients demonstrating signs of ovarian tumor malignization got the midline laparotomy with the curve around the umbilicus on the left. In six patients, diagnostic laparoscopy was performed first, and after that laparotomy and primary lesion removal (due to the suspected ovarian cancer).

The volume of the surgical procedure was determined intraoperatively in accordance with the clinical picture, reproductive history, age, ultrasonography, serum tumor marker levels and express histopathological examination results. During the intervention, surgical tumor staging was performed, as well as the abdomen and pelvic organs revision, greater omentum resection/removal, multiple peritoneal biopsies, taking swabs or ascitic fluid from the abdominal cavity. In patients with mucinous tumors, an appendectomy was carried out. The patients not interested in pregnancy maintenance and fertility underwent the radical surgery (7 patients of 76). At the first stage during pregnancy, 20 patients with BOTs underwent the organ sparing intervention preserving uterus and the healthy ovarian fragment. In two patients, the bilateral adnexectomy was performed. In one of them, the borderline tumor was found during the histopathological examination of the resected part of the visually unchanged contralateral ovary (stage IB).

It should be noted that during the histopathological examination of biopsy material or tumor preparations, errors and inaccuracies may occur. Thus, during our study, in three pregnant women with ovarian tumors, morphological examination revealed tissue features characteristic of both BOT and malignant ovarian tumors. The patients were diagnosed with well-differentiated adenocarcinoma of both ovaries against the background of the borderline serous cystadenoma. In one of those patients, bilateral ovarian tumors with signs of malignization and ascites were clinically defined during the weeks 11–12 of pregnancy. In the oncology hospital the...
The CA-125 concentration was above 8.1 pg/ml) was detected in pregnant women with malignant ovarian tumors. The VEGF level exceeded 500 pg/ml, IL6 level was above 300 U/ml. Our results were consistent with the other authors’ data [16].

When evaluating the VEGF expression level in the paraffin blocks by the semi-quantitative method, the increased immunoreactivity for the marker (score 5–7) was detected in ovarian carcinomas. The VEGF expression association with ovarian cancer has been confirmed by many studies. An increase in VEGF immunoreactivity in ovarian carcinoma (compared to BOT) has been proven, while a high VEGF expression level indicates the disease progression [17]. Increased immunoreactivity of CD31 in the malignant ovarian tumors preparations compared to BOT preparations indicates increased blood flow in the tumor tissues due to neovascularization detected in malignant tumors [18].

The main method of the BOTs treatment is surgery (organ preservation or radical approach). Researchers of the world are actively discussing the possibility of ultra-conservative interventions as an organ preservation option leaving the affected with BOT ovarian tissue unchanged after the resection/cystectomy [2, 19]. Adnexectomy on the lesion side with a morphological study of peritoneal swabs and multiple biopsies is considered the optimal intervention volume. The final surgical staging should be performed during cesarean section or after delivery (in case of vaginal birth) [20, 21]. We did not use the ultraconservative interventions in our study, 80% of patients with BOTs underwent organ preservation surgery. The restaging surgery was performed in 16% of patients.

Approximately one-third of the patients with BOTs and well-differentiated adenocarcinoma need a final postoperative morphological study using paraffin blocks [2, 22–24]. According to some reports, the high overdiagnosis rate in patients with BOTs having the suspicious for ovarian cancer foci leads to an unreasonable overestimation of the surgical interventions volume, even when performing the final histopathological examination in the specialized institutions [3]. According to our results, the morphological response interpretation discrepancies in the differential diagnosis of BOTs and ovarian cancer have been detected in 12% of patients. The diverse BOTs structure and the need for a thorough study of multiple slices are the reason for the strict requirements for the morphologist’s qualification and experience. The other researchers hold a similar opinion [3, 9, 22].

The overall recurrence rate in patients with BOTs varies from 3 to 10%, and the recurrence occurs in 25% of patients with common tumor stages. Our study has revealed recurrence in 8% of patients. According to the literature data, the 5-year survival rate of patients with I-II stage tumors is 98–99%, and
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OVARIAN NEOPLASMS, RISK FACTORS, AND THE POSSIBLE INFLUENCE OF SCREENING METHODS: A MULTICENTRE STUDY

The problem of ovarian cancer is an important one for gynecologists. The occurrence of this disease is often associated with the presence of asymptomatic growths.

The aim of the study was to assess the risk of ovarian cancer in women with different groups of growths.

Methods: A retrospective study of 306 women with growths of different sizes and locations. The data were divided into four groups: 1) women with growths of any size; 2) women with growths of size ≤ 3 cm; 3) women with growths of size > 3 cm and ≤ 6 cm; 4) women with growths of size > 6 cm.

Results: Women with growths of size > 6 cm were found to have a higher risk of ovarian cancer compared to women with growths of size ≤ 3 cm (p < 0.05). The risk of ovarian cancer increased with the size of the growths.

Conclusion: The results of the study suggest that the size of growths is an important factor in the risk of ovarian cancer. Early detection and treatment of growths are critical to improving outcomes.

Keywords: ovarian cancer, risk factors, screening methods, growth size.
can suggest their malignancy [1]. Some authors estimate that epithelial cancer accounts for 60% of all ovarian neoplasms and 80–90% of ovarian malignancies [2]. The rest of ovarian tumors arise from germ and stromal cells, are typically found in younger patients and their sonographic appearance can pose diagnostic difficulty for the clinician.

Because ovarian tumors are fast-growing and aggressive, about 60–70% of patients have advanced stages (III–IV) of the disease at the time of presentation [3]. The use of ultrasonography (US) and the improvement of its diagnostic efficacy may be a solution to the problem of early ovarian cancer detection. US is a noninvasive, cheap, widely available and reproducible modality introduced in 1970 [4–6]. The first ultrasound screening tests were offered to women in the 1980s; they consisted in the transabdominal examination of pelvic organs, which was not the best effective strategy, for anatomical reasons. In 1990, I. Jacobs included transvaginal scans in his screening model. Since then, US has been the primary diagnostic modality for suspected ovarian neoplasms. Over the years, better accuracy in discriminating between malignant and benign tumors has been achieved due to the use of Doppler US. The technique relies on the phenomenon of neovascularization: new capillaries start to develop in the tumor, promoting its further growth. In a malignant tumor, blood flow has a number of characteristics determined by the lack of vascular smooth muscle fibers and the presence of multiple vascular shunts increasing the rate of blood flow in the neoplasm [7].

The aim of this study was to evaluate the prognostic efficacy of some sonographic features in the differential diagnosis of benign and malignant ovarian tumors in reproductive-age women.

METHODS

The groundwork for this research was laid by the prospective study conducted in 168 reproductive-age women with a morphologically verified ovarian neoplastic process who underwent surgery at Samara Regional Oncology Center in 2012–2015. The following inclusion criteria were applied: age of 18 to 40 years; US findings suggestive of an ovarian mass; subsequent surgery and a histopathological examination of the excised tissue. Exclusion criteria: age below 18 and above 40 years; a medical history of cancer.

Pulsed-wave Doppler scans were performed using a Philips IU-22 scanner (Philips; USA).

The patients were divided into 3 groups according to the WHO classification (2013): 1) 101 (60.1%) patients with benign tumors; 2) 24 (14.3%) patients with borderline tumors; 3) 43 (25.6%) patients with malignant tumors.

The following parameters were evaluated: the size and the volume of the ovarian mass, fluid buildup in the pelvis, the type and the morphologic appearance of the tumor. The neoplastic process was evaluated based on the type of the ovarian mass (solid, cystic, mixed), the involvement of 1 or both ovaries (uni- or bilateral lesions), the size of the lesion, the presence of septations, the presence of projections on the external/internal surface of the capsule and the quality of the capsule surface itself, as well as blood flow in the tumor. We also measured the blood flow velocity in the tumor and the resistive index. Statistical analysis was carried out in SPSS21 (20130626-3; An IBM Company; USA) and Microsoft Excel (Microsoft; USA).

RESULTS

The maximum size of the tumors (Table 1) varied between 77.26 ± 6.94 mm and 97.06 ± 15.29 mm. No positive correlation was established between the size of the tumor and the stage of the disease. In the patients with benign tumors, the tumor volume was 99.06 ± 128.18 ml on average; in the patients with borderline tumors, it was 814.54 ± 358.32 ml, and in the patients with malignancies, 579.17 ± 196.37 ml ($p = 0.941$).

When analyzing the ultrasound appearance of the tumors, we assessed the involvement of one or both ovaries in the neoplastic process. We also identified a group of 15 patients who had undergone adnexa removal emergency surgery at the gynecological departments of general hospitals and had been subsequently referred to specialist centers for a postoperative US examination and a reexamination of histology slices. Unilateral lesions were more often observed in the patients with benign (81.2%) and malignant (86%) tumors than in the patients with borderline tumors (54.2%) ($p = 0.006$).

Based on their echotexture, the tumors were classified into 3 types (Fig. 1): cystic, solid and mixed, with both cystic and solid components ($p < 0.001$). Women with cystic ovarian masses made up 72.6% of the study participants. In this group of patients, the masses were round in shape, with well-defined smooth margins, anechoic, with single or multiple septa and without projections along the internal capsule. Cystic masses were more typical to the patients with benign tumors (87.1%), compared to the women who had borderline (54.2%) and malignant (48.8%) tumors, respectively.

Patients with mixed type tumors (with both cystic and solid components) made up 22.6% of all study participants. In this group, the tumors were round-shaped, with fairly well-defined smooth margins, anechoic, with septations or areas of echogenicity and a solid irregular or regular-shaped component. The mixed type was more prevalent in the patients with borderline and malignant tumors (37.5 and 39.5%, respectively) than in the women with benign tumors (11.9%).

Solid tumors were observed in 4.8% of the patients. Tumors of this type were either round or irregular in shape, with fairly well-defined angular margins; they were characterized by mixed echogenicity or the presence of single anechoic round-shaped components. The solid type was observed in the participants with malignancies (11.6%).

We also evaluated the surface of the tumor capsule (Fig. 2), which was either smooth or nodular ($p = 0.008$). In the patients with benign tumors, the capsule surface was smooth in 80 (79.2%) cases and nodular in 21 (20.8%) cases. In the group

<table>
<thead>
<tr>
<th>Table 1. Sizes of ovarian tumors</th>
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<tbody>
<tr>
<td>Size 1</td>
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<tr>
<td>Size 2</td>
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<tr>
<td>Size 3</td>
</tr>
<tr>
<td>Tumor volume, ml</td>
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</table>

Note: $p_{1-2}$, $p_{2-3}$, $p_{3-4}$ — intergroup comparison; $p$ — the Kruskal–Wallis H test.
of patients with borderline tumors, smooth capsule surface was observed in 17 (70.8%) cases, whereas nodular, in 7 (29.2%) women. In the group of patients with malignant tumors, the capsule surface was smooth in 23 (53.5%) patients, whereas nodular, in 20 (46.5%) patients. Additionally, we looked at the presence of projections on the external (p = 0.192) and internal (p < 0.001) surfaces of the capsule. We found that 40.6% of patients with benign tumors had projections on the external surface and 4% of women, on the internal surface. In the group of patients with borderline tumors, no projections were observed on the external surface of the capsule, and 79.2% had projections on the internal surface. In the groups of patients with malignant tumors, 65.1% had projections on the external surface, whereas 9.3% on the internal surface.

