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# Glycine influence on cerebral blood flow parameters in practically healthy individuals evaluated with transcranial Doppler sonography

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An insufficient perfusion of the brain tissue can cause a decrease in cognitive functions, and long-term ischemia also leads to emotional and motor disorders. At the same time, check-up of the state of the cerebral blood flow is an important aspect of monitoring the progression of many pathological conditions. The amino acid glycine has been widely used in neurological practice for over 30 years, which helps improve hemodynamic characteristics and metabolic processes in the brain tissue.

**The aim** of the work was to analyze the effect of a sublingual administration of glycine on the cerebral blood flow velocity in practically healthy subjects using transcranial Doppler (TCD) sonography.

**Material and methods.** The pilot randomized controlled study included 20 healthy subjects aged 25 to 65 years, equally divided into 2 groups, one of which took glycine sublingually at a dose of 300 mg/day for 30 days, and the second group was a control group and did not receive the drug. In the first group, a load testing was carried out with 1000 mg of glycine, and in the control group – with 1000 mg of placebo. All the subjects underwent an assessment of the blood flow in the extracranial and intracranial vessels using standard protocols of TCD.

**Results.** In Group I, after a month of glycine intake, the peak systolic (by 11.9 cm/s) and average maximum (by 6.3 cm/s) velocities in the left middle cerebral artery (MCA) increased significantly ($p$ <0.01), while in the right MCA there was an increase in the peak systolic (by 9.3 cm/s), and diastolic (by 2.8 cm/s) and average maximum (by 5.8 cm/s) velocities. In turn, in the control group, there was no significant increase in velocity. During the load testing with glycine / placebo, the relative increase in the peak systolic velocity in the MCA in the main group was 7.6% [1.2; 10.9], in control group was 1.5% [-3.6; 5.5] ($p$=0.03).

**Conclusion.** Glycine intake for 30 days contributes to a reliable improvement in cerebral hemodynamics in healthy individuals, such as an increase in the linear blood flow velocity in the MCA. At the same time, a single dose of 1000 mg of glycine leads to an increase in the peak systolic and average maximum intracranial blood flow velocities up to 10%.

**Keywords:** transcranial Doppler sonography; blood flow velocity; middle cerebral artery; glycine

**Abbreviations:** TCD sonography – transcranial Doppler sonography; PS – peak systolic blood flow rate; ED – end diastolic blood flow rate; TAMAX – Time Averaged Maximum Velocity; PI – pulsatility index; RI – peripheral resistance index; CCA – common carotid artery; ECA – external carotid artery; ICA – internal carotid artery; VA – vertebral artery; MCA – middle cerebral artery; ACA – anterior cerebral artery; PCA – posterior cerebral artery.

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# Оценка влияния приёма глицина практически здоровыми лицами на параметры мозгового кровотока по данным транскраниальной допплерографии

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Недостаточность перфузии мозговой ткани может вызывать снижение когнитивных функций, а длительно сохраняющаяся ишемия приводит также к эмоциональным и двигательным нарушениям. При этом контроль состояния церебрального кровотока является важным аспектом мониторинга прогрессирования многих патологических состояний. В неврологической практике уже более 30 лет широко применяется такая аминокислота, как глицин, которая способствует улучшению гемодинамических характеристик и метаболических процессов в тканях мозга.

**Цель.** Анализ влияния сублингвального приёма глицина на скоростные показатели мозгового кровотока у практически здоровых испытуемых с помощью транскраниальной допплерографии (ТКДГ).

**Материал и методы.** В пилотное рандомизированное контролируемое исследование было включено 20 здоровых испытуемых в возрасте от 25 до 65 лет, разделённых на 2 группы ($n$=10 для каждой группы). Группа I в течение 30 дней принимала препарат глицин сублингвально в дозе 300 мг/сутки, а группа II была контрольной (препарат не получала). Также в первой группе нагрузочная проба проводилась с 1000 мг глицина, а в контрольной группе – с 1000 мг плацебо. Всем испытуемым проводили оценку показателей кровотока в экстракраниальных и интракраниальных сосудах по данным ТКДГ с использованием стандартных протоколов.

**Результаты.** В группе I через месяц приёма глицина в левой средней мозговой артерии (СМА) значимо ($p$ <0,01) увеличились пиковая систолическая (на 11,9 см/с) и средняя максимальная (на 6,3 см/с) скорость, а в правой СМА наблюдалось увеличение пиковой систолической (на 9,3 см/с), конечной диастолической (на 2,8 см/с) и средней максимальной (на 5,8 см/с) скоростей. В свою очередь, в контрольной группе значимого прироста скорости не произошло. При нагрузочной пробе с глицином / плацебо относительный прирост пиковой систолической скорости в СМА в основной группе составил 7,6% [1,2; 10,9], в контрольной группе – 1,5% [-3,6; 5,5] ($p$=0,03).

**Заключение.** Приём глицина в течение 30 дней способствовал достоверному улучшению церебральной гемодинамики у здоровых лиц, которое выражалось в увеличении линейной скорости кровотока по СМА. При этом однократный приём 1000 мг глицина приводил к росту пиковой систолической и средней максимальной скоростей интракраниального кровотока до 10%.

**Ключевые слова:** транскраниальная допплерография; скорость кровотока; средняя мозговая артерия; глицин

**Список сокращений:** ТКДГ – транскраниальная допплерография; PS – пиковая систолическая скорость кровотока; ED – конечная диастолическая скорость кровотока; TAMAX – средняя по времени максимальная скорость кровотока; ОСА – общая сонная артерия; НСА – наружная сонная артерия; ВСА – внутренняя сонная артерия; ПА – позвоночная артерия; СМА – средняя мозговая артерия; ПМА – передняя мозговая артерия; ЗМА – задняя мозговая артерия.

## INTRODUCTION

The brain consumes large amounts of energy during its functioning. To maintain these high metabolic demands, a significant volume of blood is required. A cerebral blood flow, which provides oxygen and nutrients to the brain tissues, as well as a removal of metabolic products, accounts for up to 20% of the total cardiac output [1]. A blood supply to the brain is carried out by large arteries located on its surface, which form an extensive multiply branching network. Even a slight deterioration of the cerebral blood circulation leads to cognitive disorders, and a

significant impairment is one of the main causes of mortality [2].

A reduced cerebral perfusion can occur both due to microangiopathies and in case of lesions of larger caliber arteries, for example, when the elastic properties of the vascular wall deteriorate [3]. A significant factor is also an insufficiently flexible change of the blood supply to different brain regions in response to the changes in their energy requirements [4]. In addition, an impaired effective autoregulation, which ensures the constancy of the cerebral blood flow even with significant fluctuations in the systemic pressure, is often observed in patients with an arterial hypertension and atherosclerosis [5]. This fact dramatically increases the risk of a cognitive impairment progression [6].

Thus, monitoring a cerebral blood flow is an essential factor in the study of the progression dynamics of the cerebral tissue perfusion insufficiency, as well as an important component of screening in patients with risk factors without established diagnoses. PET-CT (positron emission tomography and computed tomography) is one of the ways of a direct assessment of a cerebral blood flow, as well as the “gold standard” in the study of a cerebral vascular reactivity. However, there are other methods such as near-infrared spectroscopy (NIR spectroscopy), single-photon emission computed tomography, functional magnetic resonance imaging (fMRI), and transcranial Doppler (TCD) sonography [7]. TCD sonography is a rather reliable, inexpensive, widespread noninvasive technique for assessing hemodynamic parameters of intracranial vessels, in particular, blood flow velocity indices [8, 9]. The indices calculated on the basis of the obtained values (a pulsation index and a peripheral resistance index) also provide an indirect assessment of a perfusion in the studied vascular basin [10].

In the neurological practice, neurometabolic drugs, such as ginkgo biloba, choline alphoscerate, glycine, vinpocetine, and citicoline, are widely used to reduce the activity of pathological processes in the nervous tissue arising during ischemia [4, 11]. An important aspect of therapy is to slow down the progression of a cognitive decline in various types of cerebral vascular lesions, since many drugs do not have such a vasodilating effect, but to a greater extent, affect the components of the vascular tone regulation, thus improving both hemodynamic characteristics and metabolic processes in the brain tissue [6].

Amino acid glycine has long been used to correct disorders of the autonomic nervous system, as well as in the cognitive decline. Its pharmacological properties are due to its participation in a variety of metabolic processes and a direct neurotransmitter action [12, 13]. The therapeutic effect of glycine on the clinical course of an acute ischemic stroke especially when administered early, has been shown [14]. The administration of 1000 mg of the drug in the first few days contributed to the regression of a neurologic deficit in 68.9% of cases, which significantly exceeded the similar indicator in the placebo group (31.5%) [15]. The addition of glycine to the basal therapy of newborn infants with perinatal hypoxic CNS lesions led to the normalization of neuropsychiatric development rates, as well as an improvement of the neurological status and behavioral characteristics [16]. In animal studies, the vasodilating effect of a glycine solution, expressed as a 50–80% increase in the diameter of arterioles when directly applied to the pial membranes, occurred within a few minutes [17]. It was also experimentally shown that a sublingual administration of 200 mg of glycine promotes a 1.5-fold increase in the concentration of glucose in the nervous tissue compared to the initial data, thus increasing the efficiency of its functioning [18]. In addition, the efficacy of a course glycine administration in dyscirculatory encephalopathy has been proved; both the improvement of microcirculatory processes, the improvement of the cognitive component, and a reduction of anxiety and emotional lability were observed [19].

However, until recently, no direct effect of the drug administration on cerebral vessels has been shown. In this regard, the dilating effect of glycine on human cerebral vessels has been investigated in this work. To study this phenomenon, the effect of the sublingual glycine administration on hemodynamic parameters of the cerebral blood flow in practically healthy people was analyzed using TCD sonography.

**THE AIM** of the work was to analyze the effect of a sublingual administration of glycine on the cerebral blood flow velocity in practically healthy subjects using TCD sonography.

## MATERIALS AND METHODS

### Study design

This pilot randomized controlled trial included 20 practically healthy subjects aged 25 to 65 years. The study was conducted between August and November 2022 at the Institute of Cytochemistry and Molecular

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Pharmacology (Moscow) in accordance with the Declaration of Helsinki and was approved by the Local Ethical Committee (Protocol No. 3 dated 04 July 2022). All participants signed an informed consent for the participation prior to the inclusion in the study.

### Eligibility criteria

The *inclusion criteria* were: men and women aged 25 to 65 years who had the possibility of the blood flow visualizing through the left and right middle cerebral artery (MCA) through the transtemporal window was confirmed at the entrance ultrasound. The inclusion *criteria* were as follows: presence of chronic diseases of cardiovascular system and any other diseases in the exacerbation stage; reduction of cerebral blood flow through the main vessels by more than 20% of the age norm established at the entrance examination; previously established hypersensitivity to glycine; taking glycine and other nootropic drugs within a month before the study; pregnancy, breastfeeding period; patient's refusal to participate in the study. *Exclusion criteria*: none of the patients was excluded from the study.

### Description of medical intervention

Initially, all the patients included in the study underwent a clinical examination, which comprised an assessment of the anamnestic data: a general condition of the subject, heredity, past diseases, the presence of chronic diseases; and an entrance ultrasound of the main head vessels at extra- and intracranial levels on a Mindray DC-80 device using a linear transducer L12-3E (3.0–13.5 MHz), a convex transducer C5-1E (1.3–6.0 MHz), a sectorial transducer Sp5-1E (1.0–5.0 MHz): an assessment of blood flow parameters of the carotid and vertebrobasilar insufficiency: a common carotid artery (CCA), an external carotid artery (ECA), an internal carotid artery (ICA) and a vertebral artery (VA), a middle cerebral artery (MCA), an anterior cerebral artery (ACA) and a posterior cerebral artery (PCA).

Then the patients were randomized by random number generation into two groups of 10 people each. The first group (Group I, *n*=10) took glycine, 100 mg sublingual tablets, for 30 days, 1 tablet 3 times daily. In Group I, the loading testing was performed with 1000 mg of glycine (10 tablets of 100 mg). In the control group (Group II, *n*=10), the loading test was performed with 1000 mg placebo (10 tablets containing 100 mg lactose and 0.1 mg sucralose to mimic the sweet taste of glycine); the subjects had not taken the study drug until the blood flow was reassessed. The duration of the follow-up in both groups was 30 days.

Each subject underwent 4 transcranial blood flow measurements in the MCA, ACA, and PCA at 5-minute intervals to level out individual baseline variability. The study determined the linear peak systolic (PS), end-diastolic (ED), and time-averaged maximum blood flow velocity (Time Averaged Maximum Velocity — TAMAX), a pulsatility index (PI), a peripheral resistance index (RI), and a systolic / diastolic ratio (S/D) in the MCA, ACA, and PCA in each of the patients. The flow correction angle corresponded to the direction of the vessel and was maintained in subsequent measurements.

Then the patient sublingually took 1000 mg of glycine (Group I) or 1000 mg of placebo (Group II), after which the values in the indicated arteries were taken after 5, 10, and 15 min (Fig. 1). This dose is safe and recommended[1] for a single administration in acute cerebral circulatory disorders (including those suspected to occur). Thus, the possible dilative effect of glycine on cerebral vessels in practically healthy subjects was evaluated. Subsequently, the subjects took glycine (Group I) for 30 days 3 times a day in a dosage of 100 mg (a total daily dose was 300 mg), or took nothing (Group II). At the end of the drug course, a follow-up study including the blood flow measurement in the intracranial arteries at rest, was performed. For a quantitative comparison of hemodynamic effects, a transcranial assessment of the blood flow in the MCA was chosen, since this artery is a direct continuation of the ICA and supplies a significant part of the brain. In accordance with the study design, each patient underwent multiple measurements of flow values at rest, which makes it possible to compare the effects of a glycine administration course on the blood flow parameters with the control group, as well as to evaluate the response of the cerebral arteries to a high dose compared to placebo.

### Statistical processing

Statistica 10 program (Statsoft, USA) was used for statistical data processing. The studied features could not be normally distributed, therefore the quantitative data are presented in the form of Me [Q25; Q75], where Me – median, Q25 – lower quartile, Q75 – upper quartile; nominal and categorical data – *n* (%), where *n* – absolute value, % – relative frequency of occurrence. To assess the statistical significance of differences between the quantitative data, the Mann-

[1] Registration certificate for glycine. Russian State Register of Medicines. Available from: https://grls.rosminzdrav.ru/Grls_View_v2.aspx?routingGuid=c73870d4-a6c3-41d5-aa4e-393b4a099a62

Whitney *U*-test (for independent samples) and the Wilcoxon test (for dependent samples) were used. The Fisher's exact test and the Pearson's $\chi^2$ test were used to compare the fractions (frequencies). The nonparametric Spearman's correlation coefficient was used to assess the relationship between the signs. The results were considered statistically significant at the $p$ <0.05.

## RESULTS

### Study participants

Twenty practically healthy subjects aged 27 to 46 years were included in the study. The clinical and demographic data of the volunteers are presented in Table 1. The gender and age characteristics and the number of patients with a reported history of vascular diseases did not differ significantly between the study groups.

### Main study results

The evaluation of the blood flow through the main head and neck vessels revealed no significant differences between the groups (Table 2). However, in group II, the systolic velocity was slightly higher than in group I. In general, the values of indices corresponded to the age norms [10] with insignificant differences in linear velocities on the left and right.

The initial transcranial assessment of the blood flow parameters in the MCA revealed significant differences between the left and right flows, but their values were within the physiologic range. In eight subjects, the linear peak systolic, end-diastolic, and mean maximum velocities were significantly greater on the left, in four subjects on the right, and in eight subjects, the differences were nonsignificant. In the overall statistical evaluation, the values of the indices on the right were significantly smaller ($p$ <0.05), and this trend was maintained at the second measurement (Table 3). The asymmetry of the cerebral bolod flow up to 20% is considered physiologically acceptable and can be explained by both a functional asymmetry of the cerebral hemispheres and morphological features of the paired vessels [20].

Despite the similar characteristics of the two groups, baseline blood flow values in the MCA differed significantly between the groups, and they were lower in group I (PS on the left and right, $p$ <0.01; TAMAX on the left and right, $p$ <0.01; ED on the right, $p$ <0.05). Due to the asymmetry detected, the left and right flow values were further analyzed independently. After one month of the glycine administration (in group I), there was a significant increase in velocities on the left and right (medians of both velocity values and increases in each measurement were evaluated) (Fig. 2). It was shown that peak systolic (by 11.9 cm/s) and mean maximum (by 6.3 cm/s) velocities increased on the left, while peak systolic (by 9.3 cm/s), end-diastolic (by 2.8 cm/s), and mean maximum (by 5.8 cm/s) velocities increased on the right (Table 3). In group II, there were no significant changes on the right side, with significant decreases in end-diastolic (by -2.8 cm/s, $p$ <0.05) and mean maximum (by -2.7 cm/s, $p$ <0.05) velocities on the left. The average increase in the peak systolic velocity was 10% after 30 days of the glycine administration and -2% in group I, the relative increase in one subject could be as high as 40%. It is important that in both groups, the blood flow indices after the change were within the physiologic range [10].

To assess the baseline blood flow in the 1000 mg glycine / placebo sample, the mean value on the left and right of 4 measurements during 15 min of lying at rest was calculated for each subject. The mean change in the flow relative to this value was then calculated 5, 10, and 15 min after the drug administration. In the glycine / placebo trial, the relative increase in the peak systolic velocity was 7.6% [1.2; 10.8] in group I and 1.5% [-3.6; 5.5] in group II (the significance level of differences between groups $p$=0.03). The mean peak velocity increased by 9.6% [0.6; 15.7] in group I and by 3.0% [-2.5; 8.0] in group II (the significance level of differences between groups $p$=0.08). The relative changes of the peak systolic blood flow velocity in the left and right middle cerebral artery are presented in Fig. 3.

It was found out that the baseline mean in group I was below the overall mean (for all subjects), -5.4% [-20.0; 7.0], and in group II, it was above 10.7% [-5.1; 18.0]; $p$ <0.05. The changes were multidirectional in different patients, but the maximum deviations in group I were observed at 5 and 10 min, whereas in group II they were erratic. A significant correlation ($p$ <0.05) was found out between the increase in velocity and the deviation of the individual baseline velocity value from the group mean (for PS $r$=0.68, $p$ <0.05; for TAMAX $r$=0.73, $p$ <0.05). In other words, the changes were aimed at correcting deviations from the physiological norm, and the magnitude of the changes was greater the more deviated the values were.

None of the study participants had adverse events (including allergic reactions and intolerance) associated with a sublingual administration of glycine 100 mg three times a day for 30 days, as well as a single intake of 1000 mg, which confirms a good tolerability of this drug in the indicated doses.

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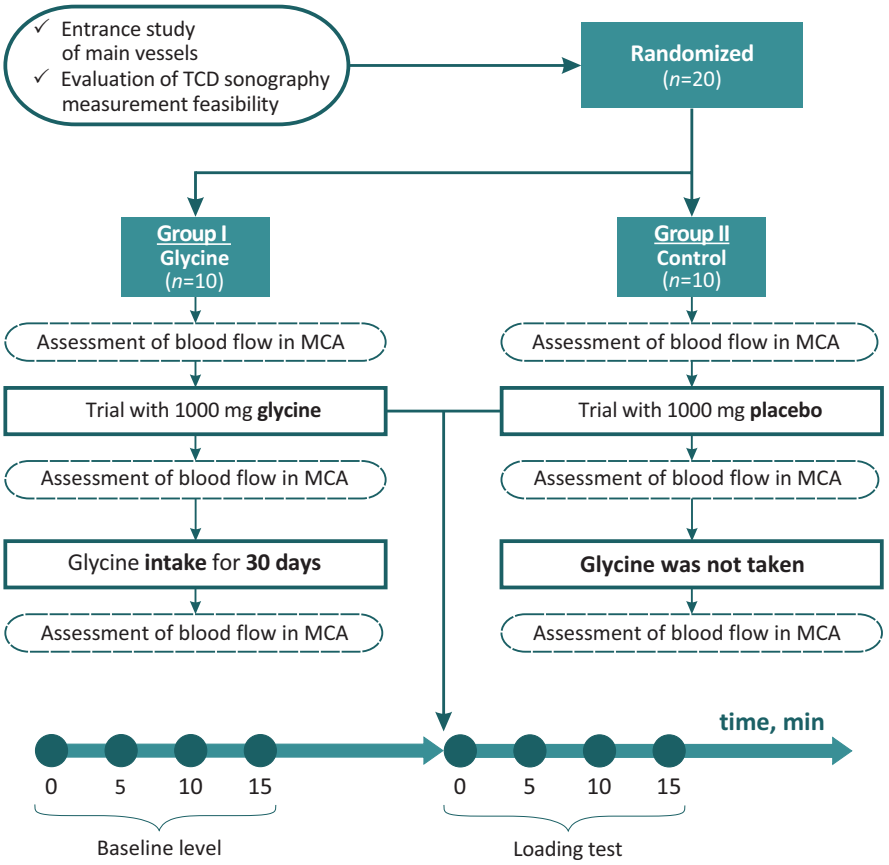


**Figure 1 – Study design**
Note: TCD sonography – transcranial Doppler sonography; MCA – middle cerebral artery.

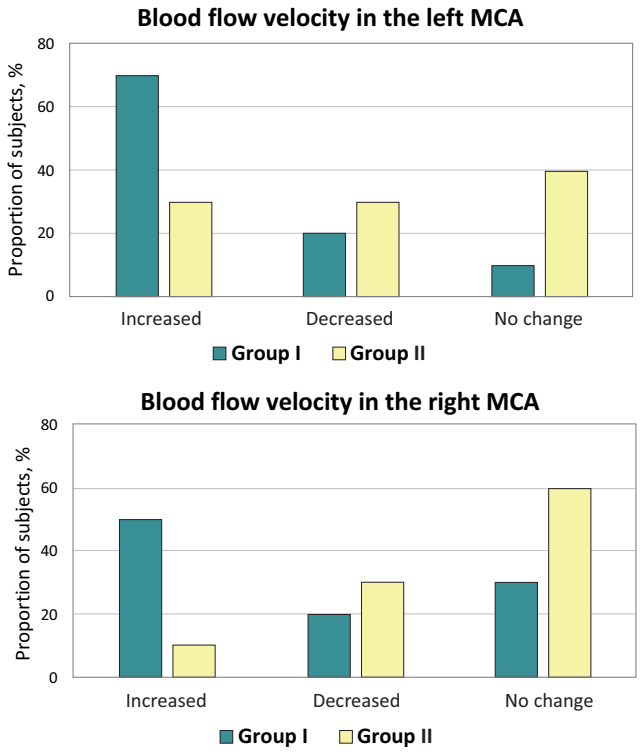


**Figure 2 – Dynamics of peak systolic blood flow velocity in the left and right middle cerebral artery at transcranial examination 30 days after the start of the study in both groups**
Note: MCA – middle cerebral artery. The observed differences between groups in the blood flow velocity changes on the left ($p$=0.02) and right ($p$ <0.001) are statistically significant.

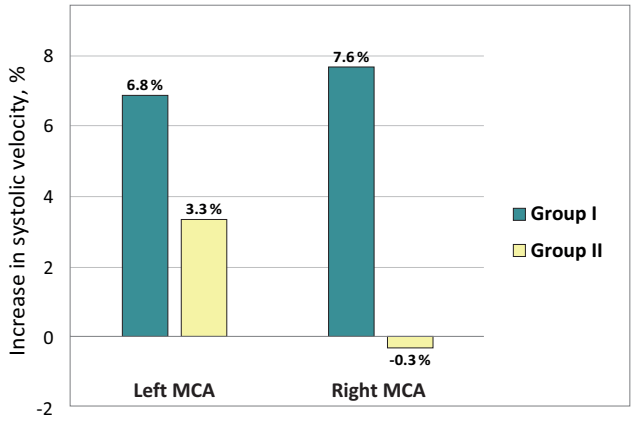


**Figure 3 – Relative changes in peak systolic blood flow velocity in the left (*p*=0.24) and right (*p*=0.09) middle cerebral artery at transcranial examination after 1000 mg glycine or placebo administration**

Note: MCA – middle cerebral artery.

**Table 1 – Clinical and demographic characteristics of participants**

| Group | | Group I (*n*=10) | Group II (*n*=10) | *p* |
|---|---|---|---|---|
| Age, years, Me [Q25; Q75] | | 35.5 [32; 43] | 38.5 [33; 42] | 0.496[1] |
| Gender, *n* (%): | | | | |
| | Male | 4 (40%) | 4 (40%) | 1,000[2] |
| | Female | 6 (60%) | 6 (60%) | |
| Atherosclerosis, *n* (%): | | | | |
| | no | 8 (80%) | 6 (60%) | 0,629[2] |
| | yes | 2 (20%) | 4 (40%) | |
| Migraine, *n* (%): | | | | |
| | no | 8 (80%) | 9 (90%) | 1,000[2] |
| | yes | 2 (20%) | 1 (10%) | |
| Arterial hypertension, *n* (%): | | | | |
| | no | 7 (70%) | 9 (90%) | 0,582[2] |
| | yes | 3 (30%) | 1 (10%) | |

Note: To assess the statistical significance of differences in quantitative data, the authors used: [1] – Mann-Whitney *U*-criterion, [2] – Fisher's exact test. The differences between the groups by the presented characteristics are statistically insignificant (*p* >0.05).

**Table 2 – Baseline peak systolic (PS) blood flow velocities through the main head and neck vessels in both groups**

| Artery | | Peak systolic blood flow velocity (PS), cm/s | | p |
|---|---|---|---|---|
| | | Group I, Me [Q25; Q75] | Group II, Me [Q25; Q75] | |
| CCA, (norm is 50-169 cm/s) | Left | 92.6 [83.7; 116.1] | 103.7 [96.7; 117.8] | 0.39 |
| | Right | 90.2 [73.7; 118.3] | 95.6 [72.5; 103.8] | 0.92 |
| ECA, (norm is 45–136 cm/s) | Left | 84.1 [78.6; 94.0] | 106.2 [85.1; 118.5] | 0.10 |
| | Right | 101.9 [73.4; 114.1] | 109.8 [95.4; 119.3] | 0.92 |
| ICA, (norm is 36–115 cm/s) | Left | 79.9 [64.5; 103.3] | 88.5 [80.8; 117.0] | 0.25 |
| | Right | 84.1 [67.2; 103.6] | 91.4 [83.1; 115.5] | 0.28 |
| VA, (norm is 28–71 cm/s) | Left | 47.2 [36.9; 53.8] | 47.4 [42.3; 58.7] | 0.76 |
| | Right | 38.5 [34.6; 50.1] | 48.1 [43.7; 51.4] | 0.13 |

Note: CCA – common carotid artery; ECA – external carotid artery; ICA – internal carotid artery; VA – vertebral artery. The Mann-Whitney *U*-criterion was used to assess the statistical significance of differences between the groups. Physiologic norms of velocities are given for persons of a corresponding average age (35±12 years) [10]. The values of velocities in each of the arteries in two groups were not statistically different (*p* >0.05).

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**Table 3 – Linear peak systolic (PS) blood flow velocities in the left and right middle cerebral artery at transcranial examination in both groups**

| Arter | Group I, Me [Q25; Q75] | | | | Group II, Me [Q25; Q75] | | | | $p^0_{I–II}$ |
|---|---|---|---|---|---|---|---|---|---|
| | Day 0 | Day 30 | $p^{0–30}$ | Δ | Day 0 | Day 30 | $p^{0–30}$ | Δ | |
| Peak systolic blood flow velocity (PS), cm/sec | | | | | | | | | |
| Left | 104.6 [93.9; 117.1] | 112.2 [105.3; 120.6] | <0.001[2] | 11.9 [-5.9; 18.4] | 117.7 [106.2; 124.7] | 114.1 [99.5; 127.0] | 0.44[2] | -4.8 [-11.6; 9.2] | 0.001[1] |
| Right | 99.8 [86.0; 110.4] | 104.3 [97.0; 116.5] | <0.001[2] | 9.3 [-3.7; 19.8] | 108.8 [97.0; 119.7] | 110.8 [94.0; 119.1] | 0.54[2] | -3.9 [-9.8; 7.8] | 0.002[1] |
| End diastolic blood flow velocity (ED) cm/sec | | | | | | | | | |
| Left | 45.6 [41.5; 5.9] | 48.5 [44.2; 53.1] | 0.22[2] | 1.1 [-4.2; 6.4] | 49.6 [45.3; 54.9] | 46.8 [43.2; 52.3] | 0.02[2] | -2.8 [-7.2; 3.8] | 0.09[1] |
| Right | 43.4 [37.9; 48.3] | 45.6 [41.1; 50.1] | 0.01[2] | 2.8 [-3.0; 6.3] | 46.4 [42.2; 50.6] | 46.3 [40.2; 51.1] | 0.61[2] | -1.5 [-6.1; 4.0] | <0.05[1] |
| Time average maximum blood flow velocity (TAMAX), cm/sec | | | | | | | | | |
| Left | 69.3 [63.1; 79.3] | 75.6 [70.1; 82.5] | <0.001[2] | 6.3 [-3.9; 13.6] | 80.0 [68.6; 86.9] | 74.2 [65.4; 86.2] | 0.04[2] | -2.7 [-8.1; 4.3] | 0.008[1] |
| Right | 65.4 [59.8; 76.6] | 73.3 [66.6; 79.0] | <0.001[2] | 5.8 [-2.7; 12.9] | 72.7 [64.1; 81.0] | 73.7 [62.9; 80.1] | 0.52[2] | -3.2 [-7.7; 5.4] | 0.007[1] |

Note: To assess the statistical significance of differences in quantitative data the authors used: [1] – Mann-Whitney *U*-criterion; [2] – Wilcoxon test. $p^{0–30}$ – significance level for the difference between the value of the indices on day 0 and day 30 in the group; $p^0_{I–II}$ – significance level for the difference between the value of the index on day 0 in both groups. The results were considered statistically significant at $p$ <0.05.

## DISCUSSION

One of the critical factors for an adequate brain function is to maintain an adequate blood supply to meet changing metabolic demands, but off the linear dependence on the systemic blood pressure [21]. Both acute and chronic kinds of cerebral ischemia are common causes of a reduced work capacity, disability and mortality of the population [22, 23].

In the brain tissue under a chronic hypoperfusion, as well as an ischemia-reperfusion, there is inevitably an imbalance of metabolic processes, antioxidant defense systems, and, as a consequence, neurotransmission disorders, a decreased neuroplasticity, the deterioration of the cognitive status and the one of the general functional status [4]. Asymptomatic or accompanied by mild cognitive decline cerebral vascular disorders are usually poorly diagnosed due to the absence of patient complaints. However, in such conditions as an arterial hypertension, atherosclerosis, diabetes mellitus, and in the presence of additional risk factors such as hypodynamia, obesity, and smoking, a cerebral circulation control is one of the most important aspects of a stroke and dementia prevention [6, 24].

Neuroprotective metabolic drugs are widely used in vascular cognitive disorders, because they have not only a nootropic effect, but also contribute to the normalization of the neuronal energy supply, exhibit antioxidant and antihypoxant properties, gently correct cerebral hemodynamic disorders [11]. One of such drugs is glycine, which has been used for more than 30 years both in severe neurological conditions and in practically healthy people for the correction of behavioral and vascular disorders, a reduction of anxiety and psychoemotional stress, an improvement of cognitive abilities [15, 19, 25, 26]. The wide range of pharmacological properties of the drug is due to the participation of this amino acid in a huge number of biochemical processes, as well as its unique neurotransmitter characteristics: an interaction with inhibitory glycine (GlyR), excitatory glutamate (NMDA-R) and metabotropic (mGlyR) receptors [13, 27].

In the present study, the effect of glycine on the state of cerebral vessels using the ultrasound was confirmed. TCD sonography is a sufficiently accurate, reproducible noninvasive method of assessing blood flow velocity parameters, as well as the reactivity of cerebral vessels [28, 29].

The course sublingual glycine administration to relatively healthy volunteers for 30 days resulted in a significant increase in the linear blood flow parameters in the intracranial vessels. The change of velocity in some subjects reached 40%, and the maximum values of the increase were observed at initially lowered indices or an expressed interhemispheric asymmetry. In group II, the input velocity values were higher than in group I, despite the fact that the hemodynamic parameters for the cerebral main vessels obtained before the randomization, did not differ. A significant interhemispheric flow asymmetry was found in more than half of the subjects, and despite individual differences, the values on the right side were significantly lower for all participants. This phenomenon may be mediated by the left carotid artery branching directly from the aortic arch and has also been repeatedly shown in animals [1]. It

is interesting that the blood flow in the right half of the brain turned out to be more stable during the study both after the glycine administration and in the control group, where after 30 days small but significant decreases in velocities on the left side were recorded. The volumetric blood flow velocity characterizes the blood filling of an organ quite completely, it depends both on the linear velocity in the vessel and on its diameter. However, due to the difficulty in estimating intracranial vessel diameters and their low variability under physiological conditions, the linear velocities are usually chosen as hemodynamic parameters [3]. Herewith, patients with different vessel calibers may have different levels of the blood flow at the same values of linear velocities. In the future, a more detailed study of the relationship between a cerebral blood flow and a functional activity of the brain, including under the influence of drugs both in the norm and in various pathological conditions, may be of scientific and clinical interest.

Nevertheless, an increase in the blood flow velocities through cerebral arteries within the physiological norms definitely indicates an increase in the nervous tissue perfusion and can be regarded as a factor in expanding the range of the brain functional activity. Since the course of the glycine administration led to an increase in the MCA indices, it can be recommended both for a mild and moderate cognitive decline and during periods of a high mental stress to maintain an adequate brain functioning.

The administration of 1000 mg of glycine as a lump sum is recommended in the first day in the therapy of an ischemic stroke and other acute cerebral accidents, as well as in a suspected acute cerebral circulatory failure [15]. The study of the MCA velocity indices after an acute test with 1000 mg of glycine or placebo allowed us to evaluate the effect of a high dose of the drug on the cerebral blood flow in practically healthy subjects. It was shown that a single administration of 1000 mg of glycine caused a short-term significant increase in the MCA linear velocities up to 10%. At the same time, the change degree was proportional to the initial deviation from the mean age-related norm: the greatest effect was observed in the case of a significant deviation of the indices. However, it is obvious that such a dilatation can occur only with a preserved vascular reactivity, the assessment of which is also of interest in a further study of the described effects on a larger sample of patients. Thus, a prophylactic administration of 1000 mg of glycine to patients with a suspected stroke is safe and can be recommended because it does not lead to critical changes in the blood flow in case of preserved intracranial hemodynamics.

### Study Limitations

According to the authors, the limitation of the study may be a relatively small sample size, so some of the results obtained could not be confirmed by statistical methods, and the analysis of the cerebral blood flow parameters was carried out in men and women in the aggregate. In addition, the effects of glycine described in this work were observed in conditionally healthy individuals, without taking into account the possible influence of possible cardiovascular diseases on the cerebral blood flow parameters. The study of the described effects on large groups of patients, including those with various vascular pathologies, will be of practical interest from the point of view of understanding the revealed regularities.

## CONCLUSION

Thus, in this study, the effect of a sublingual glycine administration on the cerebral hemodynamic parameters was studied using TCDG. It is shown that a course administration of glycine leads to a reliable improvement of the intracranial blood flow in comparison with the control group. Thus, a sublingual administration of 100 mg of glycine 3 times a day for 30 days contributed to a significant change in cerebral hemodynamics. Although the trends were multidirectional, there were many more patients in group I in whom the MCA linear blood flow velocities increased compared with group II, where the values were unchanged or decreased. In addition, a short-term significant increase in the peak systolic and mean maximal intracranial blood flow velocities up to 10% was observed after a single 1000 mg dose of glycine, whereas no effect of this kind was observed after placebo.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## AUTHORS' CONTRIBUTION

Elena V. Mashkovtseva – development of the study design and article concept, statistical processing of the results, writing the main text of the manuscript; Natalia A. Rudnikova – conducting ultrasound studies, editing the article; Veronika S. Kopylova – processing the ultrasound data, editing the article text; Yaroslav R. Nartsissov – participation in the development of the study design and article concept, approval of the final manuscript.

All the authors confirm that their authorship meets the international ICMJE criteria (all the authors have made a significant contribution to the development of the concept, research and preparation of the article, read and approved the final version before the publication).

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# Risk of secondary bacterial infections during treatment with anti-inflammatory genetically engineered biological drugs in COVID-19 patients

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**The aim** of the work was to identify the presence and strength of association between the use of anti-inflammatory genetically engineered biological drugs and the development of secondary bacterial infections in COVID-19 patients.
**Materials and methods.** We used 1 296 medical records of patients hospitalized in the infectious diseases hospital of the Volgograd region with a diagnosis of COVID-19 in September 2020, March and September 2021, March, September and November 2022, have been analyzed. A matched case-control study was performed with 275 pairs identical in gender, age (±2 years), the severity of the lung damage according to computed tomography / chest X-ray, a COVID-19 outcome, concomitant carbohydrate metabolism disorders. Patients with the signs of the secondary bacterial infection (leukocytes ≥12×109/l, procalcitonin ≥0.5 ng/ml and/or viral-bacterial pneumonia according to the autopsy data) were presented as a case. The "control" group included patients without signs of any bacterial infection (leukocytes <11×109/l, procalcitonin <0.5 ng/ml, no description of clinical signs of the bacterial infection in the medical record during the hospitalization). The prescription of 6 anti-inflammatory genetically engineered biological drugs (tocilizumab, sarilumab, olokizumab, levilimab, netakimab, secukinumab) has been studied for these groups.
**Results.** The use of any anti-inflammatory genetically engineered biological drug was associated with the development of the secondary bacterial infection signs (OR=2.41; 95% CI: from 1.54 to 3.77; $p$ <0.001): for levilimab, the OR was 3.44 (95% CI: from 1.64 to 7.23; $p$ <0.001), for tocilizumab – OR=1.75 (95% CI: from 0.73 to 4.17; $p$=0.201), for olokizumab – OR=1.28 (95% CI: from 0.81 to 2.03; $p$=0.292).
**Conclusion.** Among the three drugs (tocilizumab, olokizumab, levilimab), the Russian biosimilar olokizumab, a monoclonal antibody to circulating interleukin-6, has shown itself as the safest drug in terms of preventing the secondary bacterial infection signs. Further studies of developing bacterial complications risk in COVID-19 patients receiving anti-inflammatory genetically engineered biological drugs are required.
**Keywords**: genetically engineered biological drugs; interleukin antagonists; COVID-19; tocilizumab; olokizumab; levilimab; case-control study
**Abbreviations**: FO – fatal outcome; SBI – secondary bacterial infection; GEBDs – genetically engineered biological drugs; ARDS – acute respiratory distress syndrome; CRP – C-reactive protein; OR – odds ratio; CI – confidence interval; CT – computed tomography; XR – X-ray.

# Оценка риска возникновения вторичных бактериальных инфекций у больных COVID-19 при приёме противовоспалительных генно-инженерных биологических препаратов

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**Цель.** Выявить наличие и степень выраженности связи между применением противовоспалительных генно-инженерных биологических препаратов и развитием вторичных бактериальных инфекций у больных COVID-19.

**Материалы и методы.** Проанализировано 1296 медицинских карт пациентов, госпитализированных с диагнозом COVID-19 в сентябре 2020 года, марте, сентябре 2021 года, марте, сентябре и ноябре 2022 г. Выполнено исследование «случай-контроль» с использованием метода подбора пар «matched case-control study» (275 пар), идентичных по полу, возрасту (±2 года), степени тяжести поражения лёгких по данным компьютерной томографии / рентгенографии лёгких, исходу COVID-19, сопутствующими нарушениями углеводного обмена. В качестве «случая» были представлены пациенты с признаками вторичной бактериальной инфекции (по показателям: лейкоциты ≥12×109/л, прокальцитонин ≥0,5 нг/мл и/или вирусно-бактериальная пневмония по данным аутопсии). В качестве «контроля» были пациенты без признаков бактериальной инфекции (лейкоциты <11×$10^9$/л, прокальцитонин <0,5 нг/мл, отсутствие описания клинических признаков бактериальной инфекции в медицинской карте на протяжении всей госпитализации). Для указанных групп исследовали назначения 6 противовоспалительных генно-инженерных биологических препаратов (ГИБП): тоцилизумаб, сарилумаб, олокизумаб, левилимаб, нетакимаб, секукинумаб.

**Результаты.** Применение любого противоспалительного ГИБП было ассоциировано с появлением признаков вторичной бактериальной инфекции (ОШ=2,41; 95% ДИ от 1,54 до 3,77; *p* <0,001): для левилимаба ОШ составило 3,44 (95% ДИ от 1,64 до 7,23; *p* <0,001), для тоцилизумаба – ОШ=1,75 (95% ДИ от 0,73 до 4,17; *p*=0,201), для олокизумаба – ОШ=1,28 (95% ДИ от 0,81 до 2,03; *p*=0,292).

**Заключение**. Среди трёх препаратов (тоцилизумаб, олокизумаб, левилимаб) наибольшей безопасностью в отношении предупреждения признаков вторичной бактериальной инфекции был препарат олокизумаб. Стоит отметить, что требуется дальнейшее изучение риска развития бактериальных осложнений у пациентов с COVID-19 на фоне применения противовоспалительных ГИБП.

**Ключевые слова:** генно-инженерные биологические препараты; антагонисты интерлейкинов; COVID-19; тоцилизумаб; олокизумаб; левилимаб; исследование «случай-контроль»

**Список сокращений:** ЛИ – летальный исход; ВБИ – вторичная бактериальная инфекция; ГИБП – генно-инженерные биологические препараты; ОРДС – острый респираторный дистресс синдром; СРБ – С-реактивный белок; ОШ – отношение шансов; ДИ – доверительный интервал; КТ – компьютерная томография; РГ – рентгенография.

## INTRODUCTION

A variety of respiratory viruses often infect people during their lifetime and may lead to bacterial superinfections. Until recently, the influenza A virus has been the most dangerous among the causative agents of acute respiratory viral diseases [1]. A retrospective analysis of the preserved histological samples from the 1918 year AH1N1 influenza pandemic led to the conclusion that more than 95% of the fatal cases had been directly associated with secondary bacterial pneumonia [2]. About 70–80% of fatal cases of the 1957–1958 influenza pandemic had been also associated with bacterial pneumonia [3] and 29–55% of patients who died in healthcare facilities from influenza A (H1N1) during the 2009 outbreak, had signs of bacterial infection [4].

At the end of 2019, the first cases of COVID-19 caused by a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), were reported in China. At the beginning of the pandemic, the role of bacterial complications of the new infection was raised [5]. B.J. Langford et al. in meta-analysis [6], which covered 3 338 COVID-19 patients in 2020, studied the prevalence of the bacterial infection of the respiratory tract and/or bloodstream in patients with a confirmed COVID-19 diagnosis. Bacterial co-infections (less than 2 days from the admission to hospital) were detected in 3.5% of COVID-19 patients, and secondary bacterial infections (SBI; more than 2 days from the admission to hospital) were detected in 14.3% of patients. More than 70% of patients hospitalized with COVID-19 in 2020, received antibiotics [6].

W.H. Chong et al, in a meta-analysis of 2021 [7] examined the prevalence of secondary bacterial and fungal kinds of infection in hospitalized COVID-19 patients. The incidence of the secondary bacterial pulmonary infection in the hospitalized was 16% (580 cases out of 3 633 patients). Only 9 of 49 included studies had microbiology data. The most common bacterial agents identified in the respiratory tract sample cultures in the nine observational studies reporting the type and incidence of the SBI, were *Pseudomonas aeruginosa*, *Klebsiella species*, *Staphylococcus aureus*, *Escherichia coli*, and *Stenotrophomonas maltophilia*. According to the studies, 60 to 100% of patients received antibacterial drugs [7].

According to J.M. Farrell et al, the true prevalence of the bacterial concomitant and SBI in COVID-19 patients may be higher because of the difficulties that arise in the differential diagnosis of viral and bacterial pneumonia in

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real clinical practice, as well as the difficulties in collecting respiratory samples from patients in quarantine [3].

As with influenza, SBIs may affect the prognosis of COVID-19 patients. In a retrospective cohort study including patients in Wuhan, China, F. Zhou et al. [8] found that bacterial infections (bacteremia and pneumonia) were more common in fatal COVID-19 cases compared with recovered patients: 28/191 (15%) patients had a culture-positive bacterial infection, and all of these patients except one died. Half of the fatal cases patients (27/54) had a bacterial co-infection, while only 1 case (1/137) of the recovered patients had a bacterial co-infection [8]. Similar patterns of a high incidence of bacterial infections among the deceased patients have been reported in more recent studies [9, 10].

Recent studies have shown that an excessive interferon production and an uncontrolled inflammation are the main mechanisms which contribute to the development of bacterial infections, regardless of the type of a respiratory virus [1]. Compared to normal mice, the genetically modified mice deficient in type I interferon receptors, are more resistant to the development of an acute respiratory distress syndrome, bacterial pneumonia, or sepsis [11–13]. Thus, reducing the risk of development and severity of a cytokine storm, a characteristic complication of a new infection, may lead to a reduced risk of not only ARDS but also of a bacterial superinfection. However, anti-inflammatory genetically engineered biological drugs (GEBDs) such as tocilizumab, sarilumab, and others, used to prevent and treat a cytokine storm in COVID-19, can cause infectious complications due to the immunosuppression.

In patients with rheumatoid arthritis, the most common adverse reactions to tocilizumab in preclinincal and clinical trials were upper respiratory tract infections [14–16]. The rate of serious infections during therapy with another interleukin-6 antagonist, sarilumab, in one study in patients with rheumatoid arthritis, was the same as the similar rate during the therapy with tocilizumab, which makes it possible to conclude that this adverse reaction is class specific [17].

A.I. Rutherford et al. [18], studied the rate of serious infections among GEBDs to treat rheumatoid arthritis based on the data from the British Society of Rheumatology's Rheumatoid Arthritis Biologicals Registry. 19 282 patients were included in the prospective observational cohort study. The incidence of serious infections was 5.51 cases per 100 patient-years. Compared with the tumor necrosis factor-alpha inhibitor etanercept, tocilizumab had a higher risk of developing serious infections (adds ratio [OR]=1.22; 95% confidence interval [CI] 1.02–1.47). A 30-day mortality rate due to serious infections in patients with rheumatoid arthritis receiving biological therapy was 10.4% (95% CI 9.2–11.6%) [18]. In COVID-19 patients, SBIs may be associated with biologic drug therapy and affect the prognosis of COVID-19 considering the mechanism of the drugs action and the data obtained in patients with rheumatoid arthritis.

**THE AIM** was to identify the presence and strength of the association between the use of anti-inflammatory biological drugs and the development of secondary bacterial infections in COVID-19 patients to assess the safety of biological therapy.

## MATERIALS AND METHODS

### Study design

A single-center retrospective observational matched case-control study was conducted. Medical records of the patients hospitalized in the infectious disease departments of City Clinical Hospital No. 3 in Volzhsky, the Volgograd Region (Russia), with a confirmed PCR or a presumptive COVID-19 diagnosis during the periods of maximum hospitalization rate – in September 2020, March, September 2021 and March, September and November 2022 – were selected for the analysis. These patients were to stay in hospital for at least 5 days (1 296 patients). The "cases" were patients with the signs of the bacterial infection (leukocytosis ≥12×109/l with a left shift in the white blood cell count, procalcitonin ≥0.5 ng/mL, and/or a description of viral-bacterial pneumonia according to the autopsy data) appeared more than 48 hours after the admission to hospital. The "controls" were selected if the white blood cell count was <11×109/l, procalcitonin <0.5 ng/mL throughout the hospitalization, and there was no description of clinical signs of a bacterial infection in the medical record. For each patient with the SBI signs (a "case"), a pair was selected in a 1:1 ratio among the patients without bacterial infection signs (a "control"), matching the "case" in terms of gender, age (±2 years), a degree of the lung damage (none / 1–2 / 3–4

degrees according to the computed tomography (CT) or chest X-ray data), the outcome (recovered / died), and the presence / absence of carbohydrate metabolism disorders (Fig. 1). In the presence of several "cases" and/or "controls" matching in all parameters, the pairs were selected using a random number generator.