Small amounts of free pelvic fluid were observed in 15.8% of women with benign tumors. In this group, there were no patients with moderate or large amounts of free fluid in the pelvis. In the borderline group, fluid buildup was observed in every third patient (33.3%), of whom 16.7% had it in moderate and large volumes. However, free pelvic fluid was discovered only in 14% of women with malignancies; of them only 1% (2.3%) had in large quantities (Kruskal–Wallis H test, p = 0.007).

Doppler ultrasonography can estimate blood flow in the tumor. This facilitates timely diagnosis of a neoplastic process in the ovaries and is especially important for deciding on the treatment strategy in reproductive-age women. In our study, blood flow parameters were evaluated in several steps.

Step I. The presence of blood flow within the tumor was evaluated in all patient groups (p < 0.001). Tumor blood flow was detected by Doppler ultrasonography in 18 (17.8%) patients with benign tumors; another 27 (26.7%) patients with benign tumors had single colored spots on the dopplergram (power Doppler). In the group of patients with borderline tumors, blood flow was registered in 9 (37.5%) women; another 9 (37.5%) had single colored spots on the dopplergram (power Doppler). Of all patients with malignancies, blood flow was detected in 23 (53.5%) women, whereas single colored spots, in 15 (34.9%) women (power Doppler).

Step II. Tumor blood flow rate and resistive index (RI) were measured (Table 2). In the patients with benign tumors, the average blood flow rate was 1.45 ± 0.4 cm/s and the RI value was the lowest. For those with borderline tumors, the average blood flow rate was 4.58 ± 1.44 cm/s and the RI value was 0.21 ± 0.05. In the patients with malignancies, the maximum values for blood flow rate and RI were 6.34 ± 1.17 cm/s and 0.26 ± 0.04, respectively. We were able to identify Doppler parameters that helped us to discriminate between benign and malignant tumors: tumor blood flow rate over 1.85 cm/s (p = 0.007) and RI over 0.16 (p = 0.013).

Using stepwise logistic regression, the US findings and the calculated blood flow rate values, we built a model for early diagnosis of ovarian cancer (Table 3). The type of tumor composition was a significant predictor: solid masses were at increased (31.69-fold) risk for malignant or borderline transformation; a mixed type with cystic and solid components increased such risk 3.46-fold. Sensitivity and specificity of this diagnostic model were 87 and 68%, respectively, with a probability threshold of 0.3.

**DISCUSSION**

Considering the morphologic diversity of ovarian growths and their frequently poor outcomes, the search for early
predictors of malignancy in reproductive-age women remains a pressing concern. Algorithms predicting the risk of malignant transformation are in continuing development, aiming at detecting cancer in its early stages and thus reducing the extent of surgery. In 1996, the risk-of-malignancy index (RMI) was first proposed. It was designed to estimate the risk of malignant transformation using a scoring system [9]. Similar to our model, it relied on US features, such as the presence of septations and solid components, the involvement of 1 or both ovaries and ascites. However, unlike our model, the index also accounted for the presence of abdominal metastases, the menopausal status (premenopause/postmenopause) and the absolute values of CA 125. For the sake of convenience, each component was attributed a value (score) and the following formula was applied to calculate the index: RMI = Ultrasound features (score) · Menopausal status (premenopause/postmenopause) · Absolute values of CA 125. If the resulting RMI was below 200, the ovarian mass was assumed to be potentially benign.

The International Ovarian Tumor Analysis (IOTA) carried out in 1999–2000 aimed at formulating the guidelines and creating the models for characterizing ovarian tumors [9]. The models were developed for use by clinicians regardless of their qualifications and allowed them to better understand the etiology of ovarian cancer and the role of CA 125 and other cancer biomarkers. Later, an international team of researchers proposed 2 logistic regression models: LR1 and LR2 for differentiating between benign and malignant ovarian tumors [10, 11]. According to the models, the sonographer should evaluate over 40 different clinical and US variables. The sensitivity and accuracy of the method were 96% and 90%, respectively, but the method turned to be very time-consuming and generally demanding; it did not account for the patient’s medical history and laboratory test results. The researchers concluded that recognition of US features typical to an ovarian pathology by an experienced sonographer is the best method to characterize this pathology and that CA 125 does not improve the diagnostic accuracy in predicting the malignancy of the tumor [12–14]. Using statistical analysis, we were able to reduce the number of variables and thus to save time for and simplify the subsequent calculations without reducing the sensitivity and specificity of our diagnostic model (87 and 68%)

In 2011, it was demonstrated that blood flow in the tumor could be a sign of possible malignant transformation (p < 0.001). For reproductive-age women, Doppler parameters have been identified that can clearly discriminate between benign and malignant growths: the blood flow rate over 1.85 cm/s (p = 0.029) and projections on the internal surface of the tumor capsule (p < 0.001), moderate or significant buildup of free fluid in the small pelvis (p = 0.007) and the nodular surface quality of the tumor capsule (p = 0.008).

This study has established a correlation between the size and volume of ovarian tumors and their morphological structure. However, the analysis of tumor echotexture allowed us to identify US characteristics associated with malignancy, including the presence of a solid component (p < 0.001), septations (p = 0.029) and projections on the internal surface of the tumor capsule (p < 0.001), moderate or significant buildup of free fluid in the small pelvis (p = 0.007) and the nodular surface quality of the tumor capsule (p = 0.008).

The study demonstrates that blood flow in the tumor could be a sign of possible malignant transformation (p < 0.001). For reproductive-age women, Doppler parameters have been identified that can clearly discriminate between benign and malignant growths: the blood flow rate over 1.85 cm/s (p = 0.007) and RI over 0.16 (p = 0.013).

The identified US features (a solid or a mixed type mass, blood flow in the tumor and increased resistance index) can be used as key parameters in differentiating between various types of ovarian tumors.

Table 2. Characteristics of blood flow in ovarian tumors

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Benign</th>
<th>Borderline</th>
<th>Malignant</th>
</tr>
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<tbody>
<tr>
<td>v, cm/s</td>
<td>1.45 ± 0.40</td>
<td>4.58 ± 1.44</td>
<td>6.34 ± 1.17</td>
</tr>
<tr>
<td>RI</td>
<td>0.08 ± 0.02</td>
<td>0.21 ± 0.05</td>
<td>0.26 ± 0.04</td>
</tr>
<tr>
<td>p</td>
<td>0.007</td>
<td>&lt; 0.001</td>
<td>0.261</td>
</tr>
</tbody>
</table>

Table 3. The model for early diagnosis of ovarian cancer in reproductive-age patients

<table>
<thead>
<tr>
<th>Risk factor grading</th>
<th>Regression coefficient, b</th>
<th>OR (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cystic, reference</td>
<td>0</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>Solid</td>
<td>3.46</td>
<td>31.69 (3.16–318.11)</td>
<td>0.003</td>
</tr>
<tr>
<td>Mixed</td>
<td>1.23</td>
<td>3.40 (1.32–8.77)</td>
<td>0.011</td>
</tr>
<tr>
<td>Blood flow in tumor</td>
<td>“Yes” in comparison with “no”</td>
<td>0.98</td>
<td>2.68 (1.56–4.58)</td>
</tr>
<tr>
<td>RI</td>
<td>Increment by 1</td>
<td>2.23</td>
<td>9.34 (1.92–45.49)</td>
</tr>
<tr>
<td>Constant</td>
<td>–</td>
<td>–2.35</td>
<td>–</td>
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EEG µ-RITHYM REACTIVITY IN CHILDREN DURING IMITATION OF BIOLOGICAL AND NON-BIOLOGICAL MOTION

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The development of brain-computer interfaces based on the use of EEG sensorimotor rhythms reactivity parameters and designed for the rehabilitation of people (including children) with impaired motor functions is currently relevant. The study was aimed to analyse the EEG µ-rhythm in the individual frequency range in children during imitation of biological and non-biological motion. EEG was recorded at frontal, central and parietal cortical regions in 136 normally developing right-handed children aged 4–15, at rest and during the execution and imitation of movements using the computer mouse. When the children moved the computer mouse on their own (F(1, 132) = 31.17; p < 0.001) and executed the concentric moving of the coloured circle (F(1, 132) = 90.34; p < 0.001), the µ-rhythm desynchronization developed in the frontal, central and parietal neocortical regions. The µ-rhythm synchronization was detected during the non-biologicical motion imitation (F(1, 132) = 12.65; p < 0.001), compared to the task on the autonomous movement execution. The µ-rhythm desynchronization was observed during the biologicical motion imitation in relation to autonomous movement execution (F(1, 132) = 9.58; p = 0.002). The described effects had their features in the groups of children aged 4–6, 7–9, 10–12 and 13–15. The study results demonstrate the desirability of taking into account the µ-rhythm reactivity age-related features and the visual stimuli nature when developing software for the brain-computer interfaces.

Keywords: children; EEG; µ-rhythm; imitation; biological motion; non-biological motion

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Compliance with ethical standards: the study was approved by the Ethics Committee of VI. Vernadsky Crimean Federal University (protocol № 12 dated June 14, 2016). Informed consent to participation in the study was obtained from the parents.

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REAKTIVNOST’ µ-RIHTMA ÈEÈ U DETEY PŘÍ IMITACÍ DВÍJENÍ VIZUAΛNÝKH OBJEВOВ BИОΛOGИЧЕСКОГО I НЕБИОЛΟГИЧЕСКОГО ПРОИСХОЖДЕНИЯ

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В настоящее время актуальна разработка интерфейсов мозг-компьютер, основанных на использовании параметров реактивности сенсомоторных ритмов ЭЭГ и предназначенных для реабилитации людей с нарушениями двигательных функций, в том числе детей. Целью работы было проанализировать реактивность µ-ритма ЭЭГ в индивидуально определенном частотном диапазоне у детей при имитации движений визуальных образов биологического и небиологического происхождения. ЭЭГ регистрировали во фронтальных, центральных и парietальных областях коры у 136 нормально развивающихся детей-пациентов 4–15 лет в состоянии покоя, а также при самостоятельном выполнении и имитации движений с помощью компьютерной мыши. При выполнении детьми самостоятельных движений компьютерной мышью (F(1, 132) = 31.17; p < 0.001) и при осуществлении концентрических перемещений цветного круга (F(1, 132) = 90.34; p < 0.001) развивается десинхронизация µ-ритма во фронтальных, центральных и парietальных областях неокортика. При имитации движений визуальных образов небиологического происхождения, по сравнению с заданием на выполнение самостоятельных движений, была выявлена синхронизация µ-ритма (F(1, 132) = 12.65; p < 0.001). При подражании движениям визуальных образов биологического происхождения относительно самостоятельных движений выявлена десинхронизация µ-ритма (F(1, 132) = 9.58; p = 0.002). Данные эффекты имели свои особенности в группах детей 4–6, 7–9, 10–12 и 13–15 лет. Результаты исследования показывают целесообразность учета возрастных особенностей реактивности µ-ритма и характера предъявляемых зрительных стимулов при разработке программного обеспечения интерфейсов мозг-компьютер.