In medical records of 1 296 patients, 77 had data on possible SBIs in the first 48 h after the hospital admission; 73 had no data to confirm or exclude possible bacterial infections; 245 patients had changes in the blood count during the systemic corticosteroid therapy (leukocytosis 11–12×109/l). The data of these patients were not included in the further analysis (see Fig. 1). 512 patient had SBI signs that appeared 48 h after the hospital admission ("case"): leukocytosis ≥12×109/l, procalcitonin ≥0.5 ng/ml and/or autopsy data (viral-bacterial pneumonia). In 389 patients, the white blood cell count was <11×109/l, procalcitonin <0.5 ng/ml, and there was no description of clinical signs of bacterial infections in the medical record throughout their hospitalization ("control"). 275 pairs were matched for gender (male / female), age (a deviation of ±2 years was allowed to achieve the required sample size), a degree of the lung damage (none / 1–2 / 3–4 degrees according to the CT or chest X-ray), the outcome ("healthy" / "lethal"), and the presence / absence of carbohydrate metabolism disorders from 512 "cases" and 389 "controls". The analysis of prescriptions was performed for all patients in the "case" and "control" groups. The prescription of 6 anti-inflammatory biological drugs was identified: tocilizumab (Roche, Switzerland); sarilumab (Sanofi, France); olokizumab (R-Pharm, Russia); levilimab (Biocad, Russia); netakimab (Biocad, Russia); secukinumab (Novartis, Switzerland). One of the criteria for the use of anti-inflammatory GEBDs was the absence of bacterial infection / sepsis signs in patients before the drug administration, which was confirmed by analyzing the medical records of patients with SBI.

### Eligibility criteria

The patients met the following inclusion criteria: the age over 18 years; an informed consent of the patient for the participation in the study and a publication of personal medical information signed on the day of hospitalization; a confirmed COVID-19 diagnosis; an inpatient treatment for at least 5 days; no clinical bacterial infection signs in the first 48 hours of hospital stay. The patients' non-inclusion criteria in the study were clinical signs of a bacterial infection in the first 48 hours of hospital stay (77 patients), no blood count or procalcitonin results on the 5$^{th}$ and subsequent days after the prescribed pharmacotherapy (73 patients). The exclusion criteria from the study were as follows: leukocytosis 11–12×109/l on the 5$^{th}$ and subsequent days after the prescribed pharmacotherapy (245 patients).

### Conditions and duration of the study

The study was conducted from January 2022 to May 2024 at the Department of Clinical Pharmacology and Intensive Care of Volgograd State Medical University (Volgograd, Russia).

### Ethical approval

The study was performed in accordance with the ethical principles of medical research involving human subjects set out in the WMA Declaration of Helsinki. The study was approved by the Local Ethics Committee of Volgograd State Medical University (Protocol No. 2021/085 dated 24 December 2021). All patients had an informed consent for the use and publication of personal medical information for scientific purposes in their medical records, signed on the day of hospitalization.

### Statistical processing

The minimum sample size for the matched case-control study was calculated using an online calculator[1]. A statistical power of 95% for the expected OR of 2.0, an error probability of less than 5.0%, and an expected proportion of the "exposed" individuals among "controls" of 15% (half of the average frequency of the inpatient GEBDs use) was achieved when reaching the sample size of 272 pairs matched 1:1 (544 individuals).

Parametric and nonparametric statistics methods were used using the STATISTICA v10.0 software package (StatSoft Inc., USA), Microsoft Excel 2010 for Windows, and a statistical software for epidemiology developed by

[1] sampsize.sourceforge.net. Available from: http://sampsize.sourceforge.net/iface/s3.html

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the US Centers for Disease Control and Prevention Epi Info™ Version 7.2[2]. Quantitative characteristics (age, bed-days, percentage of the lung damage, laboratory test data) corresponded to the normal distribution according to the Shapiro–Wilk criterion. They were described as the arithmetic mean (M) ± standard deviation (σ), and the statistical significance between the study groups according to these characteristics was tested using the Student's *t*-test. Qualitative characteristics were described using absolute values (*n*) and proportions (%), and the statistical significance between the study groups according to these characteristics was tested using the Pearson's $\chi^2$ criterion. The relationship between the appearance of SBI signs and the use of biological therapy was determined based on OR and a 95% CI. In a case-control study with the use of the matched pair method, the number of pairs in which the risk factor (use of any or a specific biological drug) was present in both the case and the control (case+, control+), the number of pairs in which the risk factor was present only in the case (case+, control-), the number of pairs in which the risk factor was present only in the control (case-, control+), and the number of pairs in which the risk factor was absent (case-, control-) were determined [19]. The significance of the difference between the case and control groups in the matched case-control study was determined using the McNemar test. A difference of $p < 0.05$ was considered statistically significant.

## RESULTS

### Study participants

Among the patients with SBI signs, the rate of men and individuals with a concomitant hypertension who had suffered a myocardial infarction or stroke, was higher compared to the patients with no signs of infection (Table 1).

Patients with SBI signs had a longer period of hospitalization, a higher rate of the lung damage according to CT and/or X-ray data, and a higher mortality rate (OR for mortality 5.64; 95% CI from 3.54 to 8.98). In the structure of the main drugs used to treat COVID-19, the differences were revealed. Thus, patients with SBI signs received antiviral drugs less often and were prescribed systemic corticosteroids and antibiotics upon admission to hospital more often. No significant differences in the age of patients with and without SBI signs were found (see Table 1). The use of the pair matching method eliminated the differences between the case and control groups in the main matching indicators: gender, a degree of the lung damage and disease outcome, which was accompanied by the elimination of the statistical difference between the groups in comorbidities and the structure of the main drugs used (Table 2). However, the average number of hospital days and the rate of the lung damage were significantly higher in the case group and after pair matching. In patients with signs of nosocomial infections, both initially and after pair matching, the maximum values of leukocyte levels, procalcitonin, and CRP were higher (Tables 1 and 2).

### Assessment of the probability of detecting the prescription of anti-inflammatory GEBDs in patients with SBI signs

The prescription of at least one anti-inflammatory biologic drug was found in 38.2% (196/512) of patients who subsequently developed SBI signs (case group) and in 26.2% (102/389) of patients without signs of infection throughout their hospitalization (control group). Fourteen patients in the case group and four patients in the control group received two different anti-inflammatory biologic drugs one by another. Among the anti-inflammatory biologic drugs, olokizumab was most frequently prescribed – 173/316 prescriptions (54.7%), levilimab – 84/316 (26.6%), and tocilizumab – 49/316 (15.5%). 7 patients received Sarilumab, 2 patients – netakimab, and 1 patient – secukinumab (Table 3).

It was possible to match "control" identical in gender, age (±2 years), severity and outcome of COVID-19, the presence or absence of carbohydrate metabolism disorders for 53.7% of "cases" due to the high frequency of SBI signs in the patients hospitalized with COVID-19. The largest number of "cases" for which it was not possible to match a pair were noted among the youngest or the oldest patients. It was not possible to find a pair for patients receiving netakimab and secukinumab, and the frequency of a sarilumab prescription was too low to calculate the OR (Table 4). The probability of detecting the prescription of any anti-inflammatory GEBD and levilimab was significantly higher in patients with SBI signs both in the classic case-control study (Table 3) and in the matched case-control study (Table 4).

[2] Epi Info™. Available from: https://www.cdc.gov/epiinfo/index.html

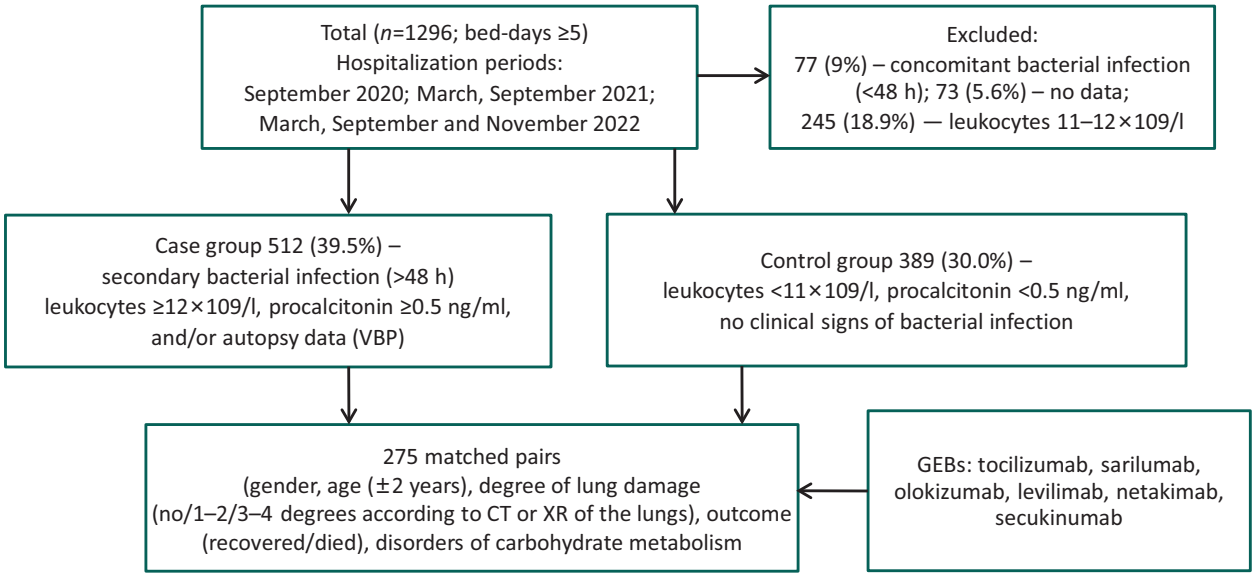


**Figure 1 – Study design**

Note: VBP – viral-bacterial pneumonia; CT – computed tomography; XR – X-ray; GEBDs – genetically engineered biological drugs.

**Table 1 – Initial characteristics of patients**

| Indicator | "case" (*n*=512) | "control" (*n*=389) | *p* |
|---|---|---|---|
| Signs of bacterial infection | present | absent | |
| Men / Women, *n* (%) | 211/301 (41.2/58.8) | 120/269 (30.8/69.2) | **0.002** |
| Age, years, M±σ | 65.5±14.6 | 64.7±15.9 | 0.432 |
| Bed-days, M±σ | 15.3±7.5 | 12.1±5.6 | **<0.001** |
| Percentage of lung damage, M±σ | 44.8±23.1 | 29.5±23.5 | **<0.001** |
| Lung damage<br>no / CT(XR)1–2 / CT(XR)3–4, *n* (%) | 35/253/224<br>(6.8/49.4/43.8) | 93/224/72<br>(23.9/57.6/18.5) | **<0.001** |
| Recovered / died, *n* (%) | 378/134 (73.8/26.2) | 366/23 (94.1/5.9) | **<0.001** |
| Hypertension, *n* (%) | 366 (71.5) | 252 (64.8) | **0.032** |
| Atrial fibrillation, *n* (%) | 74 (14.5) | 43 (11.1) | 0.133 |
| History of myocardial infarction, *n* (%) | 55 (10.7) | 25 (6.4) | **0.025** |
| History of stroke, *n* (%) | 22 (4.3) | 20 (5.1) | 0.552 |
| Disorders of carbohydrate metabolism, *n* (%) | 166 (32.4) | 114 (29.3) | 0.317 |
| White blood cells$10^9$/l, M±σ | 17.0±6.2 | 8.3±1.8 | **<0.001** |
| Procalcitonin, ng/ml | 2.1±8.2 | 0.2±0.2 | **<0.001** |
| CRP mg/ml, M±σ, | 164.2±162.3 | 76.4±111.8 | **<0.001** |
| Antiviral drugs, *n* (%) | 201 (39.3) | 220 (56.6) | **<0.001** |
| Systemic corticosteroids, *n* (%) | 478 (93.4) | 337 (86.6) | **<0.001** |
| Anticoagulants, *n* (%) | 501 (97.9) | 376 (96.7) | 0.271 |
| Antibiotics on admission, *n* (%) | 307 (59.9) | 172 (44.2) | **<0.001** |

Note: * – maximum value in the medical record during hospitalization, *p* – Student's *t*-test for quantitative characteristics, Pearson's χ2 test for qualitative characteristics, M – arithmetic mean, σ – standard deviation, CT – computed tomography, chest XR – chest X-ray, CRP – C-reactive protein; *p* <0.05 are highlighted in bold.

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**Table 2 – Initial characteristics of patients' matched pairs**

| Indicator | "case"(*n*=275) | "control" (*n*=275) | *p* |
|---|---|---|---|
| Signs of bacterial infection | present | absent | |
| Men / Women, *n* (%) | 91/184 (33.1/66.9) | 91/184 (33.1/66.9) | 1.000 |
| Age, years, M±σ | 66.3±13.9 | 66.2±13.9 | 0.898 |
| Bed-days, M±σ | 16.0±7.1 | 12.4±5.5 | **<0.001** |
| Percentage of lung damage, M±σ | 39.1±21.6 | 35.0±21.9 | **0.035** |
| Lung damage no / CT(XR)1–2 / CT(XR)3–4, *n* (%) | 19/184/72 (6.9/66.9/26.2) | 19/184/72 (6.9/66.9/26.2) | 1.000 |
| Recovered / died, *n* (%) | 253/22 (92.0/8.0) | 253/22 (92.0/8.0) | 1.000 |
| Hypertension, *n* (%) | 199 (72.4) | 180 (65.5) | 0.079 |
| Atrial fibrillation, *n* (%) | 37 (13.5) | 32 (11.6) | 0.520 |
| History of myocardial infarction, *n* (%) | 23 (8.4) | 20 (7.3) | 0.634 |
| History of stroke, *n* (%) | 9 (3.3) | 17 (6.2) | 0.108 |
| Disorders of carbohydrate metabolism, *n* (%) | 77 (28.0) | 77 (28.0) | 1.000 |
| White blood cells$10^9$/l, M±σ | 16.6±5.8 | 8.4±1.8 | **<0.001** |
| Procalcitonin, ng/ml | 1.5±4.9 | 0.2±0.2 | **0.006** |
| CRP mg/ml, M±σ, | 148.3±151.5 | 82.1±109.6 | **<0.001** |
| Antiviral drugs, *n* (%) | 117 (42.5) | 138 (50.2) | 0.073 |
| Systemic corticosteroids, *n* (%) | 257 (93.5) | 252 (91.6) | 0.417 |
| Anticoagulants, *n* (%) | 272 (98.9) | 268 (97.6) | 0.202 |
| Antibiotics on admission, *n* (%) | 142 (51.6) | 122 (44.4) | 0.088 |

Note: * – maximum value in the medical record during hospitalization, p – Student's t-test for quantitative characteristics, Pearson's χ2 test for qualitative characteristics, M – arithmetic mean, σ – standard deviation, CT – computed tomography, chest XR – chest X-ray, CRP – C-reactive protein; *p* <0.05 are highlighted in bold.

**Table 3 – Probability of detecting the prescription of anti-inflammatory genetically engineered anti-inflammatory drugs in patients with signs of secondary bacterial infection**

| Risk factor | "case" (*n*=512) | | "control" (*n*=389) | | OR | 95% CI | | *p* |
|---|---|---|---|---|---|---|---|---|
| | + | – | + | – | | | | |
| All GEBDs | 196 | 316 | 102 | 287 | **1.75** | 1.31 | 2.33 | **<0.001** |
| Tocilizumab | 33 | 479 | 16 | 373 | 1.61 | 0.87 | 2.96 | 0.126 |
| Sarilumab | 6 | 506 | 1 | 388 | 4.60 | 0.55 | 38.37 | 0.121 |
| Olokizumab | 107 | 406 | 66 | 323 | 1.29 | 0.92 | 1.81 | 0.142 |
| Levilimab | 61 | 451 | 23 | 365 | **2.15** | 1.30 | 3.54 | **0.002** |
| Netakimab | 2 | 510 | 0 | 389 | – | – | – | – |
| Secukinumab | 1 | 511 | 0 | 389 | – | – | – | – |

Note: Pearson $\chi^2$ p-test, GEBDs – genetically engineered biological drugs, OR – odds ratio, CI – confidence interval, OR and *p* <0.05 are highlighted in bold.

**Table 4 – Probability of detecting the prescription of anti-inflammatory genetically engineered anti-inflammatory drugs in patients with signs of secondary bacterial infection in matched pairs**

| Risk Factor | Number of matched pairs exposed (+) and not exposed (-) to a risk factor | | | | OR | 95% CI | | *p* |
|---|---|---|---|---|---|---|---|---|
| «case» | + | + | – | – | | | | |
| «control» | + | – | + | – | | | | |
| All GEBDs | 45 | 65 | 27 | 138 | **2.41** | 1.54 | 3.77 | **<0.001** |
| Tocilizumab | 2 | 14 | 8 | 251 | 1.75 | 0.73 | 4.17 | 0.201 |
| Sarilumab | 0 | 2 | 1 | 272 | – | – | – | – |
| Olokizumab | 22 | 41 | 32 | 180 | 1.28 | 0.81 | 2.03 | 0.292 |
| Levilimab | 2 | 31 | 9 | 233 | **3.44** | 1.64 | 7.23 | **<0.001** |

Note: McNemar p-test, GEBDs – genetically engineered biological drugs, OR – odds ratio, CI – confidence interval, OR and *p* <0.05 are highlighted in bold.

## DISCUSSION

Despite the fact that COVID-19 is characterized by a lower incidence of bacterial complications compared to the influenza virus, the widespread use of immunosuppressants to treat the cytokine storm is associated with a higher risk of secondary bacterial complications, as shown by the present study and some others [20–22]. In a retrospective single-center cohort study of 2020 [20] with a group selection in a 2:1 ratio (74 patients received tocilizumab, 148 – standard therapy), the use of tocilizumab in patients with a severe and extremely severe COVID-19 was associated with a lower mortality, but with a longer duration of hospitalization. An increase in the duration of hospitalization was associated by R. Rossotti et al., among other things, with the development of infectious complications, which were observed in 32.4% of patients receiving tocilizumab [20]. B. Minihan et al. [21] based on a retrospective analysis of medical records of patients hospitalized with a severe and extremely severe COVID-19, concluded that serious bacterial and fungal infections occurred among 41 patients who had received tocilizumab compared with 33 patients who had received standard therapy (OR=2.67; 95% CI 1.04-6.86; $p$=0.042). V. Moreno-Torres et al. [22] studied the prevalence and risk factors for bacterial infections in 1594 hospitalized COVID-19 patients. Patients with a bacterial infection (135/1594) were more likely to receive tocilizumab compared with patients without signs of bacterial infection (40 *vs.* 16.9%, $p$ <0.001) [21]. Not all the studies devoted to the investigation of bacterial infections in COVID-19 patients, describe the diagnostic methodology for these complications. However, in the study by V. Moreno-Torres et al. [21], as in the present one, it was indicated that in the individuals for whom bacteriological testing was not possible, the criteria for confirming a bacterial infection were neutrophilic leukocytosis and an increase in procalcitonin levels.

Not all studies have detected a significant association between the use of tocilizumab and bacterial complications. Thus, in a retrospective cohort study with matched groups 1:1 (59 patients received tocilizumab therapy), no reliable differences in the incidence of SBI and fungal infections were found out [23]. In the present study, despite the fact that the rate of the use of any GEBDs was higher in patients with SBI signs for tocilizumab, this was not significant (Tables 3 and 4).

Tocilizumab is the first of the interleukin-6 receptor blocking GEBDs; together with the biosimilar sarilumab, it is included in the international recommendations for the management of COVID-19 patients [24]. Most of the data on the efficacy and safety of anti-inflammatory GEBDs were obtained for tocilizumab [20–24]. In 2020, two biosimilars, olokizumab and levilimab, were approved for use in the Russian Federation and began to be used as an alternative to tocilizumab. These two GEBDs were most frequently used in the hospital under study, but a significant link between the development of SBI and the use of GEBDs was found out for only levilimab. The target of olokizumab is interleukin-6 itself, the excess of which circulates in the blood plasma during the development of a “cytokine storm”, while levilimab, similar to tocilizumab and sarilumab, blocks interleukin-6 receptors on immunocompetent cells. Perhaps the difference in the mechanism of the olokizumab action compared to other anti-cytokine drugs – a blockade of a freely circulating interleukin-6 excess, and not its receptor, on immunocompetent cells, causes a lower incidence of bacterial complications due to the immunosuppressive therapy. This fact requires a further study not only in COVID-19 patients, but also in the patients with rheumatoid arthritis and other systemic inflammatory diseases of the connective tissue.

### Study limitations

The main limitation of the present study is common to all case-control analyses compared to randomized controlled trials: although the selection of an appropriate control group seems effective, the possibility of selection bias cannot be completely excluded. The retrospective nature of the study also reduces the reliability of observations, and the detection of SBI is based primarily on clinical data rather than bacteriological examination data. Most patients received empirical antibacterial therapy upon admission to hospital, as a result of which a bacteriological examination was not performed or the results were uninformative, and procalcitonin levels may have been underestimated due to the immunosuppressant therapy, as shown in the study by E.J. Kooistra et al. [25].

## CONCLUSION

The use of any anti-inflammatory biological drug was associated with the development of nosocomial infection signs (OR=2.41; 95% CI from 1.54 to 3.77; $p$ <0.001): for levilimab OR=3.44 (95% CI from 1.64 to 7.23; $p$ <0.001), for tocilizumab – OR=1.75 (95% CI from 0.73 to 4.17; $p$=0.201), for olokizumab – OR=1.28 (95% CI from 0.81 to 2.03; $p$=0.292). According to the conducted study, among the three drugs (tocilizumab, olokizumab, levilimab), the Russian biosimilar olokizumab has the greatest safety in relation to the development of nosocomial infection signs. Further studies of the risks of developing bacterial complications in COVID-19 patients while using anti-inflammatory GEBDs, are required.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHORS' CONTRIBUTION

Vladimir I. Petrov – study design development, editing and final approval of the article; Anastasia Yu. Ryazanova – data processing, writing the article and final approval of the article; Natalia S. Tokareva – material collection, data processing and final approval of the article. All the authors confirm their authorship compliance with the ICMJE international criteria (all authors made a significant contribution to the conceptualization, research and preparation of the article, read and approved the final version before publication).

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# Analysis of mitochondrial-targeted antioxidant SkQ1 effectiveness on myocardial ischemia-reperfusion injury in a rat model: Focus on morphological and ultrastructural tissue study

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Mitochondrial-targeted antioxidant SkQ1 demonstrates a high efficiency in animal models and potentially can be used for minimizing postoperative complications in an on-pump open-heart surgery.

**The aim** of study was to the assessment of preservation degree and changes in the isolated rat heart characterized by prolonged cardioplegic ischemia, under the condition of donation of different SkQ1concentrations.

**Material and methods**. Isolated Langendorff-perfused rat hearts of Wistar line (*n*=15) were included in the presented study; the effectiveness of 12 ng/mL and 120 ng/mL of SkQ1 was analyzed. A biochemical analysis (superoxide dismutase 2 [SOD2], malondialdehyde [MDA], Troponin-I, heart-type fatty acid-binding protein [H-FABP]), a histological analysis of tissues (hematoxylin and eosin staining), scanning electron microscopy using backscattered electrons and immunofluorescence staining for cytochrome C and cytochrome P450 reductase were performed. The quantitative data were processed using GraphPad Prism 7 (Graph Pad Software, USA).

**Results**. The optimal myocardial preservation was discovered while using 12 ng/mL of SkQ1: the lowest concentrations of MDA (49.5 [41.1; 58.9] mmol/g), Troponin-I (22.3 [20.3; 23.9] pg/mL) and H-FABP (0.8 [0.6; 16.0] ng/mL) were associated with extensive areas of tissues with preserved transverse dark and light bands and a moderate interstitial edema. Moreover, non-deformed mitochondria were located mainly between the contractile fibers. Cytochrome C immunofluorescence was distributed locally, the luminescence intensity was 40% higher compared to the control group ($p$ <0.0001). Increasing SkQ1 concentration to 120 ng/mL contributed to the aggravation of oxidative stress: MDA (63.8 [62.5; 83.0] mmol/g) and H-FABP (12.8 [4.1; 15.3] ng/mL) concentrations were closer to the control group values. Myocardial tissue in this group was characterized by a pronounced edema and a fragmentation of muscle fibers. There were signs of cardiomyocyte decay, myocytolysis and an intracellular edema. Cytochrome C was distributed evenly.