Ключевые слова: дети; ЭЭГ; µ-ритм; имитация; биологическое движение; небиологическое движение

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The human's ability to understand the goal of action and imitate is necessary for effective integration into the social environment, allowing one to master various types of activities and norms of behavior in society. The mirror neuron system (MNS)
is important for recognition of movements and associated intentions. Mirror neurons are neurons able to activate in a similar way, both when executing actions, and when watching other individuals executing similar actions [1, 2]. It has been suggested that MNS plays an important role in the complex forms of social interaction. [3).

Desynchronization of the EEG sensorimotor rhythm, the µ-rhythm, is considered to be a marker of MNS activation [4]. Since modulations of the α-rhythm in the occipital region can overlap the effects of µ-rhythm desynchronization [5], to determine the individual frequency range and reactivity, the following features are taken into account: unlike the occipital α-rhythm, the µ-rhythm amplitude decreases when the subject moves, imagines movement or watches the other subjects’ movement, but does not change significantly when the subject opens or closes the eyes [6, 7].

Sensorimotor rhythm amplitude depression during the biological motion watching is more pronounced than during watching the non-biological motion [8], which is also characteristic of watching social actions, compared to actions outside the social context [9]. Regarding the movement imitation, it is assumed that imitation is associated with the activation of human MNS, and is the result of the comparison of the observed action and the internal motor plan for the execution of action [10].

The study of MNS and µ-rhythm reactivity is of special interest in a view of new methods development for rehabilitation of patients with various motor impairments using brain-computer interfaces [11, 12]. In particular, in the treatment of adult patients, the synchronous interfaces are used, based on the analysis of the EEG sensorimotor rhythms reactivity when representing the movement in response to the signal presented [13, 14]. Recently, such methods are beginning to be used for rehabilitation of children with cerebral palsy [15]. Symbols or text commands are reported to be used as the signals presented to patients. However, the concept of MNS suggests that stimuli visually representing movements and requiring the simulation of movements could be more effective for triggering reactions in the EEG µ-rhythm range. It should be noted that when working with children it is preferable to use the actions that are in the child’s motor repertoire for more effective task execution [16]. The study was aimed to analyse the EEG µ-rhythm under conditions of biological and non-biological motion imitation in children aged 4–15 using the computer pointer device, the mouse. Now, even the preschool children are familiar with the computer mouse operation.

METHODS

Characteristics of a sample

The study was performed at the Center for Collective Use of Scientific Equipment “Experimental physiology and biophysics” of V.I. Vernadsky Crimean Federal University. The study included 136 right-handed children aged 4–15 (69 boys and 67 girls). Inclusion criteria: normal (or corrected to normal) vision and hearing; preferred right hand when operating the computer mouse; sufficient degree of cognitive development (IQ at least 80 points according to the Wechsler scale, variants WPPSI and WISC). Exclusion criteria: taking the CNS affecting drugs; severe chronic somatic diseases. The children were divided into four age groups: 4–6 years (30 people), 7–9 years (46 people), 10–12 years (30 people) and 13–15 years (30 people).

EEG recording

EEG recording was performed using the Neuron-Spectrum-3 EEG System (Neurosoft; Russia). Data were obtained using the WinEEG version 2.8 software (available for free). Independent component analysis was used for the artifacts correction. The 19 monopolar EEG electrodes were used in accordance with the 10–20 system. In our study, the frontal, central and parietal neocortical regions were the area of concern (F3, F4, Fz, C3, C4, Cz, P3, P4, Pz loci). Paired electrodes attached to the ear lobes were the reference electrode. Cut-off frequencies of the high and low pass filters were 1.5 and 35 Hz, respectively, EEG digitization rate was 250 Hz. EEG recording was performed while the children performed a queue of sequential tasks, the duration of each task was 30 s. EEG segments were processed using the Fast Fourier Transform with the 4 epoch of analysis and 50% mutual overlapping of epochs.

To imitate the non-biological motion, the following tasks were used:

1) gaze fixation on the video of the computer mouse (baseline);

2) concentric moving of the coloured circle on the monitor screen using the computer mouse (Mn.1) (Fig. 1A);

3) imitation of the other coloured circle’s motion (imitation of non-biological motion, ImNB) (Fig. 1B).

When imitating the biological motion, the subject and the researcher were located at the tables next to each other (the researcher on the right), each of tables had a monitor and a computer mouse on it. Using the webcam, the working plane of the researcher’s table with the mouse on it was demonstrated on the monitor in front of the subject. The tasks queue was as follows:

1) gaze fixation on the video of the computer mouse (baseline);
2) moving the computer mouse in a circle by children on their own (Mn.2) (Fig. 1C);
3) imitation of the researcher’s movements by the children (imitation of biological motion, ImB) (Fig. 1D).

EEG was analysed in the individual μ-rhythm frequency range defined when the subject moved his right hand on his own (C3). The full frequency range of the μ-rhythm (6–13 Hz) was divided into segments of 1 Hz. As an individual frequency range, two adjacent segments were taken with maximum desynchronization in relation to baseline [17]. The μ-rhythm amplitude within the individual frequency range was calculated for each experimental situation. Log transformation was used for normalization of the amplitude values distribution.

Reactivity indices were used for comparison of μ-rhythm parameters under conditions of biological and non-biological movement imitation. These indices were calculated according to the generally accepted scheme [18] using the following formula: $k = \ln(B/A)$, where $k$ is the sensorimotor rhythm reactivity index, $B$ is the sensorimotor rhythm amplitude in the major situation, and $A$ is the sensorimotor rhythm amplitude in the initial reference situation (baseline or subjects’ moving on their own). Positive reactivity index values corresponded to synchronization of the sensorimotor rhythm, and negative values corresponded to desynchronization.

**Statistical analysis**

Statistical analysis was performed using the STATISTICA 12.0 software (StatSoft Inc., USA). To describe the non-normal distributions, median and interquartile range were used, the differences between the groups were evaluated using the Mann-Whitney U-test. For normal data distribution, the mean and standard error of the mean were used. The differences of the amplitude and reactivity indices of the μ-rhythm recorded in different experimental situations were evaluated by the repeated measures ANOVA. The 4×2×9 scheme was used for assessment of the one intersubjective factor (age group, AGE) and two intrasubjective factors (situation, SIT, and locus, LOC) influence. To calculate the statistical significance of the sensorimotor rhythm differences in relation to each of the nine EEG derivations within each age group, the ad-hoc analysis method (F-distribution estimation) was used.

**RESULTS**

**μ-rhythm frequency parameters**

The median values of the individual μ-rhythm range lower boundary were 9 Hz (8.5; 10), the extreme values were 6 and 11 Hz. The median values of the individual μ-rhythm range upper boundary were 11 Hz (10.5; 12), and the extreme values were 8 and 13 Hz. The differences between the age groups were not significant.

**EEG μ-rhythm amplitude at rest and under condition of motion execution and imitation**

The μ-rhythm amplitude differences analysis of variance in the Mn.1 situation in relation to baseline taking into account the age group and EEG locus revealed the significant influence of the μ-rhythm frequency parameters $\mu$-rhythm amplitude changes in the Mn.1 situation in relation to baseline taking into account the age group and EEG locus revealed the significant influence of the SIT ($F_{1, 132} = 90.34; p < 0.001$), AGE ($F_{3, 132} = 10.18; p < 0.001$) and LOC ($F_{8, 1056} = 73.06; p < 0.001$) factors, as well as the SIT×LOC interaction ($F_{8, 1056} = 73.06; p < 0.001$) factors, as well as the SIT×LOC interaction ($F_{8, 1056} = 41.28; p < 0.001$).

Compared to Mn.1, in the ImNB situation the μ-rhythm amplitude changes in the ImB situation in relation to baseline taking into account the age group and EEG locus revealed the significant influence of the SIT ($F_{1, 132} = 12.65; p < 0.001$), AGE ($F_{3, 132} = 14.67; p < 0.001$) and LOC ($F_{8, 1056} = 39.43; p < 0.001$) factors significantly affected the μ-rhythm amplitude changes.

The μ-rhythm amplitude differences analysis of variance in the Mn.2 situation in relation to baseline taking into account the age group and EEG locus revealed the significant influence of the SIT ($F_{1, 132} = 31.17; p < 0.001$), AGE ($F_{3, 132} = 6.46; p < 0.001$) and LOC ($F_{8, 1056} = 71.55; p < 0.001$) factors, as well as the SIT×LOC interaction ($F_{8, 1056} = 28.32; p < 0.001$) and SIT×AGE ($F_{8, 1056} = 6.35; p < 0.001$) interactions. Evaluation of the μ-rhythm amplitude changes in the ImB situation in relation to Mn.2 revealed the significant influence of the SIT ($F_{1, 132} = 9.58; p = 0.002$), AGE ($F_{3, 132} = 18.63; p < 0.001$) and LOC ($F_{8, 1056} = 54.08; p < 0.001$) factors, as well as the SIT×LOC ($F_{8, 1056} = 3.28; p = 0.001$) and SIT×AGE ($F_{3, 132} = 6.2; p = 0.001$) interactions.

![Fig. 2. EEG μ-rhythm amplitude (A, Ln µV) in children aged 4-6 during imitation of non-biological (A) and biological (B) motion. 1 — baseline, 2 — autonomous movements’ execution at arbitrary speed, 3 — motion imitation. Amplitude differences between baseline and autonomous movements’ execution: * — $p < 0.05$, ** — $p < 0.01$, *** — $p < 0.001$, when executing autonomous movements and imitating: * — $p < 0.05$](image-url)
In children aged 4–6 executing the concentric moving of the coloured circle, the significant EEG µ-rhythm desynchronization was detected (Mn.1 in relation to baseline) in most studied regions. The µ-rhythm amplitude changes in children imitating the coloured circle movement (ImNB in relation to Mn.1) were not significant (Fig. 2A). When the children of that age moved the computer mouse on their own, the significant EEG µ-rhythm amplitude increase (Mn.2 in relation to baseline) was detected in the right hemisphere central locus (C4). When the children imitated the researcher’s movements, the sensorimotor rhythm desynchronization (ImB in relation to Mn.2) was registered in the left mid-parietal locus (Pz) (Fig. 2B).