**Conclusion**. 12 ng/mL of SkQ1 demonstrates pronounced antioxidant effects in the ischemic myocardium leading to a higher degree preservation of the heart muscle compared to 120 ng/mL of SkQ1 that was associated with an aggravated oxidative stress and structural changes of the tissue.

**Keywords:** plastomitin; SkQ1; isolated rat heart; oxidative stress; ischemia-reperfusion injury; Langendorff perfusion; histology; scanning electron microscopy; immunofluorescence

**Abbreviations:** ROS – reactive oxygen species; ATP – adenosine triphosphate; SOD – superoxide dismutase; OS – oxidative stress; CVDs – coronary vascular diseases; AC – artificial circulation; SkQ1 – Skulachov's ions with plastoquinone; MDA – malondialdehyde; H-FABP – heart-type fatty acid-binding protein; ELISA – enzyme-linked immunoassay; SEM – scanning electron microscopy.

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# Анализ эффективности действия митохондриально-направленного антиоксиданта SkQ1 на модели ишемии и реперфузии миокарда крыс. Акцент на морфологическом и ультраструктурном исследовании ткани

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Митохондриально-направленный антиоксидант SkQ1 демонстрирует высокую эффективность на разных экспериментальных моделях и может занять важное место в минимизации постоперационных осложнений при проведении операций на открытом сердце в условиях искусственного кровообращения или консервации трансплантата.

**Цель.** Оценить степень сохранности и изменений изолированного сердца крысы, которое подверглось длительной кардиоплегической ишемии, при условии донации разных концентраций SkQ1.

**Материалы и методы.** Исследование эффективности SkQ1 (12 нг/мл, 120 нг/мл и контрольная группа – без донации SkQ1) проведено на модели изолированного сердца крыс линии Wistar (*n*=15) по Лангендорфу. Провели биохимический анализ (супероксиддисмутазы 2 [СОД-2], малонового диальдегида [МДА] тропонин-I, сердечного белка, связывающего жирные кислоты [с-БСЖК]), гистологию ткани (окраска гематоксилин-эозином), сканирующую электронную микроскопию в обратно-рассеянных электронах и иммунофлуоресцентную окраску на цитохром С и редуктазу цитохрома Р-450. Количественные данные обрабатывали в программе GraphPad Prism 7 (GraphPad Software, США).

**Результаты.** Наибольшая сохранность ткани миокарда выявлена при поддержке SkQ1 в концентрации 12 нг/мл: наименьшие концентрации МДА (49,5 [41,1; 58,9] мкмоль/г), тропонин-I (22,3 [20,3; 23,9] пг/мл), с-БСЖК (0,8 [0,6; 16,0] нг/мл) логично сочетались с обширными зонами с сохранением поперечной исчерченности, умеренным интерстициальным отёком. Также выявлены недеформированые митохондрии, расположенные между сократительными волокнами, иммунофлуоресценция цитохрома С была распределена локально, интенсивность свечения на 40% выше в сравнении с контролем ($p$ <0,0001). Увеличение концентрации SkQ1 до 120 нг/мл скорее способствовало усугублению окислительного стресса: концентрации МДА (63,8 [62,5; 83,0] мкмоль/г) и с-БСЖК (12,8 [4,1; 15,3] нг/мл) была ближе к контрольным значениям. Миокард данной группы охарактеризован резко выраженным отёком, фрагментацией мышечных волокон, некоторые группы кардиомиоцитов находились в состоянии глыбчатого распада, миоцитолиза и внутриклеточного отёка. Цитохром С был распределён равномерно в цитозоле кардиомиоцитов.

**Заключение.** SkQ1 в концентрации 12 нг/мл проявлял выраженные антиоксидантные свойства в отношении ишемизированного миокарда, что позволило получить более высокую степень сохранности сердечной мышцы в сравнении с применением SkQ1 в концентрация 120 нг/мл, которая усугубила окислительный стресс и структурные изменения ткани.

**Ключевые слова:** пластомитин; SkQ1; изолированное сердце крысы; окислительный стресс; ишемия-репефузия; перфузия по Лангендорфу; гистология; сканирующая электронная микроскопия; иммунофлуоресценция

**Список сокращений:** АФК – активные формы кислорода; АТФ – аденозинтрифосфат; СОД – супероксиддисмутаза; ОС – окислительный стресс; ССЗ – сердечно-сосудистые заболевания; ИК – искусственное кровообращение; SkQ1 – ионы Скулачёва с пластохиноном; МДА – малоновый диальдегид; с-БСЖК – сердечный белок, связывающий жирные кислоты; ИФА – иммуноферментный анализ; СЭМ – сканирующая электронная микроскопия.

## INTRODUCTION

Antioxidants are molecules maintaining the cellular redox homeostasis via inhibiting reactions of free radicals with other biological molecules. Finally, antioxidants maintain the cell's function and its structural integrity [1]. Free radicals in living systems are represented mainly by reactive oxygen species (ROS; hydrogen peroxide, superoxide, singlet oxygen, hydroxyl radical, etc.) forming in mitochondria during the oxidative metabolism of adenosine triphosphate

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(ATP) generation [2, 3]. Antioxidants can be classified as endogenous (superoxide dismutase (SOD), catalase, glutathione peroxidase, α-tocopherol, glutathione, etc.) [4–6] and exogenous (carotenoids, flavonoids, lycopene, lutein, vitamins) [7–10].

Many pathological processes are accompanied by the increasing ROS production and oxidative stress (OS) development via a feedback mechanism [11, 12]. OS can be involved in the pathogenesis of various cardiovascular diseases [13]. OS burdens the myocardium during an open-heart surgery under the artificial circulation (AC), contributes to the postoperative recovery of patients [14] and plays an important role in the transplant suitability: preservation methods are currently being studied and improved [15, 16]. The violation in the "antioxidant–oxidant" balance requires additional antioxidant therapy; the deposition of antioxidants in mitochondria is a promising direction in the synthesis and study of mitochondria-targeted antioxidants.

In the late 1960s, Academician V.P. Skulachev synthesized the mitochondria-accumulated molecule of triphenylphosphonium. Based on this molecule, the whole class of compounds named "Skulachev ions" was finally developed [17].

The present study is devoted to one of the "strongest Skulachev ions" including a plastoquinone (SkQ1) as an antioxidant component [18], a decamethylene linker and a lipophilic cation that contribute to the successful penetration and incorporation of the antioxidant into the mitochondrial membrane [19–21]. Conducting an *ex vivo* experimental study on an isolated rat heart made it possible to model the clinical state of the myocardium that was subjected to IR, study changes at the tissue and cellular levels, and determine the most effective concentration of SkQ1.

**THE AIM** of study was to the assessment of preservation degree and changes in the isolated rat heart characterized by prolonged cardioplegic ischemia, under the condition of donation of different SkQ1concentrations.

## MATHERIALS AND METHODS

### Study design

The design flowchart is shown in Figure 1.

### Study duration and conditions

The "Plastomitin" drug (SkQ1 concentrate – 1.7 mg/mL) was provided under a scientific cooperation agreement with Mitotech LLC (Moscow, Russia). The working solutions of SkQ1 were prepared by diluting the concentrate with a perfusion solution.

Experiments were carried out on healthy Wistar rats (♂, m=300±50 g, *n*=30) in accordance with the rules of the European Convention (Strasbourg, France, 1986). The animals were kept in standard vivarium conditions with free access to food and water. The daylight hours were 8 hours light and 16 hours without light at a humidity of 68%. The study was approved by the Local Ethics Committee of the Research Institute for Complex Issues of Cardiovascular Diseases (protocol No. 22 dated 10 Decemder 2015). The study was conducted from November to December 2022 at the scientific and technical base of the Research Institute for Complex Issues of Cardiovascular Diseases.

The perfusion of the isolated heart was performed according to Langendorff at a constant fluid column pressure of 80 cm $H_2O$. The Krebs-Henseleit solution containing 118.0 NaCl mmol/L, 25.0 mmol/L $NaHCO_3$, 11.0 mmol/L glucose, 4.8 mmol/L KCl, 1.2 mmol/L $KH_2PO_4$, 1.2 mmol/L $MgSO_4$ and 1.2 mmol/L $CaCl_2$, enriched with a gas mixture (95% $O_2$ and 5% $CO_2$, pH=7.4) with the temperature from 37 to 38°C, was used for the perfusion.

### Perfusion protocol

The cardiac contraction stabilization (perfusion with Krebs-Henseleit solution) takes 20 min; connection of two flows of the perfusion solution with SkQ1 – 10 min; hypoperfusion (20 mL/h) with a cooled (t=4°C) cardioplegic solution (custodiol, Dr. F. KOHLER CHEMIE, GmbH, Germany) – 10 min; global cardioplegic ischemia – 240 min (t=8°C); a reperfusion – 30 min. Hearts included in the first experimental group were perfused with a double flow of the Krebs-Henseleit solution with 120 ng/mL SkQ1. Hearts included in the second experimental group were perfused similarly to the first experimental group with 12 ng/mL SkQ1.

### Study groups

Three study groups were formed: the first experimental group "SkQ1 120 ng" (*n*=10), the second experimental group "SkQ1 12 ng" (*n*=10) perfused as it had been described above, and the control group (*n*=10) that was characterized by no SkQ1 supply to the perfusion solution.

### Studied parameters

### Biochemical parameters

Mitochondrial superoxide dismutase (SOD-2; EU2577, Wuhan Fine Biotech, China), heart-type fatty acid-binding protein (H-FABP; HK414, HycultBiotech,

the Netherlands), and cardiac troponin (troponin I; CSB-E08594r, CUSABIO BIOTECH Co., China) were measured by an enzyme-linked immunosorbent assay. Malondialdehyde (MDA) was determined using the OxiSelect™ TBARS Assay Kit MDA Quantitation (STA-330, Cell Biolabs, USA).

### Histology

The explanted hearts were fixed in 10% buffered formalin (B06-001/M, BioVitrum, Russia) for 24 h, washed with water to remove the fixative solution and dehydrated in IsoPrep (06-002/M, BioVitrum, Russia) for 18 h. Then the samples were permeated with three portions of paraffin at 56°C for 60 min in each portion and embedded in HISTOMIX (247, BioVitrum, Russia). After this, 8 µm thick sections were prepared using the microtome (HM 325, Thermo Scientific, Waltham, MA, USA), placed in a thermostat and dried for 18 h at 37°C. Afterwards, the samples were dewaxed in three portions of o-xylene (103118, JSC LENREACTIV, Russia) for 1–2 min and dehydrated in three portions of 96% alcohol for 1–2 min. The sections were stained with Harris hematoxylin (05-004, BioVitrum, Russia,) for 15–20 min followed by washing up to 10 min; several drops of eosin (05-011/L, BioVitrum, Novosibirsk, Russia) were applied to the section for 30 sec. Then the samples were placed in 96% alcohol. Finally, the sections were cleared in o-xylene for several minutes followed by its subsequent removal. At the last stage, the sections were embedded in the mounting medium Vitrogel (12-005, BioVitrum, Russia) and examined by light microscopy using an AXIO Imager A1 microscope (Carl Zeiss, Oberkochen, Germany) at ×50, ×200 and ×400 magnification.

### Scanning electronic microscopy

The samples were fixed in 4% buffered formalin for 24 h, postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer (10010001, Gibco, USA) and stained with 2% osmium tetroxide (7563, Sigma-Aldrich, St. Louis, MO, USA) in bidistilled water for 48 h. Next, the samples were dehydrated in a series of increasing concentrations of ethanol (50, 60, 70, 80 and 95%), stained with 2% uranyl acetate (22400-2, Electron Microscopy Sciences, USA) in 95% ethanol, dehydrated with 99.7% isopropanol (06-002/M, BioVitrum, Novosibirsk, Russia) for 5 h and acetone (OKP241811ONO, Reachim, Staraya Kupavna, Russia) for 1 h, permeated with a mixture of acetone and Epon epoxy resin (14910, Electron Microscopy Sciences, USA) in a 1:1 ratio (6 h), transferred to a fresh portion of epoxy resin for 24 h and performed its polymerization in FixiForm containers (40300085, Struers, Denmark) at 60°C. Next, the samples in epoxy blocks were ground and polished using a TegraPol-11 unit (60799, Struers, Ballerup, Denmark). Contrasting with lead citrate was carried out in accordance with Reynolds for 7 min by applying the solution to the surface of the ground sample followed by washing it with bidistilled water. Then, carbon was sprayed onto the polished surface of the epoxy blocks (coating thickness was 10–15 nm) using a vacuum sputtering post (EM ACE200, Leica, Germany). The structure of the samples was visualized using the scanning electron microscopy in backscattered electrons on the electron microscope (S-3400N, Hitachi, Japan) in the BSECOMP mode at an accelerating voltage of 10 kV.

### Confocal microscopy

Serial cryosections of 8 µm thickness were prepared from frozen rat myocardial samples (8 samples per heart) at 100 µm intervals using a cryotome (Microm HM 525, Thermo Scientific, USA). The preparations were fixed in a 4% paraformaldehyde solution for 10 min. For the permeabilization, the sections were treated with a 0.1% Triton-X100 solution (X100, Sigma-Aldrich, USA) for 15 min. Next, a mixture of primary antibodies (mouse antibodies to cytochrome C (ab13575, Abcam, UK) and rabbit antibodies to cytochrome P-450 reductase (ab180597, Abcam, UK) were applied to the sections and incubated for 18 h at 4°C. Then, the sections were washed and incubated with a mixture of secondary antibodies (goat antibodies to rabbit IgG conjugated with Alexa Fluor 488-conjugated (A11034, Thermo Fisher Scientific, USA) and goat antibodies to mouse IgG conjugated with Alexa Fluor 555-conjugated (A31570, Thermo Fisher Scientific, USA) for 1 h at room temperature. At all staining stages, phosphate-buffered saline supplied with 0.1% Tween 20 (P9416, Sigma-Aldrich, USA) was used. To remove autofluorescence, the sections were treated with Autofluorescence Eliminator Reagent (2160, Merck KGaA, Germany) according to the manufacturer's protocol. Cell nuclei were counterstained with DAPI (10 µg/mL, D9542, Sigma-Aldrich, USA) for 30 min. The prepared samples were cover-slipped using ProLong Gold Antifade Mountant (P36930, Thermo Fisher Scientific, USA). After drying, the samples were analyzed using the confocal microscope LSM700 (Carl Zeiss, Germany).

For each sample, 10 randomly selected fields of view were analyzed using ImageJ software (Wayne Rasband (NIH), USA) and the measured the average fluorescent signal intensity.

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### Statistical analysis

The obtained data were analyzed using GraphPad Prism 7 software (GraphPad Software, USA). The data distribution was assessed using the Kolmogorov-Smirnov and Shapiro–Wilk tests. Quantitative data are presented as median (Me) and quartiles [25%; 75%]. Intergroup differences were assessed using the Kruskal–Wallis criterion with Dann's correction. Differences were considered statistically significant at $p < 0.05$.

## RESULTS AND DISCUSSION

### Biochemical analysis

SOD is one of the most important metal-containing enzymes involved in the cellular antioxidant system and an integral criterion for the evaluation of the OS level. Its mitochondrial isoform (SOD-2) can characterize the local antioxidant system of cells and a mitochondrial dysfunction [22–24]. In present experiment, after modeling of 240-minute cold cardioplegia, the SOD-2 concentration had no significant differences between the experimental and control groups (Table 1).

MDA is a product of oxidation of ω-3 and ω-6 fatty acids, a reactive aldehyde that covalently binds to lipids, proteins and DNA, thereby disrupting their function, it can be suggested as another OS marker [25–27]. The maximum amount of MDA per 1 g of myocardial tissue was found in the control group without any SkQ1 support and reached 70.9 [58.7; 87.8] μmol/g. The minimum MDA concentration was observed in the "SkQ1 12 ng" group 49.4 [41.1; 58.9] μmol/g, which was statistically significantly lower than the control values ($p$=0.02). H-FABP, located in the cytoplasm of the striated muscle cells, is quickly released in response to the damage, so it can be used as a sensitive marker of an ischemic myocardial injury [28, 29]. The median reflecting the level of released H-FABP into the myocardial outflow in the end of the reperfusion was lower in the "SkQ 12 ng group" (0.8 ng/mL) in comparison with other studied groups (12.8 ng/mL in the "SkQ 120 ng" group and 9.0 ng/mL in the control group), but this decreasing was not statistically significant. Cardiac troponin is a clinical biomarker for the diagnosis of the myocardial infarction [30]. The concentration of troponin-I was statistically significantly lower in the SkQ1 12 ng and SkQ1 120 ng groups compared to the control ($p$=0.03; Table 1).

So, the additional antioxidant support from 12 ng/mL SkQ1 leads to the OS inhibition and the decreasing release of myocardial injury markers under 240-minute cold cardioplegic ischemia followed by a reperfusion.

### Histological analysis

The histological analysis data are correlated with the results of the biochemical analysis. The most significant structural changes in the myocardium (cardiomyocytes, vessels and stroma) were found in the control and "SkQ1 120 ng" groups. In the control group, a diffuse edema in the interstitium was noted (Fig. 2A). The vessels were paretically dilated, empty with swollen endothelium. The muscle fibers in the intramural zones were thinned, discomplexed, wavy deformed and unevenly stained. Extensive areas of the muscle fiber fragmentation in the subepicardial and subendocardial zones could be visualized. The cytoplasm of cardiomyocytes was homogeneous oxyphilic without a transverse striation (Fig. 2B).

Despite the established biochemical parameters, supplying of 120 ng/mL SkQ1 into the perfusion solution leads to significant structural changes. Myocardium is characterized by a pronounced edema (Fig. 2C) mainly in the perivascular spaces and intermuscular layers. Paretic dilation of vessels is accompanied with an endothelial detachment in some of them. The fragmentation and discomplexation of cardiomyocytes are more pronounced in the subepicardial and subendocardial zones. Fragmentation and depletion of the muscle fibers are combined with their waviness and polychromasia. The integrity of the sarcolemma is significantly impaired. Groups of cardiomyocytes characterized by a lumpy disintegration and myocytolysis, as well as an intracellular edema, have been discovered. The cell nuclei are deformed, unevenly enlarged and edematous, and characterized by a blurry outline and a diffuse enlightenment of chromatin. The focal lipofuscinosis has been noted (Fig. 2D).

The myocardium of the "SkQ1 12 ng" group was characterized by minimal structural changes. The extensive areas of compact cardiomyocytes arrangement alternated with the fiber fragmentation areas. Moderate edema, mainly in the perivascular zones, was noted in the interstitium. The nuclei of cardiomyocytes were clearly visible; they were located closer to the center of the cytoplasm, in which a transverse striation was preserved. However, the groups of the cells with swelling cytoplasm and poorly visible nuclei with unclear contours were visualized (Fig. 2E and 2F).

Thus, discirculatory, dystrophic and destructive changes in the myocardium were revealed in all the studied groups. However, these changes were less pronounced in the groups with an additional antioxidant support of the isolated hearts during the anoxia period by 12 ng/mL SkQ1 and had a mosaic character,

combined with extensive zones of relatively intact myocardium.

### Scanning electron microscopy in backscattered electrons

High-magnification images of the samples obtained using scanning electron microscopy made it possible to analyze the intracellular changes in details. Without antioxidant support from SkQ1, the presence of zones with "erased" transverse striations was noted. In these zones, mitochondria "stuck" together and formed electron-dense structures that did not have a clear division into individual organelles in their structure. The nuclei were compressed, usually round in shape without a clear boundary, tightly adjacent to the electron-dense contents of the cytoplasm, the chromatin was located in a disordered manner (Fig. 3A and 3B). At high magnifications, a disruption of the structure of contractile fibers was observed, which did not have a clear structure, probably due to lysis (Fig. 3C). The space between the cells was expanded and filled with fibrous contents.

In the "SkQ1 120 ng" group, enlarged spaces between cardiomyocytes containing fragments of capillaries and cells were found (Fig. 3D). Cytoplasm of cardiomyocytes in the areas of the mitochondria localization was characterized by the presence of vacuole-like structures with transparent contents (Fig. 3E). Mitochondria had various shapes and a high electron density, some of them had surface outgrowths. These organelles often formed dense clusters and were adjacent to free spaces. In some areas, mitochondria had a tight contact with contractile fibers (Fig. 3F). In this group, the nuclei of cardiomyocytes were light, elongated, the nuclear membrane was uneven, the nucleoli and euchromatin were practically absent (Fig. 3E).

In the "SkQ1 12 ng" group, the transverse striation of the myocardial samples was discovered. The contractile fibers and cardiomyocytes were highly preserved (Fig. 3G–I). The mitochondria with clear boundaries were mostly located in the layers between the contractile fibers (Fig. 3 H, I). The nuclei of cardiomyocytes were elongated, the surface was smooth. Euchromatin was located parietal; the nucleoli were registered in the center of the nuclei. Free spaces were usually located between the outer surface of the nucleus and the contractile fibers.

Thus, a high structural and intracellular preservation of the myocardium treated by 12 ng/mL SkQ, was confirmed and detailed.

### Confocal microscopy

Cytochrome C is a globular protein covalently linked to the heme group performing many important functions. It acts as an electron carrier from complex III to complex IV in the mitochondrial electron transport chain [31]. Cytochrome C, released from the mitochondria, leads to the apoptosis activation [32]. The distribution and intensity of cytochrome C fluorescence in the studied tissues differed depending on the SkQ1 concentration. The most increased immunofluorescence was noted in both groups supplied with SkQ1, the control group was characterized by the extremely low immunofluorescence (Figure 4A–C). Quantitative analysis confirmed the visual observations: cytochrome C fluorescence in the myocardial tissue from the "SkQ1 12 ng" group was statistically significantly higher compared to the control ($p$ <0.0001, Fig. 3D). At the same time, no statistically significant differences between the experimental groups were found. It should be noted that 12 ng/mL SkQ1 supplying is associated with the most local increase in the luminescence of cytochrome C in the areas of structural preservation of the tissue. 120 ng/mL SkQ1 treatments led to the relatively uniform immunofluorescence of cytochrome C that may indicate the release of this enzyme into the cytosol.

Cytochrome P-450 reductase (POR) is a part of the cytochrome P-450 system localized in the membrane of the smooth endoplasmic reticulum. This enzyme is necessary for the transfer of electrons from NADP to cytochrome P-450 in microsomes, as well as to heme oxygenase and cytochrome B5 [33]. POR was poorly visualized in the tissue, and there was no statistical differences found between the studied groups (Fig. 3A–D).

Here, the concentration-depended effectiveness of the mitochondria-targeted antioxidant SkQ1 on the isolated rat heart model according to Langendorff has been demonstrated. The chosen model can help to directly access the effect of the studied antioxidant, its cardiotoxicity, excluding the influence of hormones, the autonomic nervous system and other organs [34]. The stages of cardiac perfusion had been constructed to model the clinical conditions during heart surgeries under artificial circulation or conditions close to the transplantation. The similar studies had already been previously conducted [35].

It has been found out that the 12 ng/mL SkQ1 is associated with the greatest preservation of the myocardium at the tissue and cellular levels. A histological examination and scanning electron microscopy revealed extensive zones with undeformed muscle fibers, intact sarcolemma and organelles of cardiomyocytes. These data were associated with the decreased levels of OS

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and myocardial injury markers. This concentration is probably triggering the binding of SkQ1 to cardiolipin followed by the decreasing peroxidase activity of cytochrome C, which accordingly led to the decreasing of OS in mitochondria, their structural preservation, which ultimately did not allow cytochrome C to enter the cytosol [36]. L.E. Bakeeva et al. have performed an *ex vivo* and *in vivo* study of the dose-dependent SkQ1 effect on Wistar rats and concluded that low SkQ1 concentrations can have a pronounced antioxidant effect in case of OS [37]. In 2021, M. Hamed et al. showed that 50 nmol/L MitoQ (SkQ analogue developed in New Zealand included ubiquinone in its structure) was associated with the pronounced antioxidant effect followed by an increase in the total blood flow and diuresis in the kidneys of pigs and humans [38].