In the group of children aged 7–9, the significant depression of µ-rhythm in the Mn.1 situation was observed in most studied regions. In the ImNB situation (in relation to Mn.1) the significant EEG µ-rhythm amplitude changes were not significant (Fig. 3A).

In the Mn.2 situation the significant sensorimotor rhythm desynchronization was detected in the central (C3 and Cz) and all parietal loci. In the ImB situation (in relation to Mn.2) there were no significant µ-rhythm amplitude changes (Fig. 3B).

In children aged 10–12, in the Mn.1 situation the significant µ-rhythm suppression was detected in most studied loci. During the coloured circle movement imitation the significant sensorimotor rhythm synchronisation (ImNB in relation to Mn.1) was detected in the mid-frontal locus (Fig. 4A). In the Mn.2 situation the significant decrease in µ-rhythm amplitude was observed in the central (C3 and Cz) and all parietal loci. In the ImB situation (in relation to Mn.2) there were no significant sensorimotor rhythm amplitude changes (Fig. 4B).

In the group of teenagers aged 13–15, in the Mn.1 situation the significant µ-rhythm suppression was observed in most studied loci. During the non-biological motion imitation (ImNB in relation to Mn.1) the significant sensorimotor rhythm amplitude was the same as in Fig. 2.
synchronization was detected in most studied regions (Fig. 5A). When the subjects moved the computer mouse on their own, significant sensorimotor rhythm desynchronization (Mn.2 in relation to baseline) was registered in all studied regions. In the lmB situation, the additional (compared to previous task) μ-rhythm desynchronization was observed that was significant in all loci (Fig. 5B).

**EEG μ-rhythm reactivity comparison under conditions of biological and non-biological motion imitation**

To evaluate the differences of μ-rhythm reactivity in the lmNB and lmB situations (compared to execution of movements by children on their own at arbitrary speed), the reactivity indices analysis of variance was performed taking into account the age group and EEG locus. The mean μ-rhythm reactivity index values for children of four age groups are presented in Tables 1 and 2. The significant impact of SIT (F_{1,152} = 21.85; p < 0.001) and LOC (F_{6,1056} = 3.95; p < 0.001) factors, as well as the SIT×AGE interaction (F_{6,152} = 5.52; p = 0.001) was revealed. In the group of pre-school children, the significant μ-rhythm reactivity indices differences in the lmNB and lmB situations were detected in the parietal loci Pz and P4 (p = 0.03). In children aged 7–9, the significant differences were observed in the locus Fz (p = 0.04). In children aged 10–12, no significant μ-rhythm reactivity indices differences were detected. In the group of teenagers aged 13–15, the differences were significant in all studied regions (p ≤ 0.001).

**DISCUSSION**

According to the study results, the individual sensorimotor rhythm frequency ranges of the 4–15 years old children vary widely, and there are no significant differences in the mean values between different age groups. In the other authors’s paper [19] reporting the EEG μ-rhythm reactivity analysis in the selected frequency range in children aged 4–11, the average sensorimotor rhythm band was 9–11 Hz. High sensorimotor rhythm parameters variability among the individuals and no association with the children’s age were detected. These indicate the need to determine the children’s individual frequency range when studying the sensorimotor rhythm reactivity, as well as when attempting the correction using the μ-rhythm parameters (EEG based neurofeedback training, correction using the brain-computer interface).

Analysis of the μ-rhythm amplitude changes demonstrated that in 4–6 years old children arbitrarily moving the coloured circle (Mn.1) the significant EEG μ-rhythm desynchronization in the frontal and central loci of left hemisphere, as well as in the median frontal and all parietal loci (F3, Fz, C3, P3, Pz, P4) could be detected. The results of our study are consistent with the literature data on the sensorimotor rhythm desynchronization during the voluntary movements’ execution [20]. When the children moved the computer mouse on their own (Mn.2), no significant μ-rhythm amplitude decrease was observed. It is possible that for children of this age, the task of relatively simple circular movements’ execution with a computer mouse was simpler than the task of capturing and moving a colored circle using the computer mouse left button, and it did not require any special motor control. During the task execution, the significant EEG μ-rhythm amplitude increase in the locus C4 was registered, which could be due to inhibition of the ipsilateral hemisphere (in relation to the hand used) [21].

In the groups of children aged 7–9 and 10–12 executing the movements on their own, the μ-rhythm desynchronization was detected in most studied regions. The concentric coloured circle moving (Mn.1) unlike the mouse moving in a circle (Mn.2) was also associated with the sensorimotor rhythm desynchronization in the frontal loci (F3, Fz). It is known that the frontal cortical regions are responsible for planning and preparation of complex movements [22]. It is also assumed that more complex motor actions are accompanied by a more widespread μ-activity desynchronization [23]. Presumably, moving the color circle in the group of 7–12 years old children, as well as in the group of younger children, required considerable effort, which led to the involvement of the cerebral cortex frontal region.

The situations of biological and non-biological motion imitation in children aged 4–6, 7–9 and 10–12 were associated with almost no additional modulation of the μ-rhythm in relation to the arbitrary movements’ execution. This may indicate that in children of said age the required for processing
multimodal information additional neocortical resources are not sufficiently involved under the conditions of imitation.

In the group of teenagers aged 13–15, the significant sensorimotor rhythm desynchronization during the autonomous movements’ execution was detected in all studied regions. The µ-rhythm amplitude decrease above the frontal, central and parietal loci in elder children may be due to development of connections between the neocortical regions involved. During the non-biological motion imitation a smaller drop in the sensorimotor rhythm amplitude was observed than during the autonomous movements execution and biological motion imitation (which is especially pronounced in the frontal and central loci). It can be assumed, that the need to imitate the movements of another object (colored circle) led to the shift of attention to its perception and, as a result, to weakening of one’s own movements’ motor control. In children of this age, in the ImB situation, the additional (compared to the observed during the Mn.2 task execution) significant µ-rhythm desynchronization in all loci was detected. The more pronounced reaction in the parietal loci is noteworthy. It is known, that parietal cortical regions are involved into the information processing during watching the human’s motion (compared to watching the non-biological objects’ motion) [24]. The sensorimotor rhythm modulation, revealed by us in the described regions during the biological motion imitation, may be due to involvement of the parietal cortex MNS components responsible for coding of goals underlying the watched movements [25]. The mirror neurons are associated with the cognitive integration of visual, auditory and motor stimuli needed for social interaction in children [26] and adults [27]. Thus, it can be assumed, that the additional µ-rhythm desynchronization during the other man’s movements imitation is caused precisely by the social context to which the MNS is sensitive.

Comparison of the µ-rhythm reactivity indices for imitation tasks revealed that the biological motion imitation in elder children was associated with the greater desynchronization, compared with the situation of color circle movements’ imitation. As already noted, similar features of the sensorimotor rhythm reactivity during watching the biological and non-biological objects movements were detected in adult volunteers [8].

A sensorimotor rhythm reactivity patterns comparative analysis in children of different ages allows us to come to a number of conclusions. In the group of youngest children (4–6 years), the most pronounced activation of the frontal, central and parietal cortical regions, manifested in the µ-rhythm amplitude decrease (more pronounced in the left hemisphere), is observed during computer mouse operation associated with a non-biological motion (coloured circle) (Mn.1). Random rhythm computer mouse movements’ execution (Mn.2) does not lead to the significant decrease in the µ-rhythm amplitude, and the biological motion imitation (the other person’s hand movement) is not associated with any additional activation in most loci. Thus, in the described experimental situation, in pre-school children, the cortical center of motor analyzer is especially sensitive to manipulations with biological objects. In elder children (7–9 and 10–12 year), a similar neocortical activation pattern was revealed during execution of movements associated with non-biological objects, computer mouse moving and biological object (researcher’s hand) motion imitation. Unlike the previously described groups, the children aged 13–15 demonstrate the significant µ-rhythm desynchronization in the frontal, central and parietal cortical regions of both hemispheres during imitation of the other person’s motion.

It stands to reason, that processes of perception and other person’s movement imitation in younger children are in their infancy, and in teenagers, these processes are rather developed and similar to those in adults. In teenagers, the pronounced µ-rhythm desynchronization in all studied regions during moving on their own or imitating the biological visual images motion may be due to maturation of motor, sensorimotor and associative cortical regions involved in the execution and imitation of movements [28]. The revealed age-related sensorimotor rhythm reactivity features may be used for improvement of existing rehabilitation techniques based on the EEG-controlled robotic systems for children with cerebral palsy [15].

CONCLUSION

When children aged 4–15 move the computer mouse on their own, the µ-rhythm desynchronization develops in the frontal, central and parietal neocortical regions, which is more pronounced in the left hemisphere. When the children aged 4–6, 7–9 and 10–12 imitate the biological and non-biological motion no significant additional µ-rhythm modulation is revealed, compared to the execution of movements on their own. In children aged 13–15, the highest sensorimotor rhythm desynchronization is observed during the researcher’s hand

### Table 1. Reactivity indices mean values (together with standard error of the mean) obtained during imitation of non-biological motion

<table>
<thead>
<tr>
<th>Group</th>
<th>F3</th>
<th>F2</th>
<th>F4</th>
<th>C3</th>
<th>C2</th>
<th>C4</th>
<th>P3</th>
<th>P2</th>
<th>P4</th>
</tr>
</thead>
<tbody>
<tr>
<td>4–6 years</td>
<td>0.03 ± 0.04</td>
<td>0.04 ± 0.04</td>
<td>0.03 ± 0.03</td>
<td>0.03 ± 0.04</td>
<td>-0.02 ± 0.04</td>
<td>0.04 ± 0.03</td>
<td>0.02 ± 0.04</td>
<td>0.03 ± 0.04</td>
<td>0.04 ± 0.04</td>
</tr>
<tr>
<td>7–9 years</td>
<td>0.05 ± 0.03</td>
<td>0.06 ± 0.03</td>
<td>0.05 ± 0.03</td>
<td>0.05 ± 0.03</td>
<td>0.04 ± 0.03</td>
<td>0.00 ± 0.05</td>
<td>0.04 ± 0.03</td>
<td>-0.01 ± 0.03</td>
<td>0.03 ± 0.03</td>
</tr>
<tr>
<td>10–12 years</td>
<td>0.06 ± 0.04</td>
<td>0.09 ± 0.04</td>
<td>0.05 ± 0.04</td>
<td>0.03 ± 0.04</td>
<td>0.04 ± 0.03</td>
<td>0.06 ± 0.03</td>
<td>0.01 ± 0.04</td>
<td>0.01 ± 0.04</td>
<td>0.05 ± 0.05</td>
</tr>
<tr>
<td>13–15 years</td>
<td>0.11 ± 0.04</td>
<td>0.11 ± 0.05</td>
<td>0.07 ± 0.04</td>
<td>0.12 ± 0.03</td>
<td>0.10 ± 0.03</td>
<td>0.11 ± 0.04</td>
<td>0.09 ± 0.03</td>
<td>0.05 ± 0.03</td>
<td>0.01 ± 0.03</td>
</tr>
</tbody>
</table>

Note: positive reactivity index values correspond to sensorimotor rhythm synchronization, negative values correspond to desynchronization.