In the present experiments, a 10-fold increase in the SkQ1 concentration (120 ng/mL) triggered OS in modeling an ischemic and reperfusion injury in the isolated heart. The study showed the extensive areas with deformed and disconnected muscle fibers, edema, lumpy decay (signs of acute tissue necrosis) with relatively high-level OS markers and a myocardial injury and, at the same time, continuous intracellular immunofluorescence of cytochrome C signaling a violation of the structure and function of mitochondria and possible apoptosis triggering. It can be concluded that perfusion of an isolated heart for 10 min with 120 ng/mL SkQ1 before the global ischemia has a cardiotoxic effect. The concentration of mitochondria-targeted antioxidant plays an important role in the correction of OS.

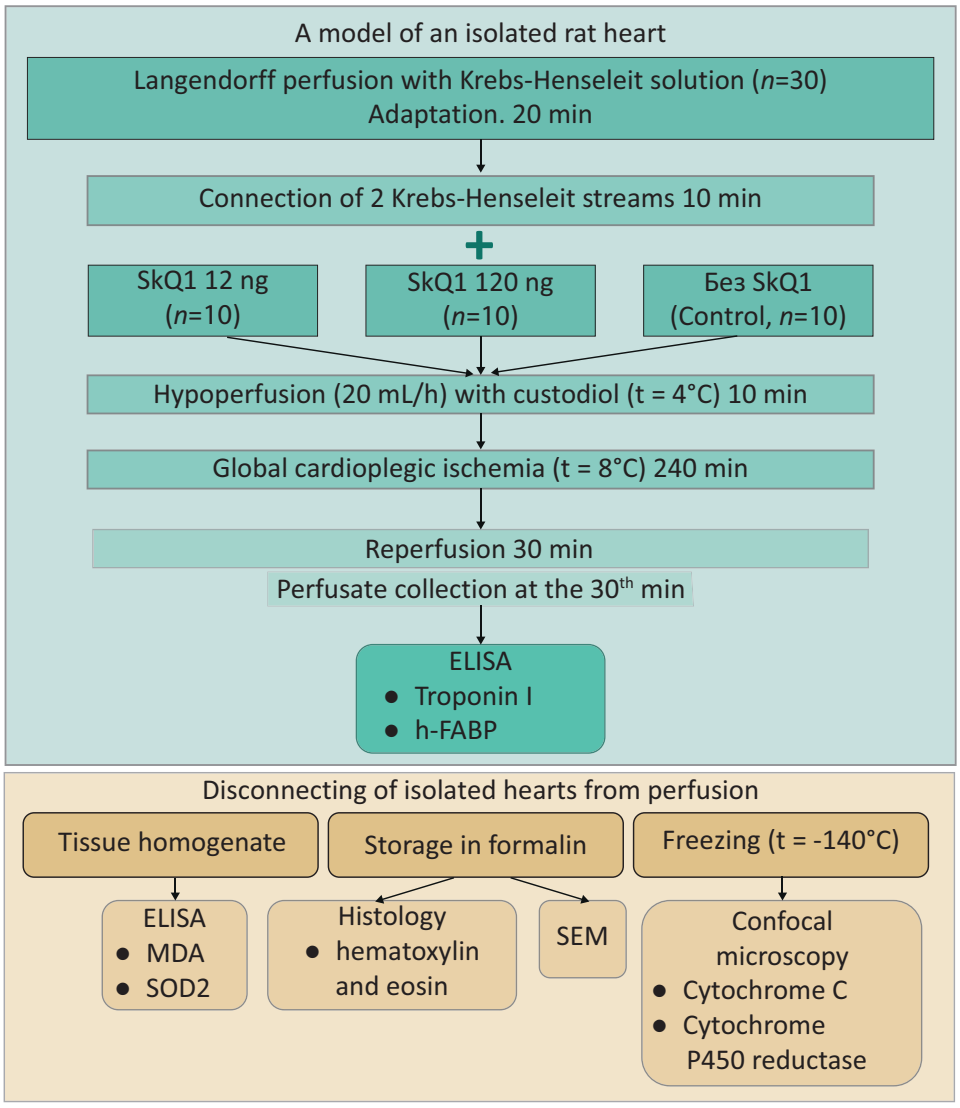


**Figure 1 – Diagram of study design**

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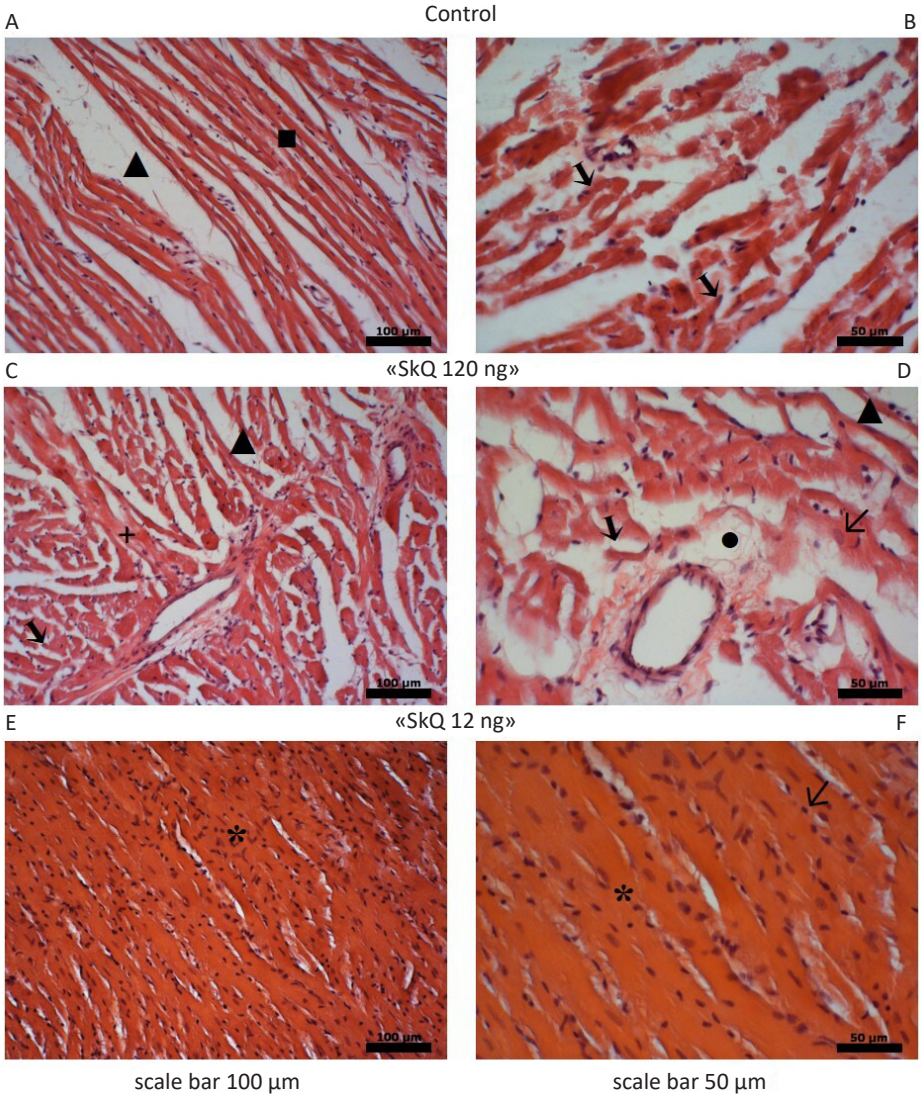


**Figure 2 – Histological staining of myocardial sections with hematoxylin and eosin**

Notes: A – diffuse interstitial edema (▲), stretching and thinning of muscle fibers (■). Magnification ×20; B ("SkQ1 120 ng") – fragmentation of muscle fibers, discomplexation of cardiomyocytes (↓), oxyphilia of the cardiomyocytes cytoplasm. Magnification ×40; C – disunion, fragmentation (↓) and polychromasia of muscle fibers, interstitial edema (▲), myocytolysis and intracellular edema of cardiomyocytes (+), magnification×20; D ("SkQ1 12 ng") – fragmentation of muscle fibers (↓), perivascular (●) and interstitial edema (▲). Swelling of cardiomyocyte nuclei (↓), magnification ×20; E – Compact arrangement of muscle fibers (*), magnification ×20; E – compact arrangement of muscle fibers (*), magnification ×20. F – compact arrangement of muscle fibers (*), well-distinguished nuclei of cardiomyocytes (↓), preservation of sarcolemma, magnification ×40.

**Table 1 – Biochemical parameters of oxidative stress and myocardial injury**

| Groups | Study parameters, Me [25%; 75%] | | | |
|---|---|---|---|---|
| | SOD-2, ng/mL | MDA, µmol/g | Troponin-I, pg/mL | H-FABP, ng/mL |
| Control | 13.0 [8.3; 18.3] | 70.9 [58.7; 87.8] | 47.7 [29.3; 54.2] | 9.0 [2.1; 17.6] |
| SkQ1 120 ng | 14.4 [11.6; 20.4] | 63.8 [62.5; 83.0] | 24.2 [23.5; 25.9]* | 12.8 [4.1; 15.3] |
| SkQ1 12 ng | 16.0 [8.4; 17.9] | 49.5 [41.1; 58.9]* | 22.3 [20.3; 23.9]* | 0.8 [0.6; 6.0] |

Note: * $p$ <0,05 compared to the control group. SOD – superoxide dismutase; MDA – malondialdehyde; H-FABP – cardiac fatty acid binding protein.

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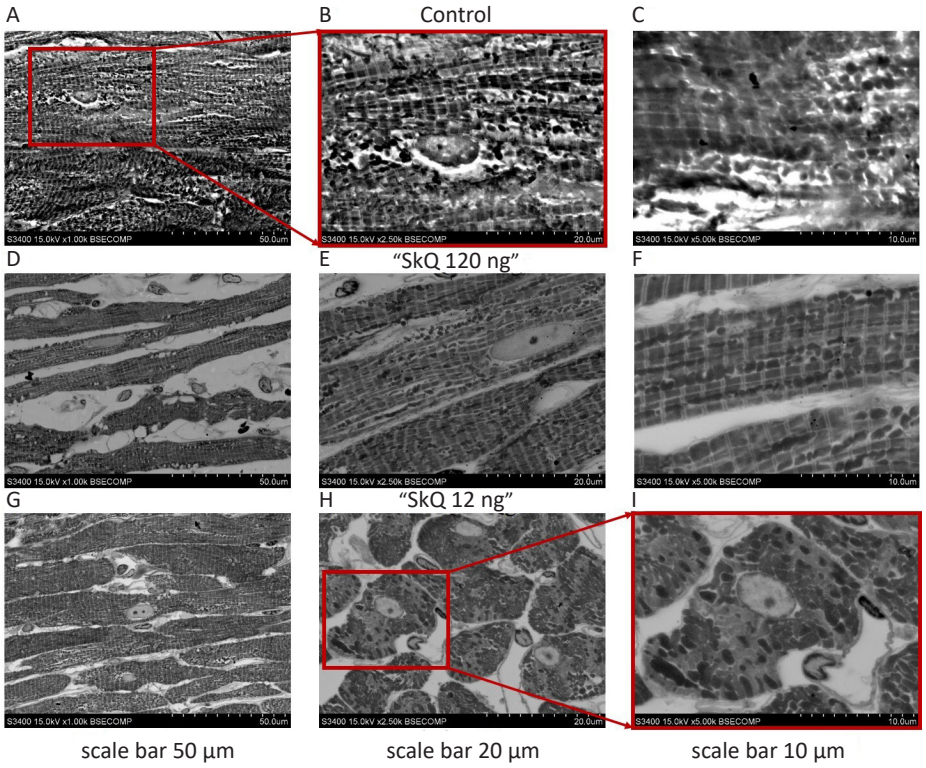


**Figure 3 – Scanning electron microscopy of the myocardium**

Notes: A – Wave-like deformation, zones of erased striation, magnification ×1000; B – Compressed nucleus, magnification ×2500; C – ("SkQ1 120 ng") – erased striation, disruption of the structure of contractile fibers, magnification×5000; D – dissociation of cardiomyocytes, magnification ×1000; E – Vacuole-like spaces around mitochondria, magnification ×2500; F – (SkQ1 12 ng) – clusters of mitochondria, magnification×5000; G – preservation of cardiomyocyte striation, magnification ×1000; H, I – layers of mitochondria around muscle fibers, elongated nuclei, magnification ×2500 and ×5000.

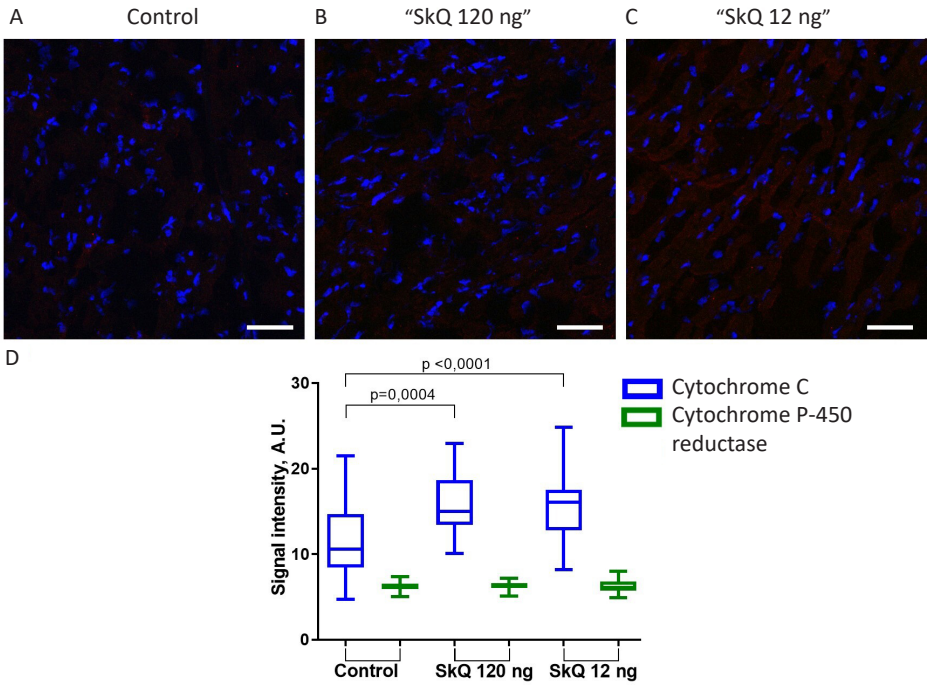


**Figure 4 – Immunofluorescent staining of myocardial tissue sections**

Notes: A – Control, weak fluorescence of immunofluorescent labels; B – SkQ1 120 ng, differentiated fluorescence of cytochrome C; C – SkQ1 12 ng, well-differentiated and zonal fluorescence of cytochrome C; D – quantitative analysis of cytochrome C and cytochrome P-450 reductase. The data are presented as a median with interquartile range and maximum and minimum values.Scale bar is 50 µm. Cytochrome C is red, cytochrome P-450 reductase is green, cell nucleus is blue.

### Study limitations

The present study is limited by a small sample size (10 hearts in each study group). It should be noted that despite all the advantages and simplicity of the isolated heart model, the cut-off of a systemic influence could be also a limitation on the regulation of the cardiac muscle activity.

## CONCLUSION

Mitochondria-targeted antioxidant SkQ1 (12 ng/mL) is associated with a pronounced antioxidant and cardioprotective effect on the model of ischemia and reperfusion on an isolated rat heart reflected in a high degree of preservation of the contractile apparatus of the myocardium and organelles. At the same time, a concentration of 120 ng/mL aggravated OS and led to destructive tissue damage. This antioxidant is extremely promising in the field of creating new cardioprotective drugs for a cardiac surgery and transplantation and requires further research.

At a concentration of 12 ng/mL, SkQ1 showed pronounced antioxidant properties against ischaemic myocardium, which resulted in a higher degree of cardiac muscle preservation compared with the use of SkQ1 at a concentration of 120 ng/mL, which exacerbated an oxidative stress and structural tissue changes.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Evgeniya A. Senokosova, Evgeniy V. Grigoriev – study conception and design; Evgeniya A. Senokosova, Elena A. Velikanova, Rinat A. Mukhamadiyarov, Olga D. Sidorova, Evgeniya O. Krivkina, Larisa V. Antonova – research conducting, results processing and analyzing; Evgeniya A. Senokosova, Elena A. Velikanova – statistical analysis; Evgeniya A. Senokosova, Elena A. Velikanova, Rinat A. Mukhamadiyarov – manuscript preparation; Larisa V. Antonova, Evgeniy V. Grigoriev – manuscript revision. All authors confirm that their authorship complies with the international ICMJE criteria (all authors made a significant contribution to the concept development, research, and preparation of the article, read and approved the final version before publication).

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# Comparative analysis of pharmacokinetic parameters, bioequivalence, safety, tolerability and immunogenicity of semaglutide-based drug for the treatment of obesity

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One of the new classes of drugs for weight loss in overweight and obesity, the safety and efficacy of which have been proven in large-scale studies, are glucagon-like peptide-1 receptor agonists (GLP-1 agonists). Separately, it is worth highlighting the main representative from the GLP-1 agonists class, semaglutide. At a dose of 2.4 mg, this drug demonstrated clinically significant results in terms of the body weight reduction and improvement of cardiometabolic health.

**The aim** of the work was to evaluate pharmacokinetic parameters, bioequivalence, safety, tolerability and immunogenicity of the Velgia® (WRYC12301) at doses of 0.25 mg (0.68 mg/mL) and 2.4 mg (3.2 mg/mL) in comparison with the reference drug Wegovy® (Novo Nordisk A/S, Denmark) at the doses of 0.25 mg (0.68 mg/mL) and 2.4 mg (3.2 mg/mL).

**Materials and methods.** The study was conducted between March and June 2024. The volunteers (*n*=60) were randomised into 4 groups (*n*=15 in each) in a 1:1 ratio to study the semaglutide dosages of 0.25 mg/dose (0.68 mg/mL) in Groups 1, 2 and 2.4 mg/dose (3.2 mg/mL) in Groups 3, 4. The study drug and the reference drug were injected subcutaneously into the anterior abdominal wall. Pharmacokinetic parameters, bioequivalence, safety, tolerability and immunogenicity of semaglutide (solution for subcutaneous administration, JSC Biochemik, Russia) were studied. Some parameters regulating the quality of the active pharmaceutical substance semaglutide, were determined.

**Results.** The obtained 90% confidence intervals (CIs) for the ratio of $C_{max}$ and $AUC_{(0-t)}$ values of the study and reference drugs (Groups 1, 2) at a dose of 0.25 mg (0, 68 mg/mL) were 85.19–114.36% for $C_{max}$ and 81.35–112.60% for $AUC_{(0-t)}$, respectively, while for Groups 3, 4, at a dose of 2.4 mg (3.2 mg/mL), $C_{max}$ was 83.18–111.3% and $AUC_{(0-t)}$ was 91.70–120.89%, respectively. The obtained 90% CI lies within the established limits, which confirms the bioequivalence of the study and reference drugs. All adverse events registered during the study were of mild severity. According to the results of the immunogenicity parameters analysis, no antibodies to semaglutide were detected in the serum of volunteers.

**Conclusion.** In the course of the study, the bioequivalence of the study and reference drugs was confirmed. A high safety profile and absence of immunogenicity were demonstrated for the Russian drug Velgia® (WRYC12301, semaglutide, solution for a subcutaneous administration, JSC Biochemik, Russia) in comparison with the reference drug (semaglutide, solution for a subcutaneous administration, Novo Nordisk A/S, Denmark) in doses of 0.25 mg/dose (0.68 mg/mL) and 2.4 mg/dose (3.2 mg/mL).

**Keywords:** semaglutide; glucagon-like peptide-1 receptor agonist; obesity; subcutaneous administration; safety; tolerability; immunogenicity; pharmacokinetic parameters

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**Abbreviations:** T2D — type 2 diabetes; MAFLD — metabolic-associated fatty liver disease; GLP-1 — glucagon-like peptide-1; GLP-1 agonists — glucagon-like peptide-1 receptor agonists; HbAlc — glycated haemoglobin; ELISA — enzyme-linked immunosorbent assay; BMI — body mass index; GI — gastrointestinal tract; BAC — biologically active compound; HR — heart rate; RR — respiratory rate; ECG — electrocardiography; AE — adverse event; SAE — serious adverse event; CI — confidence interval; API — active pharmaceutical ingredients.

# Сравнительное исследование фармакокинетических параметров, биоэквивалентности, безопасности, переносимости и иммуногенности лекарственного препарата для лечения ожирения на основе семаглутида

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Одним из новых классов препаратов для снижения массы тела при избыточном весе и ожирении, безопасность и эффективность которых доказаны в масштабных исследованиях, являются агонисты рецепторов глюкагоноподобного пептида-1 (АР ГПП-1). Отдельно стоит выделить основного представителя из класса АР ГПП-1 — семаглутид. Данный препарат в дозе 2,4 мг продемонстрировал клинически значимые результаты в отношении уменьшения массы тела и улучшения параметров кардиометаболического здоровья.

**Цель.** Оценить фармакокинетические параметры, биоэквивалентность, безопасность, переносимость и иммуногенность отечественного лекарственного препарата Велгия® (WRYC12301) в дозировках 0,25 мг/доза (0,68 мг/мл) и 2,4 мг/доза (3,2 мг/мл) в сравнении с референтным препаратом Wegovy® (Ново Нордиск А/С, Дания) в дозировках 0,25 мг/доза (0,68 мг/мл) и 2,4 мг/доза (3,2 мг/мл).

**Материалы и методы.** Исследование было проведено в период с марта по июнь 2024 года. Добровольцы (*n*=60) были рандомизированы в 4 группы (*n*=15 в каждой) в соотношении 1:1 для исследования дозировки семаглутида 0,25 мг/доза (0,68 мг/мл) — Группа 1, 2 и 2,4 мг/доза (3,2 мг/мл) — Группа 3, 4. Исследуемый препарат и препарат сравнения вводили подкожно в переднюю брюшную стенку. Были изучены фармакокинетические параметры, биоэквивалентность, безопасность, переносимость и иммуногенность исследуемого препарата семаглутида (раствор для подкожного введения, АО «Биохимик», Россия). Определены некоторые показатели, регламентирующие качество активной фармацевтической субстанции семаглутид.

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**Результаты.** Полученные 90%-ные доверительные интервалы (ДИ) для отношения значений $C_{max}$ и $AUC_{(0-t)}$ исследуемого и референтного препарата (Группа 1, 2) в дозировке 0,25 мг/доза (0,68 мг/мл) составили для $C_{max}$ — 85,19–114,36% и $AUC_{(0-t)}$ — 81,35–112,60%, соответственно, для Группы 3, 4 дозировки 2,4 мг/доза (3,2 мг/мл) — $C_{max}$ составил 83,18–111,3%, а $AUC_{(0-t)}$ — 91,70–120,89%, соответственно. Полученные 90% ДИ лежат в установленных границах, что подтверждает биоэквивалентность исследуемого и референтного препаратов. Все зарегистрированные в ходе исследования нежелательные явления были лёгкой степени тяжести. По результатам анализа параметров иммуногенности у добровольцев не были выявлены антитела к семаглутиду в сыворотке крови.

**Заключение.** В ходе проведённого исследования была подтверждена биоэквивалентность исследуемого и референтного препаратов. Был продемонстрирован высокий профиль безопасности и отсутствие иммуногенности у российского препарата Велгия® (WRYC12301, семаглутид, раствор для подкожного введения, АО «Биохимик», Россия) в сравнении с зарубежным референтным препаратом (семаглутид, раствор для подкожного введения, Ново Нордиск А/С, Дания) в дозировках 0,25 мг/доза (0,68 мг/мл) и 2,4 мг/доза (3,2 мг/мл).

**Ключевые слова:** семаглутид; агонист рецепторов глюкагоноподобного пептида-1; ожирение; подкожное введение; безопасность; переносимость; иммуногенность; фармакокинетические параметры

**Список сокращений:** СД 2 — сахарный диабет 2 типа; МАЖБП — метаболически ассоциированная жировая болезнь печени; ГПП-1 — глюкагоноподобный пептид-1; АР ГПП-1 — агонисты рецепторов глюкагоноподобного пептида-1; HbAlc — гликированный гемоглобин; ИФА — иммуноферментный анализ; ИМТ — индекс массы тела; ЖКТ — желудочно-кишечный тракт; БАД — биологически активная добавка; ЧСС — частота сердечных сокращений; ЧДД — частота дыхательных движений; ЭКГ — электрокардиография; НЯ — нежелательное явление; СНЯ — серьёзное нежелательное явление; ДИ — доверительный интервал; АФС — активная фармацевтическая субстанция.