### Table 2. Reactivity indices mean values (together with standard error of the mean) obtained during imitation of biological motion

<table>
<thead>
<tr>
<th>Group</th>
<th>F3</th>
<th>F2</th>
<th>F4</th>
<th>C3</th>
<th>C2</th>
<th>C4</th>
<th>P3</th>
<th>P2</th>
<th>P4</th>
</tr>
</thead>
<tbody>
<tr>
<td>4–6 years</td>
<td>0.04 ± 0.04</td>
<td>-0.01 ± 0.05</td>
<td>0.01 ± 0.05</td>
<td>0.01 ± 0.04</td>
<td>-0.07 ± 0.05</td>
<td>-0.07 ± 0.06</td>
<td>-0.06 ± 0.05</td>
<td>-0.09 ± 0.05</td>
<td>-0.06 ± 0.06</td>
</tr>
<tr>
<td>7–9 years</td>
<td>-0.03 ± 0.03</td>
<td>-0.04 ± 0.03</td>
<td>0.00 ± 0.03</td>
<td>-0.03 ± 0.02</td>
<td>-0.03 ± 0.03</td>
<td>-0.03 ± 0.04</td>
<td>-0.04 ± 0.04</td>
<td>-0.06 ± 0.03</td>
<td>-0.03 ± 0.03</td>
</tr>
<tr>
<td>10–12 years</td>
<td>0.04 ± 0.05</td>
<td>0.04 ± 0.04</td>
<td>0.06 ± 0.04</td>
<td>0.03 ± 0.03</td>
<td>0.01 ± 0.03</td>
<td>0.06 ± 0.04</td>
<td>0.03 ± 0.03</td>
<td>0.01 ± 0.04</td>
<td>0.05 ± 0.03</td>
</tr>
<tr>
<td>13–15 years</td>
<td>-0.11 ± 0.03</td>
<td>-0.12 ± 0.03</td>
<td>-0.11 ± 0.03</td>
<td>-0.14 ± 0.03</td>
<td>-0.12 ± 0.04</td>
<td>-0.11 ± 0.04</td>
<td>-0.18 ± 0.03</td>
<td>-0.21 ± 0.04</td>
<td>-0.20 ± 0.03</td>
</tr>
</tbody>
</table>
motion imitation. When developing the software for brain-computer interfaces designed for motor function impairment correction, elements of the non-biological objects’ movement may be used as visual stimuli, but the older the children, the more effective the presentation of moving biological objects can be for neocortical activation.

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CIRCADIAN RHYTHMS OF LEUKEMIA INHIBITORY FACTOR IN THE BLOOD OF PATIENTS WITH ESSENTIAL HYPERTENSION

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Leukemia inhibitory factor (LIF) exerts multidirectional effects in the setting of essential hypertension (EH). There is a mounting body of evidence refuting the postulate about identical STAT3 signaling in cardiomyocytes and endothelial/smooth muscle cells, which is important in the situation of extended exposure to gp 130 ligands (LIF in particular). At the same time, there are no reports on the circadian dynamics of peripheral blood LIF concentrations and possible secondary changes to the pathophysiologic effects of this cytokine. This study aimed to analyze the circadian dynamics of peripheral blood LIF concentrations in the peripheral blood serum measured at 5 different time points in patients with stage II EH in the presence/absence of antihypertensive therapy and their relationship with the frequency of complications developing within a 5-year follow-up. Blood serum LIF was measured in 60 patients with stage II EH using ELISA at 8:00, 14:00, 20:00, 2:00, and 8:00 o’clock before putting the patients on antihypertensive therapy and one year after its onset. The identified patterns of diurnal LIF concentrations (a rise by ≥15% at 20:00, p < 0,001; a further rise by ≥22% peaking at 2:00, p < 0,001 relative to the values at 8:00) can be regarded as pathologic; their persistence after one year of antihypertensive therapy is a sign of EH progression and puts the patients at 6-fold risk for cardiovascular complications, including myocardial infarction and acute cerebrovascular events.

Keywords: LIF, leukemia inhibitory factor, cytokine circadian rhythms, essential hypertension

Author contribution: Radaeva OA designed the study, analyzed the results, formulated the conclusions and wrote the manuscript; Simbirtsev AS formulated the objective of the study, revised its conclusions and the manuscript itself; Gromova EV designed the study, carried out laboratory tests, and contributed to writing the manuscript; Iskandiarova MS analyzed the literature, supervised blood collection, followed up with the patients, contributed to writing the manuscript; Belyaeva SV analyzed the literature, supervised blood collection, followed up with the patients.

Compliance with ethical standards: the study was approved by the Ethics Committee of National Research Mordovia State University (Protocol No. 12 dated December 14, 2008). Written informed consent was obtained from all study participants. Blood samples were collected in compliance with the Declaration of Helsinki (2008), the protocol of European Convention on Human Rights and Biomedicine (1999) and the additional protocol to the Convention on Human Rights and Biomedicine concerning Biomedical Research (2005).

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Leukemia inhibitory factor (LIF) exerts a vast variety of physiological effects through specific LIF receptors located on the membranes of endothelial cells, monocytes, neurons and other cells [1] in physiologically relevant quantities [2]. Although LIF signaling pathways through JAK/STAT (Janus kinase/signal transducer and activator of transcription), MAPK (mitogen-activated protein kinases) and PI3K (phosphoinositide 3-kinases) are stable, the effects LIF induces in different cell types can be opposite, including both stimulation and inhibition of cell differentiation and survival. There is a lot of debate as to how LIF affects the arterial wall in patients with essential hypertension (EH) since the mechanism underlying STAT3 activation is redox-sensitive [3] and its directionality changes in the setting of chronically elevated blood pressure, distorting LIF effects. There is a growing body of evidence refuting the postulate about identical STAT3 signaling in cardiomyocytes and endothelial/smooth muscle cells, which is important in the situation of extended exposure to gp 130 ligands (LIF in particular) [4]. Research has demonstrated that factors implicated in EH progression and the risk of EH complications are dependent on time of day [5], proving the significance of investigating both the levels of cytokines involved and diurnal variations in their concentrations.

The aim of this study was to analyze the circadian rhythms of LIF concentrations in the peripheral blood serum measured at 5 different time points (8:00, 14:00, 20:00, 2:00, and 8:00 o’clock) in patients with stage II EH in the presence/absence of antihypertensive therapy and their relationship with the frequency of complications developing within a 5-year follow-up.

METHODS

In 2008 through 2019, a study called Cytokines in the pathogenesis of essential hypertension was carried out at the Institute of Medicine (National Research Mordovia State University) and the Regional Vascular Center (Republican Clinical Hospital № 4).

As part of the study, a group of 60 patients with stage II EH (30 men and 30 women) was formed to explore how LIF production changed over a 24-hour cycle. The following inclusion criteria were applied: individuals of stage II EH, born in 1955–1956, who had a 10- to 14-year history of the disease, were not receiving any antihypertensive therapy at the beginning of the study but were subsequently put on therapy (ACE inhibitors ± diuretics) to achieve a target blood pressure, as recommended by the Russian guidelines on the diagnosis and treatment of hypertension (2010) [6], which they did within a year that followed; total cholesterol < 5.0 mmol/L, LDL < 3.0 mmol/L, HDL > 1.0 mmol/L, triglycerides < 1.7 mmol/L, glucose < 5.5 mg/dl, BMI < 30 kg/m²; comparable risk of developing EH-related complications. Patients with hypertension-associated comorbidities, types 1 or 2 diabetes mellitus, autoimmune disorders, allergies, or symptomatic hypertension were excluded from the study. The control group consisted of 30 seemingly healthy individuals (15 men and 15 women) with systolic BP of 100 to 130 mmHg and diastolic BP of 70 to 89 mmHg; the groups were comparable in terms of age and blood biochemistry.

Blood samples (2 ml) were collected prior to the onset of antihypertensive therapy (2014) and one year after the start of treatment (2015) at 8:00, 14:00, 20:00, 2:00, and 8:00 o’clock (the fasting period was at least 6 hours). The time points were selected based on the results of our pilot study (blood samples had been collected from 7 individuals at 7:00, 8:00, 10:00, 12:00, 14:00, 16:00, 18:00, 20:00, 22:00, 00:00, 2:00, 4:00, 6:00, 7:00, and 8:00 o’clock). Time elapsed from sample collection to sample freezing was 60 min. Serum LIF concentrations were measured using ELISA kits (Bender MedSystems; USA).

Follow-up phone interviews were conducted annually (2014–2019) to obtain information about possible complications, such as myocardial infarction (MI), acute cerebrovascular events (ACVE) and transient ischemic attacks (TIA), which were subsequently confirmed by clinical and diagnostic tests, including ECG, echocardiography, troponin tests, brain CT scans.