## INTRODUCTION

Obesity has become one of the most pressing public health problems in the world today. According to the World Health Organization (WHO) in 2022, one in eight people on the planet was obese. According to the forecast, by 2025, about 46% of the world's adult population will be overweight [1].

Obesity (body mass index [BMI] >30 kg/m$^2$) and overweight (BMI=25–29.9 kg/m$^2$) are chronic diseases characterized by an excessive accumulation of the adipose tissue in the body as a result of an excessive energy value (calories) of the diet over physiological needs of a person. The presence of obesity and excess body weight are considered to be the main risk factors for the development of a number of chronic diseases, including type 2 diabetes (T2D), cardiovascular diseases (CVDs), metabolically associated fatty liver disease (MAFLD), cancer, musculoskeletal diseases, mental health disorders, etc. [2].

A 5–10% reduction in body weight has been shown to improve glycemic control, reduce risk factors for CVDs, as well as an insulin resistance, an arterial hypertension, lipid metabolism disorders, including cholesterol and triglyceride concentrations, inflammatory markers and an endothelial dysfunction. The treatment of obesity and overweight is based on a comprehensive approach, including a proper nutrition, a physical activity, pharmacological therapy and, if necessary, endoscopic procedures or, in some cases, bariatric surgery [3]. At the same time, a lifestyle modification without additional therapy provides clinically significant weight loss only in a small category of patients. According to the statistical studies, only 10% of people manage to maintain the achieved weight after weight loss, while the rest return to their previous habits and initial weight parameters [4, 5]. It should be noted that the treatment of obesity and overweight with bariatric surgery is associated with a number of limitations and possible complications both during and after the surgery [1, 6, 7].

Recent scientific advances in the study of the obesity pathogenesis have made it possible to develop and introduce into clinical practice new promising drugs for reducing excess weight and maintaining the achieved results. Taking into account the role of glucagon-like peptide-1 receptor (GLP-1) in the regulation of glucose metabolism and energy balance, as well as significant effects on other organs and systems, drugs capable of stimulating GLP-1 receptors similar to the native hormone — GLP-1 agonists have been developed [7, 8].

Semaglutide is a 94% homologous analogue of human GLP-1. The half-life of semaglutide (about 1 week) allows its use subcutaneously once a week. Its use in patients with T2D not only contributed to the effective glycaemic control, but also resulted in a significant weight loss and control of CVD risks. Semaglutide was initially approved for the treatment of T2D at a dose of 0.5 or 1.0 mg per week; in 2021, the FDA[1] approved a dosage of 2.4 mg [9, 10].

[1] U.S. Food and Drug Administration. FDA Approves New Drug Treatment for Chronic Weight Management, First Since 2014 (For Immediate Release: June 04, 2021). Available from: https://www.fda.gov/news-events/press-announcements/fda-approves-new-drug-treatment-chronic-weight-management-first-2014

The dose-dependent effect of semaglutide on weight loss in patients with T2D was observed in the SUSTAIN clinical trial as well as in real clinical practice. Therefore, phase 2 clinical trials were conducted to evaluate the effect of semaglutide on weight in patients without diabetes, in which effective doses and concentrations of semaglutide corresponding to 1.7 and 2.4 mg have been identified [11].

According to the results of the STEP 1 clinical trial, in the semaglutide group, 9 out of 10 patients achieved a clinically significant weight loss of 5% or more, and every third patient achieved a 20% or more reduction in body weight. The mean weight reduction was 16.9% from baseline (17.2 *vs.* 2.7 kg in the placebo group). The body weight reduction was mainly due to the adipose tissue — minus 10.4 kg in the semaglutide (2.4 mg) group and minus 1.17 kg in the placebo group. Semaglutide therapy at a dose of 2.4 mg had a more favourable effect on the cardiometabolic health: this dosage contributed to a reduction in waist circumference (-15.22 cm), a normalization of lipid profile and blood pressure [12, 13].

The STEP 2 clinical trial evaluated the effect of semaglutide therapy on weight loss (0.25, 0.5, 1.0, 1.7 and 2.4 mg) compared to semaglutide for the treatment of T2D (0.25, 0.5 and 1.0 mg) in patients with diabetes (HbA1c: 7–10%, the diabetes duration averaged 8 years). The study participants receiving semaglutide for the treatment of obesity demonstrated a clinically significant reduction in body weight of 9.64% from baseline compared to 6.99% with a 1.0 mg dose. The proportion of patients achieving a weight reduction of 10% or more was 45.6% in the semaglutide (2.4 mg) group *vs.* 28.7% in the semaglutide (1.0 mg) group. The results of the clinical trial obtained at weeks 8–12, when the patients took the same doses of the drug (0.5–1.0 mg), are noteworthy: the reduction in their body weight has statistical differences ($p$ <0.05) in favour of the obesity drug. This study shows that not only the dose, but also a specially selected and scientifically substantiated concentration determines the dynamics of weight loss, because it allows the drug to overcome physiological barriers and affect GLP-1 receptors as effectively as possible, in particular, in the brain structures of the reward system. This is what determines the pronounced effect of the drug on the formation of the rational eating behaviour and the reduction of cravings for sweet and fatty foods [14].

In the two-year STEP 5 clinical trial, the use of semaglutide for obesity contributed not only to a significant weight loss (-15.2 vs. -2.6%), but also to the maintenance of the achieved result throughout the follow-up period. According to the results of therapy in the cohort of patients receiving semaglutide at a dose of 2.4 mg, 83.3% of the patients reduced weight by ≥5% or more, 67.4% by ≥10%, 56.8% by ≥15%, and 39.4% by ≥20% [15].

In the multicentre randomized placebo-controlled SELECT study involving 17 604 patients from 41 countries, it was demonstrated that the administration of semaglutide at a dose of 2.4 mg to patients with CVDs and obesity / overweight without diabetes leads to a 20% reduction in the risk of Major Adverse Cardiovascular Events (MACEs), including a reduction in fatal outcomes, a reduction in the risk of non-fatal infarctions, which fundamentally changes the scenario of the obesity treatment in the long term [13]. The accumulated data on the use of semaglutide at a dosage of 2.4 mg made it possible to include the drug in clinical guidelines for a stroke prevention, which proves the effectiveness of this type of therapy in achieving the main goal of the obesity treatment — reducing the risks of complications and restoring metabolic health [16, 17].

It is important to note that due to its similarity to physiological hormones, an increase in the dosage does not correlate with an increase in the incidence of side effects. Semaglutide at a dosage of 2.4 mg has a favourable safety profile similar to that of semaglutide 1.0 mg [18, 19].

At a dosage of 2.4 mg for the treatment of obesity and overweight, including in patients with T2D, semaglutide is registered in Europe and the USA under the trade name Wegovy®[2,3]. This drug is not registered in Russia, and until recently, Russian specialists have not been able to use this drug in clinical practice.

Promomed Company has been providing the healthcare system with reliable drugs for the treatment of obesity and overweight with a high level of evidence for more than 20 years, and was the first in the country to develop its own full-cycle technology for GLP-1 agonists (liraglutide, semaglutide) — from the chemical synthesis and isolation of active pharmaceutical substances to the production of a finished dosage form. The preparations based on liraglutide 3.0 mg (Enligria®) and semaglutide 1.0 mg (Quinsenta®) were registered in the second half of 2023 and became full substitutes for their foreign predecessors that had left the market.

[2] Ibid.
[3] EMA. Wegovy. Overview. Available from: https://www.ema.europa.eu/en/medicines/human/EPAR/wegovy

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**THE AIM** of the work was to evaluate pharmacokinetic parameters, bioequivalence, safety, tolerability and immunogenicity of the domestic drug Velgia® (WRYC12301) at doses of 0.25 mg (0.68 mg/mL) and 2.4 mg (3.2 mg/mL) in comparison with the reference drug Wegovy® (Novo Nordisk A/S, Denmark) at the doses of 0.25 mg (0.68 mg/mL) and 2.4 mg (3.2 mg/mL).

## MATERIALS AND METHODS

### Study design

An open randomized parallel bioequivalence study was conducted in a single subcutaneous administration of the study and reference drugs on an empty stomach in healthy volunteers. The block diagram of the design (duration wise) is shown in Fig. 1.

### Randomization

The volunteers who had successfully completed the screening procedure (met the eligibility criteria) were randomized into the clinical trial. Each randomized volunteer was assigned according to the randomization plan using WinPepi 11.65 software (ETCETERA module 3.26) by a random number generation (assigning numbers to volunteers — 01 to 60). The volunteers (*n*=60) were randomized into 4 groups in a 1:1 ratio.

If a volunteer dropped out of the clinical trial before time, their randomization number was not reused and the volunteer could not subsequently re-enter the study.

### Study subjects and eligibility criteria

The study randomized 60 healthy male and female volunteers aged between 18 and 65 years inclusive with a body mass index of 25–30 kg/m$^2$ inclusive, who met all the inclusion criteria and did not meet any of the non-inclusion criteria.

All the participants signed an informed consent form and were able and willing to comply with the study protocol.

The main *inclusion criteria* were:

- men and women aged 18 to 65 years;
- body mass >50 kg;
- BMI=25–30 kg/m$^2$ inclusive;
- verified diagnosis of “healthy” according to the standard clinical, laboratory and instrumental examination methods;
- negative results of tests for alcohol, psychotropic and narcotic substance use, and willingness to give up an alcohol use during the participation in the study.

The participants were warned to use reliable contraceptive methods and to abstain from a sperm donation throughout the study and for 2 months after the end of the study.

The main *non-inclusion criteria* were:

- presence of an aggravated allergological anamnesis, drug intolerance, chronic diseases of various organ systems;
- mental illnesses;
- hypersensitivity to the study drugs;
- history of use of semaglutide or other human GLP-1 analogues (within less than 6 months before screening);
- taking medications with significant effects on haemodynamics and/or a liver function for less than 2 months prior to screening;
- taking other medications, including herbal and homeopathic preparations, vitamins and/or dietary supplements, for less than 4 weeks prior to screening;
- inability to perform subcutaneous injections;
- any history of difficulties with blood collection or any vasovagal attacks during a blood collection;
- Gastrointestinal Surgical Procedures (except appendectomy).

The participants were also not considered for the study inclusion if they had the following diseases and conditions: a history of medullary thyroid cancer, including a family history; a history of multiple endocrine neoplasia type 2; a severe depression; suicidal thoughts or behaviour, including a history; acute infectious diseases or symptoms of acute respiratory infections for less than 4 weeks prior to screening.

The volunteers were excluded from the study if they refused to participate in the clinical trial, if they were taking illegal drugs and tested positive for the use of alcohol, psychotropic and/or narcotic substances, if there were gross violations of the requirements and procedures of the study protocol, if there were adverse events (AEs), or if during the study, the volunteer developed any diseases or conditions that made their further participation in the study impossible. The research physician may decide to exclude a volunteer in the volunteer’s own interests.

Concomitant therapy and exclusion criteria were

assessed throughout the study. The total duration of the study for each volunteer was no more than 35 days (including the screening period).

### Study conditions and duration

The study was conducted on the basis of the state budgetary health care institution of the Yaroslavl Clinical Hospital No. 3 (Yaroslavl, Russia) in the period from March to June 2024.

### Characteristics of drugs

The following preparations were used in the investigation — the investigational new drug (Velgia®; WRYC12301; IND; semaglutide, a solution for a subcutaneous administration, JSC "Biochemik", Russia) at doses of 0.25 and 2.4 mg/mL) and a reference drug (Wegovy®; RD; semaglutide, a preparation for a subcutaneous administration, Novo Nordisk A/S, Denmark) at doses of 0.68 and 2.4 mg/mL.

### Drug administration

The volunteers received the drugs in the morning on an empty stomach subcutaneously into the anterior abdominal wall. Group 1 (*n*=15) and Group 2 (*n*=15) received the study and reference drugs at a dose of 0.5 mg (a single administration of 2 doses of 0.25 mg/dose), Group 3 (*n*=15) and Group 4 (*n*=15) — 2.4 mg (a single administration of 1 dose of 2.4 mg/dose).

### Sample preparation and collection

To assess pharmacokinetic and immunogenicity parameters, after the randomization and before the initial blood sampling, a cubital heparinized catheter was placed in the volunteers and removed after the blood sampling at 12 h point (day 1). After the removal of the catheter, the blood was collected from the volunteers by venipuncture.

Blood samples for the determination of pharmacokinetic parameters were collected at the following time points: 1, 0.5 and 0 h (day 1) before the administration of the IND / RD and then on and off at 2, 8, 12 (day 1), 24 (day 2), 36 (day 2), 48 (day 3), 72 (day 4), 96 (day 5), 144 (day 7), 192 (day 9), 240 (day 11), 360 (day 16) and 480 h (day 21) after the administration of the IND / RD.

The blood samples for immunogenicity were collected from the volunteers receiving the IND at a dosage of 2.4 mg/dose (3.2 mg/mL) (Group 3) and the RD at a dosage of 2.4 mg/dose (3.2 mg/mL) (Group 4) no more than 15 min before the administration of the study/reference drug (baseline (0) sample) and 480 h (day 21) after their administration. The blood samples for the analysis of immunogenicity parameters were collected separately from the blood samples for the estimation of pharmacokinetic parameters.

Thus, the study involved the collection of 16 blood samples for each volunteer (6 mL each) for pharmacokinetic parameters and 2 blood samples for the volunteers from Groups 3 and 4 (6 mL each) to carry out immunogenicity studies.

At the screening, a blood sample of no more than 25 mL was taken for standard clinical, biochemical, serological analyses and a blood glucose level determination using a glucometer.

The blood samples were collected into tubes to obtain serum with a coagulation activator. Then, the samples were gently mixed 5–8 times for a better contact between the blood and clotting activator. The tubes with the blood samples were then left on the table in an upright position at 18–25°C until a complete coagulation (clot formation). After the clot formation, the tubes were centrifuged at 1500 g for 10 min at 18–25°C (Eppendorf 5702 R medical centrifuge No. 0006208, Eppendorf, Germany). The obtained serum was carefully transferred into pre-labelled cryotubes, dividing the serum into three 500 µl aliquots — two for the main assay (aliquots A and B), the third - for repeat assays (aliquot C). The serum samples were frozen immediately after the receipt, transferred to cryotubes and stored at -70°C or less.

### Analytical method

Calculations of pharmacokinetic parameters were performed by a serum semaglutide concentration. A quantification of semaglutide was performed by a high-performance liquid chromatography with a tandem mass spectrometry (HPLC-MS/MS). A chromatographic separation and detection were performed on an LC-20 Prominence Nexera XR liquid chromatograph (Shimadzu, Japan) and a LCMS-8040 tandem mass spectrometric detector (triple quadrupole) using a Phenomenex Kinetex C18, 100×3.0 mm, 5 µm column. An enzyme-linked immunosorbent assay (ELISA) was used to determine the immunogenicity parameters.

Immunogenicity was assessed in the volunteers with antibodies to semaglutide. A quantitative determination of antibodies to semaglutide in calibration

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and quality control samples was performed using a HiPo MPR-96 microplate photometer (Biosan, Latvia) with the ELISA kit KRIBIOLISA™ Anti-Semaglutide (Ozempic™) ELISA (competitive) (Krishgen BioSystems, USA).

### Safety and tolerability assessment

To assess the safety of the IND, the frequency and severity of the AEs registered according to the data of deviations from normal results of the laboratory tests, physical examination, assessment of basic vital signs, electrocardiogram (ECG); the number of cases of an early participation termination in the study due to the development of AEs and/or serious AEs (SAEs), including those associated with IND / RD, were taken into account. Tolerability of the drug was assessed by the study physician using a Likert scale.

Among the criteria for assessing immunogenicity, the number (%) of the volunteers with detected antibodies to semaglutide were considered.

### Ethical approval

The conduct of the study was approved by the Local Ethical Committee of the Yaroslavl Clinical Hospital No. 3 (extract from Minutes No. 202 dated 21 March 2024).

### Statistical analysis

#### Principles of sample size calculation

The calculation was based on the interindividual variability ($CV_{inter}$) of semaglutide as reported in Clinical Review (S) Semaglutide [1].

As the $CV_{inter}$ value of semaglutide had not been published directly, it was calculated from the submitted data in the R software environment with the PowerTOST package using the "CV from CI" function. The calculation of the required abundance was performed using the PASS 11 programme (PASS 11 PLUS, UK). For standard two-parallel-group design conditions, assuming a 90% CI of 80.00–125.00%, $CV_{inter}$=17%, α=0.05, the study power of 80%, and the IND / RD ratio of 0.95, the inclusion of at least 26 healthy volunteers (13 in each dose group) who will fully complete the study and be accepted the for statistical analysis, is required.

#### Methods of statistical data analysis

The aim of the work was to evaluate pharmacokinetic parameters, bioequivalence, safety, tolerability and immunogenicity of the IND at doses of 0.25 mg (0.68 mg/mL) and 2.4 mg (3.2 mg/mL) in comparison with the RD.

The primary database was created in MS Excel 19 (Microsoft Corp., USA) by processing the registration cards received from the research centre. The calculation of pharmacokinetic parameters, a statistical analysis of safety parameters and the presentation of results were performed using statistical packages (StatSoft Statistica version 10.0/13.3, IBM SPSS Statistics 22 and using the R project software (current version, GPL-2/GPL-3 licence) with a bear extension). The following pharmacokinetic variables were calculated: $C_{max}$ — the maximum concentration of the substance in serum; $t_{max}$ — the time to reach $C_{max}$; $AUC_{(0-t)}$ — the area under the concentration-time curve from the time of a drug administration to the last detectable concentration at time point t; $AUC_{(0-\infty)}$ — the area under the pharmacokinetic curve from time zero to infinity; $AUC_{(0-t)}/AUC_{(0-\infty)}$ — a ratio of $AUC_{(0-t)}$ to $AUC_{(0-\infty)}$; $K_{el}$ — a terminal elimination rate constant; $t_{1/2}$ — elimination half-life; $V_d$ — volume of distribution; $AUC_{(t-\infty)}$ — residual (extrapolated) area under the curve, determined by the formula $AUC_{(0-\infty)}$– $AUC_{(0-t)}/AUC_{(0-\infty)}$. The conclusion about bioequivalence of the compared drugs was made by the ratio of $C_{max}$ and $AUC_{(0-t)}$ parameters of the study drug to the reference drug, which should lie in the range of 80.00–125.00% at the 90% CI.

The following statistical parameters were calculated for all pharmacokinetic parameters: arithmetic mean (Mean), standard deviation of mean (SD), coefficient of variation (CV), median (Me), minimum (Min) and maximum (Max) values, and spread.

To analyze frequency indicators, the fractions using a two-sided version of Fisher's exact test (or the $\chi^2$ (chi-square) criterion), were compared. For the comparison of quantitative continuous indicators, the Students' t-test (in case of a normal distribution) or the Mann-Whitney test (in case of the distribution other than normal) were used. The test for normality of the data distribution was carried out using generally accepted methods (the Shapiro-Wilk test or the Kolmogorov-Smirnov test). The differences at $p$ <0.05 were considered statistically significant.

### Quality control of semaglutide active pharmaceutical substance

The appearance of all tested samples of the semaglutide active pharmaceutical ingredient (API) was assessed visually. A specific optical rotation values were determined in accordance with the requirements of the Pharmacopoeia of Eurasian Economic Union (PhEAEU), general monograph (GM) 2.1.2.7 "Optical rotation".

High-resolution mass spectra were recorded on the LCMS-9030 instrument (Shimadzu, Japan) by an electrospray ionization mass spectrometry method (ESI-MS). The following parameters were used: capillary voltage — 4.0 kV; mass scanning range — 500–2000 m/z; external calibration with sodium iodide solution in MeOH / $H_2O$; drying and heating gases — nitrogen (10 L/min each); atomizing gas — nitrogen (3 L/min); interface temperature — 300°C. Molecular ions in the spectra were analyzed using LabSolutions v.5.114 software (LabSolutions Series, Shimadzu, Japan).

The control method for the indicator "Peptide mapping" included an enzymatic cleavage of proteins to form peptide fragments with their subsequent separation and identification by HPLC (PhEAEU GM 2.1.2.39. "Peptide mapping"). A high-pressure liquid chromatograph with Agilent UV detector (Agilent Technologies, USA) and a 150×4.6 mm column filled with XBridge Peptide BEH C18 sorbent (4.6×150 mm, 3.5 µm) were used for the study; the elution mode was gradient. The detection was performed at a wavelength of 214 nm.

The determination of the quantitative content and the identification of related impurities in the samples of the semaglutide substance was carried out by HPLC (PhEAEU GM 2.1.2.28. "High-performance liquid chromatography"). A high-pressure liquid chromatograph with Agilent UV detector, Kinetex 2.6 µm C18 100 Å column, LC Column 150×4.6 mm, elution mode — gradient, were used for the study. The detection was carried out at a wavelength of 210 nm.

The determination of the acetic and trifluoroacetic acid content was carried out by a HPLC method (PhEAEU GM 2.1.2.28 "High-performance liquid chromatography"). A high-pressure liquid chromatograph with an Agilent UV detector, Luna C18(2) 5 µm 4.6×250 mm column, the elution mode — gradient, were used for the study. The detection was carried out at a wavelength of 210 nm.

The content of bacterial endotoxins evaluated according to PhEAEU GM 2.1.6.8 "Bacterial endotoxins". The total amount of aerobic bacteria, yeasts and moulds was determined according to PhEAEU GM 2.3.1.2 "Requirements for microbiological purity of medicinal preparations, pharmaceutical substances and excipients for their production", and GM 2.1.6.8 "Bacterial endotoxins". The total amount of aerobic bacteria, yeasts and moulds was determined according to PhEAEU GM 2.3.1.2 "Requirements for microbiological purity of medicinal preparations, pharmaceutical substances and excipients for their production".

## RESULTS

The primary outcome of the study was to evaluate the pharmacokinetic parameters, bioequivalence, safety, tolerability and immunogenicity of the Velgia® at doses of 0.25 (0.68 mg/mL) and 2.4 mg (3.2 mg/mL) in comparison with the reference drug Wegovy® (Novo Nordisk A/S, Denmark) at doses of 0.25 (0.68 mg/mL) and 2.4 mg (3.2 mg/mL).

### Population

Sixty male and female volunteers participated in the study. The main anthropometric characteristics and gender distribution are shown in Table 1.

### Bioequivalence assessment

The mean values of basic and additional pharmacokinetic parameters for the study and reference drugs are presented in Table 2.

Fig. 2 shows the averaged pharmacokinetic profiles after the administration of the study and reference drugs at a dosage of 0.25 mg/dose (0.68 mg/mL). As can be seen from the compared curves, the comparability of the nature of the "concentration-time" dependence for the studied drugs is observed.

Figure 3 shows the averaged pharmacokinetic profiles with and without standard deviations after the administration of the study and reference drugs at a dosage of 2.4 mg/dose (3.2 mg/mL). As can be seen from the compared curves, the nature of the "concentration-time" dependence for the compared drugs did not practically differ either.

### Time after drug administration, hours

According to the results of the statistical analysis, the obtained 90% CIs for the ratio of $C_{max}$ and $AUC_{(0-t)}$ values of the Velgia® and the reference drug Wegovy® in the dosage of 0.25 mg/dose (0.68 mg/mL) — Group 1, 2 — were for $C_{max}$ — 85.19–114.36% and $AUC_{(0-t)}$ — 81.35–112.60%, respectively; for the dosage of 2.4 mg/dose (3.2 mg/mL) —Group 3, 4 — $C_{max}$ was 83.18–111.3% and $AUC_{(0-t)}$ — 91.70–120.89%, respectively. The obtained confidence intervals lie within the established limits, which confirms the bioequivalence of the studied drugs.

### Safety and tolerability assessment

All the volunteers completed the study in full compliance with the approved study protocol. During the study, Group 1 and Group 2 (0.25 mg/dose (0.68 mg/mL)) volunteers did not experience any AEs.

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In Groups 3 and 4 (the dosage of 2.4 mg/dose (3.2 mg/mL)), single cases of AEs, similar in type (nausea or vomiting), frequency of occurrence and severity, were reported in both the study and reference drugs groups. All the AEs registered during the study were of mild severity. The nature and frequency of AEs were consistent with the known profile for semaglutide and did not require withdrawal of therapy.

The tolerability of IND / RD at 0.25 mg/dose (0.68 mg/mL) and at 2.4 mg/dose (3.2 mg/mL) was rated as "good" and "satisfactory" in 100% of cases and was comparable to the reference drug.

No SAEs were observed in the volunteers during the study and after its completion. No deaths were observed. No cases of pregnancy of the sexual partner of the study participant during the study and after its completion were registered. No abnormalities were detected in the results of clinical and biochemical blood tests, blood glucose levels, a general urinalysis, parameters of basic vital signs, a physical examination and ECG.

### Immunogenicity evaluation

According to the results of the immunogenicity parameters analysis, no antibodies to semaglutide were detected in the serum of the volunteers, which indicated the absence of immunogenicity of the drug. Unexpected results were not observed in the study, which supports the concept of the advantage of the drug chemical "origin".

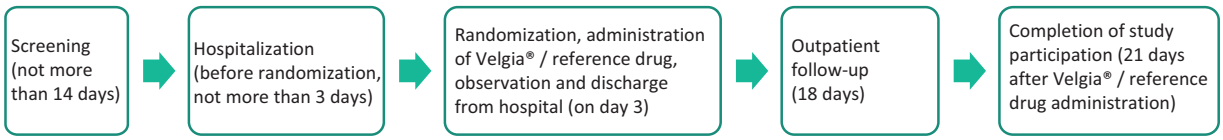


**Figure 1 – Study design**

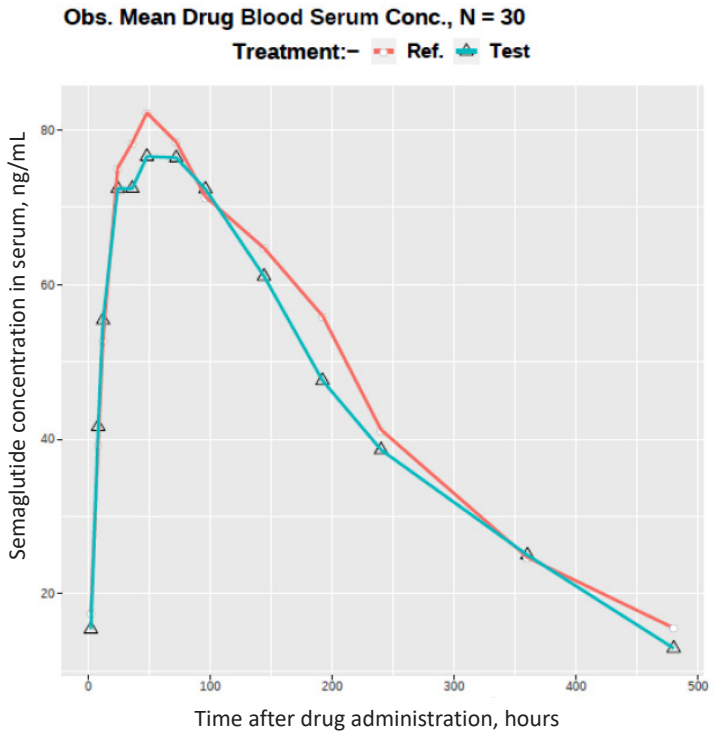


**Figure 2 – Averaged pharmacokinetic profiles after administration of the study drug Velgia® and the reference drug Wegovy® at a dosage of 0.25 mg/dose (0.68 mg/mL)**
Note: Ref. — reference drug; Test — study drug.

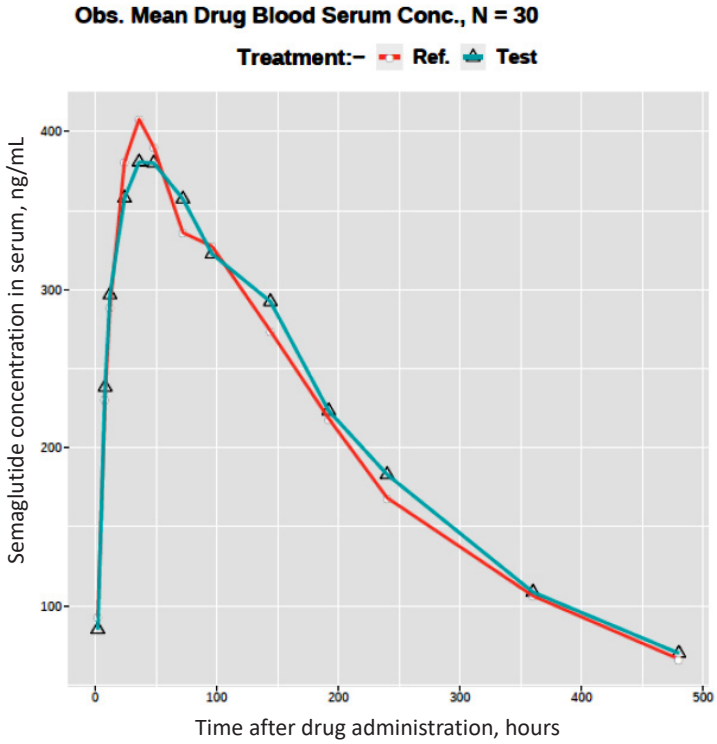


**Figure 3 – Averaged pharmacokinetic profiles after administration of the study drug Velgia® and the reference drug Wegovy® at a dosage of 2.4 mg/dose (3.2 mg/mL)**
Note: Ref. – reference drug; Test – study drug.

**Table 1 – Descriptive characteristics of demographic and anthropometric data of volunteers' study and reference drugs groups**

| Indicator | | 0.5 mg dose (2 doses of 0.25 mg/dose), *n*=30 | | | 2.4 mg dose, *n*=30 | | |
|---|---|---|---|---|---|---|---|
| Gender, *n* (%): | | | | | | | |
| | Male | 8 (26.67%) | | | 21 (70%) | | |
| | Female | 22 (72.33%) | | | 9 (30%) | | |
| – | | Mean | -95% CI | 95% CI | Men | -95% CI | 95% CI |
| Age, full years | | 45.13 | 41.76 | 48.51 | 43.87 | 40.60 | 47.13 |
| Body weight, kg | | 75.71 | 73.05 | 78.37 | 82.89 | 80.37 | 85.41 |
| Height, cm | | 169.40 | 166.58 | 172.21 | 175.77 | 172.82 | 178.71 |
| BMI, $kg^2/m$ | | 26.35 | 25.97 | 26.72 | 26.81 | 26.42 | 27.21 |

**Table 2 – Pharmacokinetic parameters calculated for the studied drugs dosages**

| Parameter, units | Dose 0.25 mg/dose (0.68 mg/mL), Mean±SD | | Dose 2.4 mg/dose (3.2 mg/mL), Mean±SD | |
|---|---|---|---|---|
| | Velgia® | Wegovy® | Velgia® | Wegovy® |
| $C_{max}$, ng/mL | 85.63±22.14 | 86.75±16.12 | 417.54±83.16 | 433.85±125.47 |
| $AUC_{(0-t)}$, ng×h/mL | 19458.26±5374.17 | 20330.32±4374.67 | 94426.48±18947.4 | 89684.6±21791.3 |
| $AUC_{(0-\infty)}$, ng×h/mL | 22168.60±7014.38 | 23829.36±6296.99 | 112034.65±25345.73 | 106204.16±25136.1 |
| $k_{el}$, $h^{-1}$ | 0.005±0.003 | 0.005±0.001 | 0.004±0.001 | 0.004±0.001 |
| $t_{max}$, h | 52.46±35.19 | 51.47±54.2 | 40.53±26.24 | 45.67±22.16 |
| $t_{1/2}$, h | 139.7±43.1 | 149.77±38.16 | 167.68±43.24 | 167.77±39.34 |
| $AUC_{(t-\infty)}$, % | 9.73±5.51 | 12.55±7.62 | 13.99±6.78 | 14.51±5.8 |
| $V_d$, L | 4.55±1.37 | 4.53±1.49 | 5.18±1.55 | 5.47±1.96 |
| $AUC_{(0-t)}$ / $AUC_{(0-\infty)}$, % | 87.77±5.51 | 85.32±7.62 | 84.28±6.78 | 84.45±5.8 |

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**Table 4 – Comparison of norms and actually obtained control results quality of semaglutide (production of JSC "Biochemik", Russia and Zhejiang Peptites Biotech Co., Ltd, China)**

| Parameter | Semaglutide (JSC Biochemik, Russia) | | Semaglutide (Zhejiang Peptites Biotech Co., Ltd, China) | |
|---|---|---|---|---|
| | Standardised indicator | Control result | Standardised indicator | Control result |
| Appearance | White or almost white amorphous powder | White amorphous powder | White or almost white powder or a loose lump | Almost white powder |
| Specific rotation | -2° to -20° in terms of anhydrous and acetic acid free | -14.6° | This indicator is not controlled | |
| Identification (by HPLC) | In the chromatogram of the standard sample, the retention time of the main peak of the test solution shall correspond to the retention time of the semaglutide peak | Corresponds. | The retention time of the main peak of the test sample should correspond to the retention time of the main peak of the standard sample | Corresponds |
| Identification (MS) | M.W.: 4113.6±1.0 | M.W.: 4112.8 | MW: 4113.6±1.0 | M.W.: 4112.8 |
| Peptide mapping | The chromatographic profile of the lysate solution of the test sample shall correspond to the chromatographic profile of the lysate solution of the semaglutide standard sample. | Corresponds. | This indicator is not controlled. | |
| Related impurities (by HPLC method) | Impurity A: Not more than 0.15% | 0.02% | G06-IM37: Not more than 0.1% | Not detected |
| | Impurity A: Not more than 0.15% | 0.09% | G06-IM42: Not more than 0.1% | Not detected |
| | Impurity A: Not more than 0.15% | 0.03% | G06-IM10: Not more than 0.1% | Not detected |
| | Impurity A: Not more than 0.15% | 0.02% | G06-IM60: Not more than 0.2% | 0.10% |
| | Any single impurity: Not more than 0.10% | 0.03% | G06-IM18: Not more than 0.2% | Not detected |
| | Total impurities: Not more than 1.0% | 0.23% | G06-IM01: Not more than 0.2% | 0.07% |
| | | | G06-IM59: Not more than 0.2% | 0.10% |
| | | | G06-IM28: Not more than 0.2% | 0.09% |
| | | | G06-IM03: Not more than 0.2% | Not detected |
| | | | Any single impurity: Not more than 0.10% | Not detected |
| | | | Total impurities: Not more than 2.0% | 0.36% |
| Acetic acid | Not more than 0.25 % | 0.01% | Not more than 0.5% | 0.03% |
| Trifluoroacetic acid | Not more than 0.25 % | Not detected | Not more than 0.25 % | 0.02% |
| Bacterial endotoxins | Not more than 5 EUs/mg | Less than 5 EU/mg | Less than 10 EUs/mg | Less than 10 EUs/mg |
| Total aerobic bacteria | Not more than 100 CFUs/g | Absent | Not more than 100 CFUs/g | Less than 10 CFUs/g |
| Total yeasts and moulds | Not more than 50 CFUs/g | Absent | Not more than 100 CFUs/g | Less than 10 CFUs/g |

## DISCUSSION

The broad evidence base for the efficacy of semaglutide with respect to the weight loss, a restoration of metabolic health and a reduction of risks of complications presents semaglutide as a priority drug for a widespread use in routine clinical practice for the therapy of obesity and overweight [4, 10, 11, 24]. Moreover, the potential of the molecule is not limited to metabolic diseases. Semaglutide shows positive results in preclinical and clinical studies for the treatment of MAFLD, apnea, Alzheimer's disease, CVD prevention [12, 17], depression, etc. [23, 24, 26]. In 2024, semaglutide was included in the latest Russian clinical guidelines[4] for the treatment of obesity (revision 2024) for a sustained reduction of a body weight and cardiovascular risks in overweight and obese patients. Availability of the above-mentioned medicines for patients in Russia is an important task for the introduction of health-saving technologies into medical practice. An adequate drug supply is critical for achieving the main goals of the healthcare system — preservation and promotion of public health.

At the moment, Velgia® is the only domestic preparation based on semaglutide INN containing all necessary dosages and concentrations of the active substance for the treatment of obesity and overweight, taking into account the need for a proper transition from the first use of the drug through the titration period to the therapeutically effective dosage.

The authors' own original technology of obtaining and purification of the active pharmaceutical substance ensures a high level of quality control of the obtained substance, an exclusion of undesirable impurities and isomerization. As a consequence, it contributes to the achievement of high efficiency and safety of the therapy. The developed technology of the chemical synthesis and product isolation allows Promomed Company to create peptide molecules with precisely defined properties without a spontaneous replacement of amino acids in the peptide structure, the absence of products of producers' life activities (as in case of their production from bacteria or yeast), a high degree of purity, which increases the safety profile of the drug. The literature describes the advantages of the developed technology compared to foreign precursors [14, 15].

Moreover, the production of semaglutide by this method is highly productive, scalable and economically feasible compared to the biotechnological route used by a foreign company, which ensures that the need for the drug is met for a wide range of patients [20, 21, 25, 26]. The registration procedure for the medicinal product Velgia® (semaglutide) was completed on 3 October 2024. Biological, pharmaceutical, functional and consumer characteristics of this drug meet all the requirements for the drugs for the treatment of obesity and overweight:

- high efficacy and safety parameters, taking into account the specifics of a long-term use for the treatment of chronic obesity;
- full range of specially selected dosages and concentrations of the active ingredient to ensure the required dose titration pattern from the first use (0.25 mg/week) to the therapeutic dose (2.4 mg/week);
- syringe pens, which are easy to use and do not require any effort and specialized skills during their use.

The introduction of Velgia® (semaglutide) into the practice of Russian physicians represents an access to the drug therapy proven worldwide, which will reduce the burden of the obesity pandemic and ensure an effective and safe weight loss.

The syringe pen specially designed for this drug does not require specialized skills and is compatible with needles of any manufacturer, which is important in case of a possible decrease in the availability of foreign-made needles.

For the production of semaglutide, Promomed Company uses the solid-phase peptide synthesis (SPPS) method. It allows to automate the process and accurately reproduce the specified structure of the peptide, which mitigates the risks of changes in the obtained substance and, consequently, adverse immune reactions when using the drug.

[4] Clinical Guidelines for Obesity (revised 2024). Available from: https://cr.minzdrav.gov.ru/view-cr/28_3. Russian

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### Comparative evaluation of quality indicators of semaglutide active pharmaceutical substance

The main characteristics affecting the quality of semaglutide substance (Biochemik JSC, Russia) and semaglutide substance manufactured by Zhejiang Peptites Biotech Co., Ltd, China, which is a part of one of the medicinal preparations registered in Russia, were analyzed. The indices pledged by both manufacturers for the control of their APIs, were taken into account. The results of the study are presented in Table 4.

The above data show that the semaglutide API produced by Zhejiang Peptites Biotech Co., Ltd, China is not controlled by the indicators “Specific rotation” and “Peptide mapping”. That indicates the lack of a systematic authenticity verification of the obtained preparation and the risk of reducing the effectiveness of the finished drug as a result of, for example, an amino acid substitution. Moreover, in comparison with the semaglutide API produced by JSC Biochemik (Russia), the Chinese manufacturer lays down underestimated quality requirements for the indicators “Sum of impurities”, “Acetic acid content”, “Bacterial endotoxins” and “Total yeast and moulds”. This fact may indirectly indicate a coarser technology of the APIs purification, which may affect the safety parameters and lead to the rejection of the entire series of the finished dosage form according to the indicator “Sterility” and, consequently, interruptions in the supply of the vital drug.

Increased requirements for the API purity of the semaglutide produced by JSC Biochemik, Russia, in particular, reflected the lack of immunogenicity at the stage of clinical trials.

Thus, thanks to the efforts of domestic pharmaceutical companies, in particular Promomed Company, all the most effective tools to combat overweight and obesity are available in our country and the new drug Velgia® will be the next step towards a metabolically healthy society.

## CONCLUSION

The study confirmed the bioequivalence of the study drug Velgia® (WRYC12301, semaglutide, a solution for a subcutaneous administration, JSC Biochemik, Russia) and the reference drug (semaglutide, a solution for a subcutaneous administration, Novo Nordisk A/S, Denmark), both at doses of 0.25 mg (0.68 mg/mL) and 2.4 mg (3.2 mg/mL), their high safety profile, good tolerability and lack of immunogenicity.

Velgia® is available in ergonomic syringe pens, in 5 doses, each containing one of the following prescribed doses of semaglutide: 0.25, 0.5, 1, 1.7 and 2.4 mg/dose. Each syringe pen contains four doses of the drug to be used once a week for a month.

A step-by-step titration of Velgia® promotes a safe and effective weight loss. The drug is produced in Russia according to the full cycle — from the chemical synthesis of the substance to the finished dosage form. The prescription of the drug does not require in-depth diagnostics, and can be implemented by doctors of various specialities as an addition to a low-calorie diet and physical activities to reduce a body weight for the indication “obesity and overweight”, which will allow effective and safe therapy of patients regardless of their gender, age and presence of comorbidities.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHORS' CONTRIBUTION

Aleksander S. Ametov — development of the clinical study concept, analysis and description of the results, text correction; Kira Ya. Zaslavskaya — analysis and selection of literature sources, writing the text of the article; Ekaterina A. Rogozhina — discussion of the study design and results; Petr A. Bely — implementation of the study design, processing of the study data; Victoria S. Shcherbakova — development of the study design and concept, writing the text of the article; Yurii G. Kazaishvili — development of the clinical study design and concept; Alexey V. Taganov — analyzing and describing the results, searching and analyzing literature sources; Tatiana G. Bodrova, Ekaterina S. Mishchenko, Ksenia N. Koryanova, Larisa I. Shcherbakova — processing the study data, editing the text of the article.

All the authors confirm their authorship compliance with the ICMJE international criteria (all the authors made a significant contribution to the conceptualization, conduct of the study and preparation of the article, read and approved the final version before publication).

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# Evaluation of the relationship between the minimum steady-state concentration of angiotensin II receptor blockers and polymorphic markers of *CYP2C9 (Arg144Cys)*, *CYP2C9 (IIE359Leu)*, *AGTR1 (A1166C)*, *AGT (Met235Thr, C4072T)*, *ACE (I/D)*, *CYP11B2 (C-344T)* genes and office arterial pressure

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**The aim** of the work was to study the relationship of the minimum steady-state concentration of angiotensin II receptor blockers with polymorphic markers of *CYP2C9 (Arg144Cys)*, *CYP2C9 (Ile359Leu)*, *AGTR1 (A1166C)*, *AGT (Met235Thr, C4072T)*, *ACE (I/D)*, *CYP11B2 (C-344T)* genes and the office blood pressure (BP) indices.

**Materials and methods.** The study included 179 patients of the Moscow region with newly diagnosed hypertension of stages 1–2, among whom there were 141 (78.8%) women and 38 (21.2%) men aged from 32 to 69 years (mean age — 58.2±6.4, median age — 60 (57–63 years), who had been randomized into treatment groups with valsartan and irbesartan in the form of mono- or combination therapy with hydrochlorothiazide. After 3 weeks of pharmacotherapy, polymorphic markers CYP2C9 *(Arg144Cys)*, *CYP2C9 (Ile359Leu)*, *AGTR1 (A1166C)*, *AGT (Met235Thr*, *C4072T)*, *ACE (I/D)*, *CYP11B2 (C-344T)* were genotyped and the minimum steady-state concentrations of irbesartan and valsartan were determined. The office BP measurements were performed on each visit.

**Results.** The carriers of alleles *2 and *3 of the *CYP2C9* gene, the genotype T/T of the *AGT* gene, the genotype I/I of the *ACE I/D* polymorphism achieved higher values of the minimum steady-state concentration of irbesartan after 3 weeks of pharmacotherapy. Homozygotes A/A for the genetic polymorphism of the *AGTR1* gene *(A1166C)*, homozygotes D/D for the *ACE I/D* polymorphism reached significantly higher values of the minimum-steady concentration of valsartan after 3 weeks of pharmacotherapy. In the patients taking irbesartan, a more pronounced decrease in the office systolic (SBP) and diastolic (DBP) BP was detected with an increase in the concentration for every 100 ng/mL after 3 weeks of therapy. Any association of the indicators with the valsartan concentration was found out.

**Conclusion.** The effects of irbesartan and valsartan indicate a maximum modulation of pharmacodynamic effects during 3 weeks of pharmacotherapy, followed by a consolidation in the therapeutic range and a stop in the increasing the effectiveness with a further increase in the steady-state concentration, which can be used to predict therapy, personalize it, a better control and a high safety profile.

**Keywords:** arterial hypertension; *CYP2C9; AGTR1; AGT; ACE; CYP11B;* irbesartan; valsartan; plasma concentration

**Abbreviations:** AH — arterial hypertension; BP — blood pressure; RAAS — renin-angiotensin-aldosterone system; *CYP2C9* — cytochrome P450, family 2, subfamily C, polypeptide 9; *AGTR1* — angiotensin II type 1 receptor gene, *AGT* — angiotensinogen gene; *ACE* gene — angiotensin-converting enzyme gene; *CYP11B2* — aldosterone synthase gene; GFR — glomerular filtration rate; ACE — angiotensin-converting enzyme; ARB — angiotensin II receptor blockers; CI — confidence interval; OR — odds ratio; SBP — systolic blood pressure; DBP — diastolic blood pressure; HR — heart rate.

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# Оценка взаимосвязи минимальной равновесной концентрации блокаторов рецепторов ангиотензина II с полиморфными маркерами генов *CYP2C9 (Arg144Cys), CYP2C9 (Ile359Leu), AGTR1 (A1166C), AGT (Met235Thr, C4072T), ACE (I/D), CYP11B2 (C-344T)* и показателями офисного артериального давления

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**Цель.** Изучить взаимосвязь минимальной равновесной концентрации блокаторов рецепторов ангиотензина II с полиморфными маркерами генов *CYP2C9 (Arg144Cys), CYP2C9 (Ile359Leu), AGTR1 (A1166C), AGT (Met235Thr, C4072T), ACE (I/D), CYP11B2 (C-344T)* и показателей офисного артериального давления (АД).

**Материалы и методы.** В исследование включено 179 пациентов Московского региона с впервые выявленной артериальной гипертензией (АГ) 1–2 степени, среди которых 141 (78,8%) женщина и 38 (21,2%) мужчин в возрасте от 32 до 69 лет (средний возраст — 58,2±6,4, медианный возраст — 60 (57–63 лет), которые были рандомизированы по группам лечения валсартаном и ирбесартаном в виде моно- или комбинированной терапии с гидрохлортиазидом. Через 3 нед. фармакотерапии проводили генотипирование по полиморфным маркерам *CYP2C9 (Arg144Cys), CYP2C9 (Ile359Leu), AGTR1 (A1166C), AGT (Met235Thr, C4072T), ACE (I/D), CYP11B2 (C-344T)* и определение минимальной равновесной концентрации ирбесартана и валсартана. Офисное измерение АД выполняли на каждом визите.

**Результаты.** Носители аллелей *2 и *3 гена *CYP2C9*, генотипа T/T гена *AGT*, генотипа I/I по I/D-полиморфизму *ACE* достигали более высоких значений минимальной равновесной концентрации ирбесартана через 3 нед. фармакотерапии. Гомозиготы A/A по генетическому полиморфизму гена *AGTR1 (A1166C)*, гомозиготы D/D по I/D полиморфизму *ACE* достигали более высоких значений минимальной равновесной концентрации валсартана через 3 нед. фармакотерапии. У пациентов, принимавших ирбесартан, было выявлено более выраженное снижение офисного систолического (САД) и диастолического (ДАД) АД при увеличении концентрации на каждые 100 нг/мл через 3 нед. терапии. Ассоциации показателей с концентрацией валсартана установлено не было.

**Заключение.** Эффекты ирбесартана и валсартана свидетельствуют о максимальной модуляции фармакодинамических эффектов в течение 3 нед. фармакотерапии с последующим закреплением в терапевтическом диапазоне и остановкой в увеличении эффективности при дальнейшем увеличении равновесной концентрации, что может быть использовано для прогнозирования терапии, её персонализации, лучшего контроля и высокого профиля безопасности.