The obtained data were processed in Statistica 10.0 (Stat Soft; USA). Normality of data distribution was analyzed using the one-sample Kolmogorov–Smirnov test. Based on the obtained results, we used the paired t-test to compare the results of pre-treatment blood tests taken at 8:00, 14:00, 20:00, 2:00, and 8:00 o’clock in the group of patients with stage II EH; the Wilcoxon test was applied to compare the data in the group of patients on antihypertensive therapy one year after its onset and also in healthy controls. Intergroup comparison was carried

Table 1. LIF concentrations (pg/ml) in the peripheral blood serum in patients with stage II EH at 8:00, 14:00, 20:00, 2:00, and 8:00 o’clock (next day) in the absence/presence of antihypertensive therapy in patients with or without cardiovascular complications developed in the 5-year follow-up period (M±(Q25%–Q75%))

<table>
<thead>
<tr>
<th>Groups</th>
<th>8.00 (day 1)</th>
<th>14.00 (day 1)</th>
<th>20.00 (day 1)</th>
<th>2.00 (day 2)</th>
<th>8.00 (day 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with EH (before therapy), n = 60</td>
<td>a</td>
<td>7.51(6.58–8.34)</td>
<td>7.58(6.47–8.41)</td>
<td>9.02(7.52–9.73)</td>
<td>10.1(9.44–11.8)</td>
</tr>
<tr>
<td>Healthy controls, n = 30</td>
<td>b</td>
<td>1.41(1.02–1.83)</td>
<td>1.38(1.04–1.79)</td>
<td>1.45(1.06–1.78)</td>
<td>1.42(1.03–1.81)</td>
</tr>
<tr>
<td>p(b–a) &lt; 0.001</td>
<td>p(b–a) &lt; 0.001</td>
<td>p(b–a) &lt; 0.001</td>
<td>p(b–a) &lt; 0.001</td>
<td>p(b–a) &lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>Patients with EH (one year in treatment), n = 60, of them:</td>
<td>c</td>
<td>7.54(6.57–8.38)</td>
<td>7.61(6.53–8.44)</td>
<td>8.95(7.63–9.58)</td>
<td>7.62(6.84–8.63)</td>
</tr>
<tr>
<td>Healthy controls = 30</td>
<td>d</td>
<td>1.46(1.08–1.89)</td>
<td>1.39(1.05–1.85)</td>
<td>1.46(1.09–1.84)</td>
<td>1.43(1.08–1.83)</td>
</tr>
<tr>
<td>p(c–a) &gt; 0.05</td>
<td>p(c–b) &gt; 0.05</td>
<td>p(c-d) &gt; 0.05</td>
<td>p(c–d) &gt; 0.05</td>
<td>p(c–d) &gt; 0.05</td>
<td>p(c–d) &gt; 0.05</td>
</tr>
<tr>
<td>p(c2–c1) &gt; 0.05</td>
<td>p(c2–c1) &gt; 0.05</td>
<td>p(c2–c1) &gt; 0.05</td>
<td>p(c2–c1) &gt; 0.05</td>
<td>p(c2–c1) &gt; 0.05</td>
<td></td>
</tr>
</tbody>
</table>

Note: significant for comparisons with the specified time of blood collection or the group (a, b, c, c1, c2); * — p < 0.001; † — p < 0.01; ‡ — p < 0.05. The paired t-test was applied for intragroup comparison of pretreatment results obtained at 8:00, 14:00, 20:00, and 8:00 o’clock. The Wilcoxon test was applied for intragroup comparison in the group of patients after one year of treatment and in the healthy controls. The Mann–Whitney U test was applied to compare independent samples. The Wilcoxon test was applied to compare dependent samples.
out using the Mann–Whitney U (for independent samples) and the Wilcoxon test (for dependent samples). Below, the data are presented as a median (Me) and percentiles (Q0.25–Q0.75). When comparing the subgroups, the Bonferroni correction for multiple comparisons was applied, ensuring the reliability of the statistical data. We calculated the absolute and relative risks of developing MI and ACVE, 95% CI, sensitivity and specificity. The analysis was aided by Fisher’s exact test ($\phi$) and Pearson’s correlation coefficient ($C'$) were used.

RESULTS

The analysis revealed significant qualitative and quantitative differences in the circadian rhythms of blood serum LIF between the control group and the patients with stage II EH and a 10–14-year history of the disease who were not on antihypertensive therapy at the beginning of the study. In patients with stage II EH, LIF levels measured at 8:00, 14:00, 20:00 and 2:00 o’clock were 5–7.5 times higher (p < 0.001) than in the healthy individuals (Table 1). In the group of patients with EH, a significant increase in LIF levels relative to 8:00 measurements (by 20.1% (16.7–24.3%); $\rho < 0.001$) was observed at 20:00, peaking at 2:00 (an increase by 34% (25.7–43%); $\rho < 0.001$). Importantly, in the group of healthy controls, LIF levels did not change at 14:00, 20:00 and 2:00 o’clock relative to their initial values at 8:00 ($\rho > 0.05$). After being on antihypertensive therapy for one year, the patients with stage II EH who had achieved their target blood pressure demonstrated no decline in LIF concentrations at 8:00, 14:00 and 20:00 o’clock in comparison with pretreatment values ($\rho < 0.01$), but the circadian rhythm of the cytokine was different. In the patients who had been receiving antihypertensive therapy and had achieved the desired blood pressure, serum blood serum LIF peaked at 20:00; measurements taken at 2:00 showed a decline in LIF concentrations (Table 2) in comparison with the pretreatment period. In the group of patients undergoing treatment, the distribution of data differed from Gauss–Laplace distribution, which prompted us to analyze LIF circadian rhythms for each individual patient in order to identify the criteria for heterogeneity. We found that 22 patients undergoing antihypertensive therapy who had achieved the target blood pressure had the same circadian rhythms of blood serum LIF as before therapy (a rise at 20:00 with a peak at 2:00 and a decline at 8:00; see Table 2).

The analysis of data obtained during the follow-up observation from the patients undergoing antihypertensive therapy who had partially recovered normal LIF dynamics (a decline in LIF concentrations at 2:00) revealed that only 4 of 42 patients had developed ACVE or MI within a 5-year follow-up period (the absolute risk of complications was 9.5% (0.63–18.4%)). In the group of patients with persisting pathological diurnal rhythms of serum LIF (a rise at 20:00, a peak at 2:00 and a return to morning levels at 8:00), 11 of 18 patients had developed complications (ACVE, MI); in this group, the absolute risk of complications was 61.1% (38.6–83.6%). The risk ratio between these two groups was 6.41 (2.35–17.5%); specificity, 0.84; sensitivity, 0.73; $\phi = 0.0000 (p < 0.05), C' = 0.67$ (the correlation was very strong).

DISCUSSION

The rise in serum LIF concentrations observed in the patients with stage II EH relative to the healthy controls can be explained by impaired integration of ILFR/CD118 and gp130 signaling under oxidative stress accompanying EH, which affects the catalytic activity of JAK [7] and can stimulate LIF secretion. The elevation of LIF levels at 20:00 o’clock, with a further rise peaking at 2:00 observed in the study participants prior to antihypertensive therapy and also in some patients who had reached the desired blood pressure and were still on antihypertensive drugs is pathogenically relevant: there are

<table>
<thead>
<tr>
<th>Groups</th>
<th>8.00 (day 1)</th>
<th>14.00 (day 1)</th>
<th>20.00 (day 1)</th>
<th>8.00 (day 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with EH (before therapy), n = 60</td>
<td>a</td>
<td>0.91 [-1.67–2.54]</td>
<td>20.1 [16.7–24.3] *14.00</td>
<td>34 [25.7–33] *14.00, 20.00</td>
</tr>
<tr>
<td>Healthy controls, n = 30</td>
<td>b</td>
<td>-1.08 [-2.03–1.19]</td>
<td>2.81 [1.93–3.22]</td>
<td>0.98 [-0.13–2.03]</td>
</tr>
<tr>
<td>Patients with EH (one year in treatment), n = 60, of them</td>
<td>c</td>
<td>0.93 [-0.61–2.03]</td>
<td>18.9 [15.9–24.1] *14.00</td>
<td>6.32 [2.08–14.4] *14.00, 20.00</td>
</tr>
<tr>
<td>5-year follow-up — MI, ACVE, n = 15</td>
<td>c1</td>
<td>-0.91 [-2.83–1.8]</td>
<td>19.9 [15.1–23.8] *14.00</td>
<td>26.5 [22.3–28.1] *14.00, 20.00</td>
</tr>
<tr>
<td>5-year follow-up — no complications, n = 45</td>
<td>c2</td>
<td>0.52 [-0.47–1.67]</td>
<td>18.1 [14.8–23.8] *14.00</td>
<td>2.29 [-0.35–5.44] *14.00, 20.00</td>
</tr>
</tbody>
</table>

Note: significant for comparisons with the specified time of blood collection or the group (a, b, c, c1, c2), ‘—’ $\rho < 0.001$; ‘–’ $\rho < 0.01$; ‘—’ $\rho < 0.05$. The paired t-test was applied for intragroup comparison of pretreatment results obtained at 8:00, 14:00, 20:00, 2:00, and 8:00 o’clock. The Wilcoxon test was applied for intragroup comparison in the group of patients after one year of treatment and in the healthy controls. The Mann–Whitney U test was applied to compare independent samples. The Wilcoxon test was applied to compare dependent samples.
reports that LIF-dependent stimulation of STAT3 in endothelial cells triggers the inflammatory cascade [8] and IL1 activation; in turn, this causes a more pronounced EH progression in the evening (20:00) and at night (2:00), when proinflammatory activity of IL1α and IL10 is low, through the activation of protein arginine methyltransferase and the inhibition of dimethylarginine, leading to an imbalance in the NO synthesis system. Previously [9], we reported an increased left ventricular mass index, a low mean fiber shortening fraction and a reliable association with pronounced concentric left ventricular hypertrophy in patients with EH and elevated LIF (>7.5 pg/ml). The observed pathophysiological process led us to hypothesize that patients whose LIF levels were growing between 20:00 and 2:00 in the setting of antihypertensive therapy were at increased risk for cardiovascular complications. The hypothesis was confirmed in the course of this study. LIF-induced cardiac hypertrophy can be characterized by an early reduction in myocardial contractility resulting from the transmural changes in cardiomyocytes [10, 11]. In the early stages of the pathology, elevated LIF serves as a mechanism of compensatory adaptation that stimulates contractility of cardiomyocytes by increasing the activity of T-type Ca²⁺-channels [12]. Besides, elevated LIF could be potentially protective against the inflammation-induced loss of axons and also promotes survival of oligodendrocytes by stimulating the expression of IGF-1 [insulin-like growth factor 1] [13]. However, the further rise in LIF levels and its circadian fluctuations reported in this study promote poor outcomes in patients with stage II EH, including potential damage to the myocardium or the brain.

CONCLUSIONS

The identified patterns of circadian rhythms of blood serum LIF in patients with stage II EH, namely the rise by 15% at 20:00 and the further rise by 22% peaking at 2:00, relative to LIF levels at 8:00, can be regarded as pathologic. Their persistence in the setting of antihypertensive therapy could contribute to the progression of hypertension and put the patient at increased risk for cardiovascular complications, in spite of seemingly clinically favorable course of the disease and the success in achieving the target blood pressure. Our findings might lay the groundwork for further research into the role of LIF aimed at establishing a personalized approach to interpreting its dynamics in individual patients. The analysis of LIF circadian rhythms is a candidate diagnostic approach for the assessment of occult progression of the disease in patients with essential hypertension who have managed to achieve their target blood pressure.

References


PREVENTIVE PHARMACOTHERAPY OF TYPE 2 DIABETES MELLITUS IN PATIENTS WITH EARLY CARBOHYDRATE METABOLISM DISORDER: LONG-TERM EFFICACY AND CLINICAL OUTCOMES

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2 Pirogov Russian National Research Medical University, Moscow, Russia

Prevention of type 2 diabetes mellitus (T2DM) in prediabetic patients is a pressing concern due to its increasing prevalence. The aim of this study was to evaluate the efficacy of preventive pharmacotherapy in delaying progression of incident impaired glucose tolerance (IGT) and impaired fasting glycemia (IFG) to T2DM. The participants of the study (1,136 subjects) found healthy by a regular annual checkup underwent repeat screening for T2DM. Blood samples were processed following the guidelines for good preanalytical sample preparation. Patients with incident IGT/IFG were prescribed medication therapy with metformin or/and acarbose. The rate of IGT/IFG conversion to T2DM was evaluated in years 3 and 10 of observation. Carbohydrate metabolism disorders were detected in 18.5% (n = 210) of the re-screened patients: 5.0% had T2DM, 5.5% had IGT, 8.0% had IFG. Patients with incident T2DM were prescribed blood sugar lowering therapy and they were excluded from further analysis. Patients with IGT/IFG (n = 151) were given recommendations on lifestyle modification and prescribed metformin (77%) or a combination of metformin and acarbose (23%). Three years after the start of observation, the rate of conversion to T2DM was 6.8% in patients undergoing monotherapy with metformin and 11.4% in patients undergoing combination therapy with metformin and acarbose. After the active follow-up phase was over, the majority of the patients (n = 85) decided to discontinue preventive therapy without consulting their physicians. Ten years after the active follow-up phase, the rate of NGT/IFG conversion to T2DM was 38.8% in patients who had discontinued their treatment and 0% in patients still taking metformin (p < 0.01). Long-term therapy with metformin prevented progression to T2DM in the long run in 83.3% (p < 0.05).

Keywords: type 2 diabetes mellitus, impaired fasting glucose, impaired glucose tolerance, screening, metformin, acarbose, prevention, fasting plasma glucose

Author contribution: Boeva VV planned the study; analyzed the literature; collected, analyzed and interpreted study results; wrote the manuscript; Zavyalov AN analyzed the literature; analyzed and interpreted study results; wrote the manuscript.

Compliance with ethical standards: the study was approved by the Ethics Committee of Pirogov Russian National Research Medical University (Protocol № 176 dated June 25, 2018), informed consent was obtained from all study participants.

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МЕДИКАМЕНТОЗНАЯ ПРОФИЛАКТИКА САХАРНОГО ДИАБЕТА 2-ГОТИПА У ПАЦИЕНТОВ С РАННIMИ НАРУШЕНИЯМИ УГЛЕВОДНОГО ОБМЕНА: ЭФФЕКТИВНОСТЬ И КЛИНИЧЕСКИЕ ИСХОДЫ ПРИ ДЛИТЕЛЬНОМ НАБЛЮДЕНИИ

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Актуальность профилактики сахарного диабета (СД) 2-го типа у пациентов с предиабетом увеличивается из-за неуклонного распространения заболевания. Целью работы было оценить эффективность медикаментозной профилактики в замедлении темпов конверсии впервые выявленных нарушенной толерантности к глюкозе и нарушенной гликемии натощак (НТГ/НГН) в СД 2-го типа. Участниками исследования (1136 человек), счищающимся здоровыми после диспансеризации, повторно провели скрининг СД 2-го типа с соблюдением правил преаналитической подготовки образцов крови. Пациенты с впервые выявленными НТГ/НГН были назначены терапия митформином или акарбозой, частоту конверсии НТГ/НГН в СД 2-го типа оценивали через 3 и 10 лет наблюдения. У 18,5% (n = 210) обследованных выявили различные категории нарушения углеводного обмена: СД 2-го типа — у 5,0%, НТГ — у 5,5%, НГН — у 8,0%. Пациентам с впервые выявленным СД 2-го типа была назначена сахароснижающая терапия, они были исключены из последующего наблюдения. Пациентам с НТГ/НГН (n = 151) рекомендовали изменение образа жизни и назначили терапию митформином (77%) или комбинацией митформина и акарбозы (23%). Частота конверсии СД 2-го типа в течение 3 лет активного наблюдения составила 6,8% на фоне монотерапии митформином и 11,7% — на фоне комбинированной терапии митформином и акарбозой. По окончании периода активного наблюдения большинство пациентов (n = 85) самостоятельно прекратили терапию. Частота конверсии НТГ/НГН в СД 2-го типа через 10 лет после окончания активного наблюдения в группе без медикаментозной профилактики составила 38,8% и 0% — в группе принимающих митформин (p < 0,01). Показано, что длительное применение митформина предупреждает развитие СД 2-го типа в отдаленном периоде у 83,3% (p < 0,05).

Ключевые слова: сахарный диабет 2-го типа, нарушение гликемии натощак, нарушенная толерантность к глюкозе, скрининг, метформин, акарбоза, профилактика, глюкоза венозной плазмы натощак

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Type 2 diabetes mellitus (T2DM) imposes a huge burden on society. This disease has a high, steadily rising prevalence and increases the risk of disabilities and early death in the affected individuals. It was reported that 4.1 million people in Russia were living with T2DM in 2018 [1, 2]. But according to epidemiologic surveillance, the actual number of such patients could be as high as at least 8 million. Based on extrapolation from the NATION study data, it is estimated that about 20.7 million Russians with prediabetes are undiagnosed [3] and, therefore, do not receive therapy or counseling on lifestyle modification.

Currently, it is not mandatory for healthcare providers to report patients who test positive for impaired glucose tolerance and impaired fasting glycemia (IGT/IFG); such patients are overlooked by statistical reports and are not followed up, so the actual prevalence of prediabetes remains understudied.

The primary cause of delay in the diagnosis of T2DM and detection of carbohydrate metabolism disorders in their early stages is preanalytical errors, specifically failure to comply with standard procedures for blood sample collection and handling aimed at inhibiting glycolysis in the sample. Upon sample collection, blood cells in the test tube undergo glycolysis, which causes glycol levels to decline and thus skews the result of the test. This is the reason why screening tests reveal normal glycolc levels in some patients with carbohydrate metabolism disorders (18.5% in our study); as a result, such patients are not followed up by their physicians.

Primary care physicians do not always attach due importance to IGT/IFG. They give their patients some perfunctory advice on lifestyle modification and do not prescribe any preventive pharmacotherapy; in turn, the patients do not find it necessary to follow the recommendations. Importantly, the efficacy of medication therapy in prediabetic individuals has already been confirmed by multiple studies [4–7] and meta-analyses [8].

Today, there is a need for implementing effective strategies for active case-finding of early carbohydrate metabolism disorders and their treatment. The aim of this study was to assess the long-term efficacy of preventive pharmacotherapy in delaying conversion of incident IGT/IFG to T2DM in the real clinical setting.

**METHODS**

**Study participants**

The study enrolled 1,136 adult residents of Tambov region presenting at Tambov Central Regional Hospital for an annual medical checkup under the annual health screening program in 2007. All patients were found to be healthy. Inclusion criteria: no history of carbohydrate metabolism disorders; no history of glucose-lowering therapy. Patients who screened positive for types 1 or 2 DM were excluded from the study.

**Study phases**

In the first phase, individuals found healthy after the annual medical checkup were screened for carbohydrate metabolism disorders; preventive pharmacotherapy was prescribed to those at risk for T2DM; the rate of IGT/IFG conversion to T2DM or normoglycemia within 3 years of follow-up was evaluated.

In the second phase, long-term outcomes were analyzed, i.e. the rate of IGT/IFG conversion to T2DM or normoglycemia within 10 years after active follow-up (Fig. 1).

The first phase is essentially a nonrandomized continuous prospective interventional study; the second phase should be regarded as a non-randomized retrospective observational controlled study.

**Study duration**

The active follow-up phase, which included preventive pharmacotherapy for T2DM, lasted for 3 years. Some patients continued their therapy for as long as 13 years. Its long-term efficacy was evaluated 10 years after the active follow-up phase was over.

**Description of medical intervention**

Disorders of carbohydrate metabolism were identified based on the screening data, including the results of the oral glucose tolerance test (OGTT). Patients with IGT/IFG received

**Fig. 1. A study plan. FPG — fasting plasma glucose test; OGTT — 75-gram oral glucose tolerance test**
counseling on lifestyle modification and were prescribed medication therapy. Ten years after the active follow-up phase, the patients we were able to contact were re-examined ($n = 115$). The primary focus was on the rates of IGT/IFG conversion to T2DM or normoglycemia during the active follow-up phase in the setting of preventive pharmacotherapy (3 years) and in the 10 years that had followed.

Analysis of subgroups

In the screening stage, the participants were stratified in groups by the presence of the metabolic syndrome and risk factors for T2DM.

In 10 years, the outcomes for metabolic syndrome were stratified by adherence to long-term T2DM prevention therapy. The patients available for the analysis ($n = 115$) were divided into 2 subgroups: subgroup 1 had discontinued their medications without consulting their physicians ($n = 85$), subgroup 2 had been taking metformin throughout the entire observation period ($n = 30$).

Evaluation of outcomes

Disorders of carbohydrate metabolism were diagnosed and classified following WHO guidelines published in 1999. Carbohydrate metabolism was evaluated using a conventional 2-hour 75-gram OGTT. For all samples, glucose measurements were conducted at the laboratory of Tambov Central Regional Hospital. Blood samples were collected into test tubes containing sodium fluoride. Sample preparation was performed following a standardized analytical technique.

Statistical analysis

The obtained data were processed in Statistica 6.1 (TIBCO; USA). For the analysis, we used nonparametric statistics, Pearson's $\chi^2$, Fisher's exact test, and Yates' correction for contingency tables. Differences were considered significant at $p < 0.05$.

RESULTS

Initial screening was conducted in 1,136 individuals who denied any health complaints. The fasting plasma glucose test (FPG) revealed plasma glucose levels $\geq 7.0$ mmol/l in 73 (6.4%) individuals. The test was repeated in those patients, confirming T2DM in 37 cases. They were prescribed medication therapy and excluded from further observation. The remaining 36 patients had glycemia ranging from 6.1 to 6.9 mmol/l. Those 36 patients and other 181 subjects of 1,136 study participants who tested positive for blood glucose in the same range underwent OGTT.

In 882 of 1,136 (77.6%) subjects, FPG was below 6.1 mmol/l, which WHO interprets as normal and does not recommend testing for DM in the short run. However, according to IDF guidelines (2005), OGTT should be performed on all patients with the metabolic syndrome (MS) whose fasting blood sugar levels are $\geq 5.6$ mmol/l. Of all examined study participants, 430 had FPG within the normal range (5.6–6.0 mmol/l). Of them, MS signs were detected in 169 (39.3%) individuals, who subsequently underwent OGTT, as recommended by IDF. The rest 261 subjects had not developed a full clinical picture of MS, but 108 still had risk factors for T2DM and underwent OGTT (Fig. 2). In total, OGTT was performed on 494 people.

OGTT revealed that 20 participants (4.0%) had T2DM, 62 participants (12.6%) had impaired glucose tolerance, 91 participants (18.4%) had impaired fasting glucose, and 321 participants (65.0%) had normal glucose tolerance.

Characteristics of the patients included in the study were previously published in [9]. Of 153 prediabetic patients, 2 (1.3%) had contraindications for metformin and 26 (16.9%) refused to take acarbose because of its high cost. Thus, in the first year of active follow-up, there

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**Fig. 2.** Stratification of patients based on screening data for further T2DM diagnostic tests. FPG — fasting plasma glucose test; MS — metabolic syndrome; OGTT — 75-gram oral glucose tolerance test; RF — risk factors for T2DM
were 90 patients with IFG undergoing therapy with metformin (500 mg/day), 26 patients with IGT undergoing therapy with metformin (500 mg/day), 35 patients with IGT undergoing combination therapy with metformin (500 mg/day) and acarbose (titrated from 50 mg to a maximum dose of 150 mg following the titration scheme provided by the manufacturer). OGTT was repeated in prediabetic patients and those with normal glucose tolerance in years 2 and 3 of active follow-up. In year 3, T2DM was detected by OGTT in 4 subjects (1.4%) with previously normal glucose tolerance; in 1 patient (1.1%) with IFG undergoing therapy with metformin; in 2 patients (8.7%) with IGT undergoing treatment with metformin; in 2 patients (6.1%) with IGT undergoing treatment with metformin and acarbose. By the end of year 3, there were a total of 156 people with IGT/IFG (Fig. 3).

When the active follow-up phase, which included visits to the endocrinologist, was over, the majority of the patients discontinued their medications, in spite of having been recommended not to.

Of 156 patients with IGT/IFG, the analysis of long-term (10 years) outcomes of preventive pharmacotherapy for T2DM was done in 115 individuals. Causes for not including some patients in the analysis are shown in Fig. 4.

Of 115 people available for the analysis, 30 (26.1%) were still taking 500 mg/day metformin at the time of data collection for our study (2018). Eighty-five patients had chosen to terminate their treatment; of them 74 did it almost immediately after 3 years of active follow-up. Distribution of patients by type and duration of preventive pharmacotherapy after the end of the active follow-up phase is shown in Fig. 5.

Fig. 3. Stratification of patients by the initial state of carbohydrate metabolism and prescribed therapy. M+A – therapy with metformin and acarbose

Causes of poor adherence to treatment were not analyzed in detail in our study, but it should be noted that the patients reported not only health-related factors affecting their adherence but also organizational and financial issues. For example, the most common cause of non-compliance was the fact that the patients had not been followed up by their local endocrinologists and as a result had been refused prescriptions for preventive medications at their local healthcare facilities.

Results of preventive pharmacotherapy

During the active observation phase, 12 (7.9%) of 151 prediabetic patients progressed from a carbohydrate metabolism disorder to T2DM.

In the setting of preventive therapy, OGTT was repeated in years 2 and 3 of active follow-up. In the IFG group undergoing treatment with metformin, significant positive outcomes were achieved by the patients with initial FPG of 5.6–6.0 mmol/l who were able to normalize their carbohydrate metabolism in 47.8% cases in year 2 and in 72.9% of cases in year 3 of observation ($\chi^2 = 6.195; p = 0.013$). Outcomes of preventive pharmacotherapy for T2DM in the IFG group achieved during the active follow-up phase are detailed in [9].

In the IGT group, the patients undergoing combination therapy with metformin and acarbose demonstrated better results than the group undergoing treatment with metformin: normal glucose tolerance was observed in the majority of patients in combination therapy in year 3 of active follow-up ($\chi^2 = 7.222; p = 0.007$). Of 115 individuals available for the analysis 10 years later, 30 (26.1%) were still taking metformin

Fig. 4. Stratification of patients with IGT/IFG 10 years after the end of the active follow-up phase
The study had some limitations related to its design. The study was observational and the use of only routine medical interventions did not allow us to form positive and negative control groups. The patients had been undergoing diagnostic procedures and receiving medical care at their local healthcare facilities and not under the supervision of the study authors, meaning that we were unable to fully rule out the impact of external factors on the study outcomes.

Adverse effects

No unpredicted adverse effects of preventive pharmacotherapy against T2DM were observed during the study. The analysis of other adverse events was beyond the scope of this study and was not implied by the study design.

Limitations of the study

The study had some limitations related to its design. The study was observational and the use of only routine medical interventions did not allow us to form positive and negative control groups. The patients had been undergoing diagnostic procedures and receiving medical care at their local healthcare facilities and not under the supervision of the study authors, meaning that we were unable to fully rule out the impact of external factors on the study outcomes.

DISCUSSION

The issue of adherence to preventive pharmacotherapy is closely linked to screening issues. The lack of motivation for early detection of carbohydrate metabolism disorders on part of healthcare providers leads to the lack of awareness on part of patients and results in noncompliance with prescribed medication therapy.

According to ADA standards of medical care (2019) [10, 11], the use of metformin, α-glucosidase inhibitors, orlistat, GLP-1 agonists, and thiazolidinediones helps to reduce the incidence of T2DM, but so far, prediabetes is not an approved indication for any of these drugs. The protocol of the SiMePred study intended to evaluate the efficacy of sitagliptin (a DPP-4 inhibitor) in preventing T2DM has already been published but the results of the study are not available yet [12]. ADA experts believe that metformin should be a preferred drug for secondary prevention of T2DM. For other drugs listed above, the risks and benefits should be thoroughly considered in each individual case [10]. However, IDF guidelines formulated in 2019 recommend both metformin and acarbose (an α-glucosidase inhibitor) for T2DM prevention in patients with early stages of carbohydrate metabolism disorders [13]. In Russia, information leaflets for metformin and acarbose list prediabetes as an indication for use [14, 15].

In 2005, the Diabetes mellitus state-funded program was launched in the Russian Federation under the nationwide Health project [16]. The key parameters it focused on were: life expectancy of patients with diabetes mellitus and the rate of complications in these patients, i.e. tertiary prevention. The existing electronic state registry of diabetes mellitus can also be regarded as a tool for tertiary prevention, but there is still a dearth of data on its long-term efficacy.

In our opinion, regular medical checkups and screening tests are the key measure for primary T2DM prevention [17]. Regular checkups help to identify risk factors for T2DM and subsequently take measures to reduce the contribution of modifiable risk factors to the development of the disease. Screening should be used both for regular checkups and in patients with identified risk factors. In screening tests, preanalytical errors are not rare, leading to the underdiagnosis of carbohydrate metabolism disorders. According to WHO report (2006), the whole blood sample collected from a patient...
Table 1. Long-term outcomes in patients who had and had not been receiving preventive pharmacotherapy

<table>
<thead>
<tr>
<th>Carbohydrate metabolism</th>
<th>Number of re-examined patients</th>
<th>Preventive pharmacotherapy n = 30</th>
<th>No preventive pharmacotherapy n = 85</th>
<th>χ²</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>abs.</td>
<td>%</td>
<td>abs.</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>25</td>
<td>83.3</td>
<td>22</td>
<td>25.8</td>
<td>30.28</td>
</tr>
<tr>
<td>IFG</td>
<td>1</td>
<td>3.3</td>
<td>13</td>
<td>15.2</td>
<td>3.0</td>
</tr>
<tr>
<td>IGT</td>
<td>4</td>
<td>13.3</td>
<td>17</td>
<td>20.0</td>
<td>0.66</td>
</tr>
<tr>
<td>T2DM</td>
<td>0</td>
<td>0</td>
<td>33</td>
<td>38.8</td>
<td>16.3</td>
</tr>
</tbody>
</table>

Note: SI — statistically insignificant.

Table 2. Long-term results of OGTT in patients with different disorders of carbohydrate metabolism undergoing long-term preventive pharmacotherapy

<table>
<thead>
<tr>
<th>OGGT results in 10 years</th>
<th>Initial condition of patients in the long-term pharmacotherapy group (n = 30)</th>
<th>χ²</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IFG</td>
<td>IGT</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>21</td>
<td>4</td>
<td>0.250 &gt; 0.05</td>
</tr>
<tr>
<td>IFG</td>
<td>1</td>
<td>0</td>
<td>1 &gt; 0.05</td>
</tr>
<tr>
<td>IGT</td>
<td>2</td>
<td>2</td>
<td>0.169 &gt; 0.05</td>
</tr>
<tr>
<td>T2DM</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Long-term results of OGTT in patients with different disorders of carbohydrate metabolism who did not have long-term preventive pharmacotherapy

<table>
<thead>
<tr>
<th>OGGT results in 10 years</th>
<th>Initial condition of patients in the group without preventive pharmacotherapy (n = 85)</th>
<th>χ² (with Yates’ correction)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IFG</td>
<td>IGT</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>17 (20.0%)</td>
<td>5 (5.9%)</td>
<td>6.4</td>
</tr>
<tr>
<td>IFG</td>
<td>9 (10.5%)</td>
<td>4 (4.8%)</td>
<td>1.14</td>
</tr>
<tr>
<td>IGT</td>
<td>8 (9.4%)</td>
<td>9 (10.5%)</td>
<td>0.027</td>
</tr>
<tr>
<td>T2DM</td>
<td>10 (11.8%)</td>
<td>23 (27.1%)</td>
<td>8.6</td>
</tr>
</tbody>
</table>

should be placed in a blood collection tube containing a glycolysis inhibitor (collection tubes with gray caps) if immediate plasma separation is not possible. The collection tube should be kept on ice until plasma separation or the test itself. The cap color has been approved by the International Organization for Standardization (2000) [18].

Secondary prevention of T2DM includes early detection of the disease, as well as measures for slowing conversion of the initial pathology to T2DM. Secondary prevention is closely related to primary prevention: for patients with one or more risk factors for T2DM who have been covered by primary prevention, secondary prevention should consist in screening tests. Currently, there is no state-funded program on secondary prevention of T2DM (progression of prediabetes to diabetes) in Russia. Secondary prevention with pharmacotherapy has demonstrated its long-term efficacy both in research work and in international studies [19, 20] and could be a cost-effective way of reducing the burden of early stage carbohydrate metabolism disorders and T2DM.

CONCLUSIONS

1. Currently, healthcare providers are not required to report patients with early disorders of carbohydrate metabolism; these patients are not followed up, which results in the underestimating the danger of IGT/IFG, delayed diagnosis of type 2 DM and poor adherence to treatment. 2. We have demonstrated that metformin can significantly delay progression to type 2 DM in the actual clinical setting. 3. Recommendation on active case-finding of IGT/IFG and early start of preventive pharmacotherapy should be included in the diagnostic algorithms and healthcare standards used in clinical routine. 4. In the absence of counterindications, all patients with early disorders of carbohydrate metabolism should be prescribed long-term medication therapy with metformin.

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