**Ключевые слова:** артериальная гипертензия*; CYP2C9; AGTR1; AGT; ACE; CYP11B;* ирбесартан; валсартан; равновесная концентрация

**Список сокращений:** АГ — артериальная гиперетензия; АД — артериальное давление; РААС — ренеин-ангиотензин-альдостероновая система; *CYP2C9* — цитохром P450, семейство 2, субсемейство C, полипептид 9; *AGTR1* — ген рецептора 1-го типа ангиотензина II, *AGT* — ген ангиотензиногена; *ACE* — ген ангиотензинпревращающего фермента; *CYP11B2* — ген альдостерон синтазы; СКФ — скорость клубочковой фильтрации; АПФ — ангиотензинпревращающий фермент; БРА — блокаторы рецепторов ангиотензина II; ДИ — доверительный интервал; ОШ — отношение шансов; САД — систолическое артериальное давление; ДАД — диастолическое артериальное давление; ЧСС — частота сердечных сокращений.

## INTRODUCTION

Arterial hypertension (AH) is one of the most significant global health problems [1, 2], affecting 16 to 37% of the adult population worldwide [3–5]. A high prevalence of this disease causes its significant impact on the mortality and morbidity, as it is a leading risk factor for cardiovascular accidents [6], including a coronary heart disease [7], acute cerebrovascular disorders [8], a heart failure and a peripheral vascular disease [9, 10].

A blood pressure (BP) regulation is a complex multifactorial process involving the renin-angiotensin-aldosterone system (RAAS) [11, 12], natriuretic peptides [13], endothelial mechanisms [14], a sympathetic nervous system and immune processes [15,

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16]. The current studies show that a genetic predisposition to it covers a wide range of genetic variations, from rare monogenic mutations to polygenic associations involving more than 1500 single-nucleotide polymorphisms [18, 19].

The development of pharmacogenomics has significantly expanded the understanding of the complex causal relationships between the BP levels, genetic and epigenetic factors, and the risk of hypertensive complications. The first advances in this field have opened new perspectives for a personalized approach to the antihypertensive therapy aimed at optimizing the treatment efficacy and reducing the risk of side effects. The introduction of the personalized medicine principles in the management of hypertension patients can contribute not only to a more accurate selection of antihypertensive drugs, but also to the improvement of a long-term prognosis through an individualized control of risk factors.

**THE AIM** of the work was to study the relationship of the minimum steady-state concentration of angiotensin II receptor blockers with polymorphic markers of *CYP2C9 (Arg144Cys)*, *CYP2C9 (Ile359Leu)*, *AGTR1 (A1166C)*, *AGT (Met235Thr*, *C4072T)*, *ACE (I/D)*, *CYP11B2 (C-344T)* genes and the office blood pressure indices.

## MATERIALS AND METHODS

The study design was an open randomized controlled pharmacogenetic and pharmacokinetic clinical trial.

The study included 179 patients of the Moscow region with newly diagnosed hypertension of stages 1–2, among whom there were141 (78.8%) women and 38 (21.2%) men aged from 32 to 69 years (mean age — 58.2±6.4, median age — 60 (57–63 years).

### Eligibility criteria

The patients met the following *inclusion criteria*: hypertension of stages 1–2; the age between 18 and 74 years; a signed written informed consent from the patient to participate in the study.

The *non-inclusion criteria* of the patients in the study were as follows: hypertension of stage 3; the uncontrolled arterial hypotension; hypersensitivity to irbesartan and valsartan or excipients of the drugs; an active liver disease or an increase in the serum transaminase activity more than 3-fold, the liver failure (Child-Pugh classes A and B); a chronic kidney disease of stages 4–5 (glomerular filtration rate less than 30 mL/min/1.73 $m^2$; creatinine clearance <30 mL/min); decompensated diabetes mellitus; pregnancy and lactation; the age under 18 years and over 75 years; patients with primary hyperaldosteronism; angioedema, including Quincke's oedema; during treatment with the drugs affecting the RAAS, including angiotensin-converting enzyme (ACE) inhibitors, a concomitant use of aliskiren and the drugs containing aliskiren in the patients with diabetes mellitus and/or a moderate or severe renal dysfunction (glomerular filtration rate (GFR) less than 60 mL/min/1.73 $m^2$ body surface area); a concomitant use with ACE inhibitors in the patients with diabetic nephropathy; galactose intolerance, lactase insufficiency and a glucose-galactose malabsorption syndrome; an established diagnosis of malignancy at the inclusion time in the study; the need for a continuous intake of non-steroidal anti-inflammatory drugs and/or the drugs metabolized by cytochrome P-450 *CYP2C9*, which can affect the efficacy and safety profile of irbesartan.

*Exclusion criteria:* pregnancy, a development of serious adverse drug reactions, an acute myocardial infarction, an acute cerebral circulatory failure. No patient dropped out of the study.

### Study duration

The patients were recruited from 1 July 2021 to 28 August 2022. The selection of the study participants was carried out in the outpatient treatment and preventive care institutions of Moscow, clinical bases of the Department of Clinical Pharmacology and Propaedeutics of Internal Medicine of Sechenov First Moscow State Medical University (Sechenov University), Mukhin City Clinical Hospital, Hospital for War Veterans No. 3, Central Clinical Hospital of Civil Aviation, Municipal Polyclinic No. 2.

### Randomization procedure

All the patients included in the study had not previously received regular antihypertensive therapy and were randomly allocated to the irbesartan and valsartan groups by a simple randomization (an envelope method).

### Study methodology

The study participants received ARBs irbesartan (Aprovel, Sanofi Winthrop Industries, France) and valsartan (Diovan, Novartis Pharma GmbH, Germany) in monotherapy or in the combination with hydrochlorthiazide (Coaprovel, Sanofi Winthrop Industries, France; Co-Diovan, Novartis Pharma GmbH, Germany) for 3 months. Four groups of patients were formed: Group 1 (irbesartan 150 mg once daily) — 32 patients; Group 2 (irbesartan 150 mg+hydrochlorothiazide 12.5 mg once daily) — 51 patients; Group 3 (valsartan 80 mg once daily) — 8 patients; Group 4 (valsartan 80 mg+hydrochlorothiazide 12.5 mg once daily) — 88 patients. When the target

BP values were achieved after 3 weeks of therapy (<140/90 mmHg, if well tolerated <130/80 mmHg, but not <120/70 mmHg), the patients continued to follow the prescribed therapy during 3 months of treatment. In case of the insufficient BP control, the intensification of therapy was performed by increasing the dose of irbesartan or valsartan to 300 and 160 mg, respectively, as a part of mono- or combination therapy.

Three weeks after the inclusion in the study, the blood was drawn by a Vacuette vacuum system (Shandong Chengwu Medical Products Factory, China) by a venipuncture of the middle elbow or saphenous vein to determine the genetic polymorphisms of *CYP2C9*2* (*Arg144Cys*) and *CYP2C9*3* (*Ile359Leu*), rs5186 (*A1166C*) of angiotensin II type 1 receptor gene *AGTR1*, rs699 (*Met235Thr*) of the angiotensinogen gene *AGT*, rs4646994 of Alu Ins/Del of the angiotensin-converting enzyme gene *ACE*, rs1799998 (*C-344T*) of the aldosterone synthase gene *CYP11B2*. The determination of the minimum-steady concentration of ARBs was carried out. The office BP measurements were performed at each visit: at the study inclusion, at intermediate stages after 3 weeks and after 3 months of therapy. BP was measured on both arms using the Korotkoff method after a 10-minute rest of the patient in the sitting position, and was determined as the average of three measurements taken at 1-minute intervals [20–22].

### Determination of genetic polymorphism

To determine genetic polymorphisms, a real-time PCR method on the DNA amplifier "CFX96 Touch Real Time System" with software "CFX Manager" from BioRad (USA) and commercial kits were used.

The detection of the *CYP2C9*2* (*Arg144Cys*) and *CYP2C9*3* (*Ile359Leu*) alleles was performed using the RealBest-Genetika Warfarin kit manufactured by VectorBest (Russia), based on PCR followed by analysis of the melting curves of the resulting amplicons.

The genetic polymorphism rs5186 (*A1166C*) of the angiotensin II type 1 receptor gene *AGTR1* was determined using a reagent kit for a real-time polymorphism detection in the human genome "SNP-EXPRESS" manufactured by Litech Research and Development Company LLC (Russia).

The genetic polymorphism rs699 (*Met235Thr*) of the angiotensinogen *AGT* gene was determined using a reagent kit for a real-time polymorphism detection in the human genome "SNP-EXPRESS" produced by Litech Research and Development Company LLC (Russia).

The genetic polymorphism rs4646994 Alu Ins/Del of the angiotensin-converting enzyme *ACE* gene was determined using the "SNP-SHOT" Two-step kit produced by Litech Research and Development Company LLC (Russia) and its accompanying instructions.

The genetic polymorphism rs1799998 (*C-344T*) of the aldosterone synthase *CYP11B2* gene was determined using the reagent kit for a polymorphism detection in the human genome "SNP-Screen" ZAO NPK Syntol (Russia).

### Irbesartan and valsartan concentration study

Irbesartan and valsartan concentrations in blood plasma were studied using HPLC on an Agilent 1290 Infinity II LC liquid chromatograph coupled with the 6470 Triple Quadrupole LC/MS (Agilent Technologies, USA) using standard calibration solutions with concentrations of 2500, 1000, 500, 250, 100, 50, 25 and 10 ng/mL, trifluoroacetic acid acid, acetonitrile and purified Milli-Q water for HPLC. The additional equipment included ME54 METTLER TOLEDO analytical scales (USA), single-channel mechanical pipettes with variable volumes of 100–1000 and 20–200 µl (Thermo Scientific Black), a centrifuge (Eppendorf, Germany), C-18 column, 50×2.1 mm, 1.7 µm, a liquid chromatograph. 250 µl of each calibration standard solution of irbesartan was transferred into a 1.5 mL microtube, 250 µl of plasma was added. The final concentration of irbesartan calibration standard solutions were 2500, 1000, 500, 500, 250, 250, 100, 50, 25 and 10 ng/mL respectively. The sample preparation was carried out in such a way — 500 µl of acetonitrile was added to 500 µl of the sample. It was carefully pipetted and centrifuged for 10 min at 13 200 rpm. The supernatant was collected in individual microtubes and used for the analysis.

250 µl of each valsartan calibration standard solution was transferred to a 1.5 mL microtube and 250 µl of the plasma obtained by centrifugation was added. The final concentrations of the valsartan calibration standard solutions were 2500, 1000, 500, 500, 250, 100, 50, 25, 5 and 1 ng/mL, respectively. The sample preparation was carried out in such a way — 500 µl of acetonitrile was added to 500 µl of the sample. The sample was carefully pipetted and centrifuged for 10 min at 13 200 rpm. The supernatant was collected in individual microtubes and used for the analysis.

### Ethical approval

The study was approved by the Local Ethical Committee of the Sechenov First Moscow State Medical University (Sechenov University) (Protocol No. 05-21 dated 10 March 2021). A written voluntary informed consent for the participation in the study was obtained from all the patients.

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### Statistical analysis

The sample size had not been pre-calculated. The statistical analysis and visualisation of the obtained data were performed using an R 4.2.3 statistical computing environment (The R Foundation, Austria). The descriptive statistics for quantitative variables without a pronounced asymmetry of conditional sampling distributions are presented as a mean (±standard deviation, M+SD), for quantitative variables with a pronounced asymmetry (absolute values of the asymmetry coefficient >1.96) — as a median ($1^{st}$ and $3^{rd}$ sample quartiles), Me (Q1–Q3). Descriptive statistics for the qualitative variables are presented as a number of observations (relative frequency, %). The Fisher's exact test was used to compare the groups with respect to the qualitative variables. The likelihood ratio test was used to analyze the correspondence of the empirical distribution of genotypes to the theoretical one defined by the Hardy-Weinberg equilibrium. For the correlation analysis, the Spearman's rank correlation coefficient ρ with corresponding 95% confidence intervals (95% CIs) was used, and regression coefficients (with corresponding 95% CIs) in single-factor regression models were estimated if there was a statistically significant correlation between quantitative indices. To assess the strength and statistical significance of the association of quantitative predictors with binary outcomes, single-factor logistic regression models were used, with coefficients estimated with the Firth (1993) adjustment for rare outcomes.

Linear regression models with the inclusion of an interaction term between the genotype and drug used and robust standard errors of the regression coefficients were used to compare the effects of the genotypes with changes in systolic (SBP), diastolic (DBP) BP and heart rate (HR). To assess a relative contribution of the drug concentration as a mediator of the identified genotype effects, two linear regression models were constructed: a two-factor outcome model including the genotype and concentration and a single-factor genotype-dependent concentration model. They were used to estimate the total and partial genotype effects and the ratio of coefficients (the sum of coefficients) to calculate the proportion of the genotype effect mediated by the concentration (a standard error was estimated using a nonparametric estimator). The association was considered statistically significant at $p$ <0.05 [20–22].

## RESULTS

Table 1 and Figure 1 show comparative analysis results of the minimum steady-state drugs concentrations depending on the use of hydrochlorthiazide. Higher concentrations of irbesartan ($p$ <0.001) and valsartan ($p$=0.011) were achieved with monotherapy.

In the comparative analysis, a difference regarding the effect of the *CYP2C9* (*Arg144Cys*) genotype in the use of irbesartan and valsartan was found out ($p$=0.002; Fig. 2); a carriage of *2 allele in the patients receiving irbesartan, was statistically significantly associated with a higher concentration of irbesartan ($p$ <0.001). Among the patients receiving valsartan there was no statistically significant association of a steady-state drug concentration with a genotype at this locus ($p$=0.854).

Regarding the effect of the *CYP2C9* (*Ile359Leu*) genotype, a difference in the concentration was also found out between irbesartan and valsartan ($p$ <0.001; Fig. 3). The carriage of allele *3 (33 patients) in the patients receiving irbesartan was statistically significantly associated with a higher concentration of irbesartan ($p$ <0.001). Among the patients receiving valsartan there was no statistically significant association of the steady-state drug concentration with a genotype at this locus ($p$=0.854).

There was a trend towards an association between the genotype at the *AGTR1* (*A1166C*) locus and the steady-state concentration achieved with irbesartan and valsartan ($p$=0.086; Fig. 4). No statistically significant association of this polymorphic site with the irbesartan concentration was found out ($p$=0.55). The heterozygotes at this locus taking valsartan were characterized by a statistically significantly lower drug concentration compared to AA ($p$=0.001) and CC ($p$=0.032) homozygotes.

The effect of the *AGT* (*Met235Thr C4072T*) genotype on the steady-state concentration was statistically significantly different between the patients receiving irbesartan and valsartan ($p$=0.001; Fig. 5). The use of irbesartan by the TT homozygotes was associated with a higher steady-state concentration compared to the heterozygotes ($p$=0.019) and TT homozygotes ($p$=0.017), the group of patients receiving valsartan showed a trend towards a lower concentration in the TT homozygotes compared to the heterozygotes and CC homozygotes ($p$=0.058).

The genotype of the *ACE* polymorphic locus had a different effect on the steady-state concentrations of irbesartan and valsartan ($p$=0.003; Fig. 6). The D/D homozygotes receiving irbesartan showed a statistically significantly lower drug concentration compared to I/I homozygotes ($p$=0.013), the other pairwise comparisons showed no statistically significant differences ($p$=0.176). Among the patients receiving valsartan, the lowest concentration was characterized by the homozygotes

I/I and heterozygotes, with differences being statistically significant when comparing the drugs concentration between the homozygotes D/D and heterozygotes ($p$=0.024).

There was a tendency to the association between the steady-state concentration of the studied sartans and the *CYP11B2* (*C344T*) genotype, and the association was statistically significantly dependent on the drug ($p$=0.007; Fig. 7). The heterozygotes taking irbesartan were characterized by the highest concentration of the drug, the lowest concentration of valsartan was found out in the CC homozygotes, and the highest — in the TT homozygotes.

Table 3 shows the results of changes in the office SBP, DBP and HR during the therapy and the minimum steady-state drug concentration.

The patients receiving irbesartan showed statistically significantly greater reductions in the office SBP and DBP (a mean of 1.26 [95% CI: -1.51; -1] mmHg and 0.86 [95% CI: -1.16; -0.55] mmHg for every 100 ng/mL increase) at the interim assessment. No statistically significant association of these parameters with a valsartan concentration was found out. However, among the patients receiving valsartan at the interim assessment, a statistically significantly less pronounced decrease in the office HR was found out with a mean of 0.25 [95% CI: 0; 0.5] bpm for every 100 ng/mL increase in the concentration. When assessed at the end of the study, an increase in the irbesartan concentration for every 100 ng/mL was associated with a mean of 0.25 [95% CI: -0.14; 0.64] mmHg less than the pronounced decrease in the office DBP; an increase in the valsartan concentration for every 100 ng/mL was statistically significantly associated with a smaller decrease in the office SBP by a mean of 0.41 [95% CI: 0.02; 0.79] mmHg and the office HR — by a mean of 0.38 [95% CI: 0.21; 0.55] bpm.

In a single-factor regression analysis, an increase in irbesartan and valsartan concentrations for every 100 ng/mL was associated with a mean of 1.21 [95% CI: 1.08; 1.37] fold ($p$=0.001) and 1.3 [95% CI: 1.16; 1.46] ($p$ <0.001) fold increase in the odds of achieving the target BP numbers at the interim study (Fig. 8), and a reduced need for the intensification of therapy (OR=0.51 [95% CI: 0.36; 0.7] and 0.78 [95% CI: 0.7; 0.88] ($p$ *<0.001* respectively; Fig. 9). At the end of the study, there was an inverse association between the irbesartan concentration and the odds of the achieving target BP (OR=0.64 [95% CI: 0.42; 0.99] ($p$=0.043). No statistically significant association was found among the patients receiving valsartan (OR=0.96 [95% CI: 0.79; 1.16], $p$=0.651) (Fig. 10). As among the patients receiving irbesartan there was also a statistically significant association of the steady-state concentration with increased odds of the developing arterial hypotension (OR=1.72 [95% CI: 1.15; 2.56], $p$=0.008), the association was not statistically significant with valsartan (OR=1.05 [95% CI: 0.86; 1.27], $p$=0.651) (Fig. 11).

## DISCUSSION

Currently, the number of studies investigating the association of genetic polymorphisms and the ARBs plasma concentration and its possible effects, is limited by a number of genetic polymorphisms studied, the sample size, and race.

In one of the first studies on the relationship between the concentration of an antihypertensive drug and its efficacy depending on the genotype, L. Kurland et al. [23] found out a relationship between the concentration of irbesartan in plasma and the dynamics of the BP reduction in the patients' homozygous T/T for the genetic polymorphism *C5245T* of the *AGTR1* gene. The study included 42 patients with hypertension of stages 1–2 and left ventricular hypertrophy, who were prescribed irbesartan 150 mg as monotherapy for 12 weeks. They were measured BP and the irbesartan concentration in its minimum value (24 h after the last dose), determined the genotype by five genetic polymorphisms of the *AGTR1* gene. The authors obtained the following results: neither an irbesartan concentration nor the genetic polymorphisms were associated with a BP response to the irbesartan treatment. However, there was an interaction between the plasma irbesartan concentration and the *AGTR1 C5245T* gene polymorphism with a decreased SBP ($p$=0.025). The irbesartan concentration was associated with an SBP change in the T/T homozygotes of the *AGTR1 C5245T* gene — $r$=-0.56, $p$=0.03).

In the study conducted in China by G. Chen et al. [24], the effect of the genetic polymorphism *CYP2C9* on the plasma concentration of irbesartan 30 min, 2 and 6 h after the administration, and the pharmacodynamic efficacy of irbesartan, were analyzed. A total of 196 patients participated in the study. The authors obtained the following results: the patients with the *CYP2C9*1 / CYP2C9*3* genotype had significantly higher plasma concentrations of irbesartan compared to the *CYP2C9*1 / CYP2C9*1* genotype (beta±SE=81±36) and a more pronounced DBP response (beta±SE=5.6±2.5 mmHg) at the 6 h time point, which correlates with the authors' findings. The authors concluded that the *CYP2C9*3* gene variant significantly alters the plasma concentration and the DBP response at the 6 h time point after the irbesartan treatment in the Chinese patients with hypertension.

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**Table 1 – Minimum steady-state concentration of irbesartan and valsartan depending on the pharmacotherapy regimen, ng/mL, Me (Q1-Q3)**

| All patients (*n*=179) | Monotherapy (*n*=40) | Combination therapy (*n*=139) | *p* |
|---|---|---|---|
| **Irbesartan** | | | |
| 2007 (1732–2554) | 2438 (1990–2672) | 1776 (1680–2293) | <0.001 |
| **Valsartan** | | | |
| 1163 (727–1537) | 1504 (1428–1580) | 1048 (688–1529) | 0.011 |

**Table 2 – Minimum steady-state concentration of irbesartan and valsartan according to *CYP2C9* (*Arg144Cys*), *CYP2C9* (*Ile359Leu*), *AGTR1* (*A1166C*), *AGT* (*C4072T*), *ACE* (I/D polymorphism), *CYP11B2* (*C-344T*) genotype, ng/mL, Me (Q1-Q3)**

| Gen | Genotypes | Irbesartan, ng/mL | Valsartan, ng/mL |
|---|---|---|---|
| *CYP2C9* (*Arg144Cys*) | *1/*1 | 1856 (1684–2303) | 1231 (688–1562) |
| | *1/*2 \| *2/*2 | 2557 (2472–2630) | 1095 (776–1327) |
| *p* | – | <0.001 | 0.854 |
| *CYP2C9* (*Ile359Leu*) | *1/*1 | 1914 (1690–2346) | 1095 (586–1580) |
| | *1/*3 | 2798 (2689–2950) | 1231 (1002–1355) |
| *p* | – | <0,001 | 0.854 |
| *AGTR1* (*A1166C*) | AA | 2049 (1745–2573) | 1428 (740–1580) |
| | AC | 1914 (1720–2488) | 776 (505–1355) |
| | CC | 1982 (1693–2508) | 1315 (1002–1629) |
| *p* | – | 0.55 | <0.001 |
| *AGT* (*C4072T*) | CC | 1788 (1633–2341) | 1380 (1172–1568) |
| | CT | 1909 (1707–2346) | 1095 (740–1428) |
| | TT | 2476 (1969–2672) | 688 (519–1562) |
| *p* | – | 0.009 | 0.058 |
| *ACE* (*I/D* polymorphism) | I/I | 2346 (1973–2616) | 1095 (746–1580) |
| | I/D | 2007 (1682–2559) | 1002 (519–1428) |
| | D/D | 1789 (1690–2026) | 1492 (740–1685) |
| *p* | – | 0.016 | 0.03 |
| *CYP11B2* (*C-344T*) | CC | 1945 (1747–2410) | 889 (688–1428) |
| | CT | 2229 (1755–2629) | 1231 (505–1629) |
| | TT | 1830 (1655–2307) | 1355 (994–1580) |
| *p* | – | 0.055 | 0.072 |

**Table 3 – Results of correlation analysis of changes in SBP, DBP and HR during therapy and minimum steady-state drugs concentration**

| Index | Irbesartan | Valsartan |
|---|---|---|
| **3 weeks of pharmacotherapy** | | |
| Δ office SBP | -0.65 [-0.76; -0.51]; *p* <0.001 | -0.08 [-0.27; 0.13]; *p*=0.465 |
| Δ office DBP | -0.48 [-0.63; -0.29]; *p* <0.001 | -0.14 [-0.33; 0.06]; *p*=0.181 |
| Δ office HR | 0.02 [-0.19; 0.24]; *p*=0.846 | 0.21 [0.01; 0.4]; *p*=0.036 |
| **3 months of pharmacotherapy** | | |
| Δ office SBP | 0.18 [-0.04; 0.38]; *p*=0.115 | 0.25 [0.05; 0.43]; *p*=0.013 |
| Δ office DBP | 0.22 [0.01; 0.42]; *p*=0.045 | 0.1 [-0.1; 0.3]; *p*=0.312 |
| Δ office HR | 0.18 [-0.04; 0.38]; *p*=0.111 | 0.47 [0.3; 0.61]; *p* <0.001 |

Note: SBP — systolic blood pressure; DBP — diastolic blood pressure; HR — heart rate.

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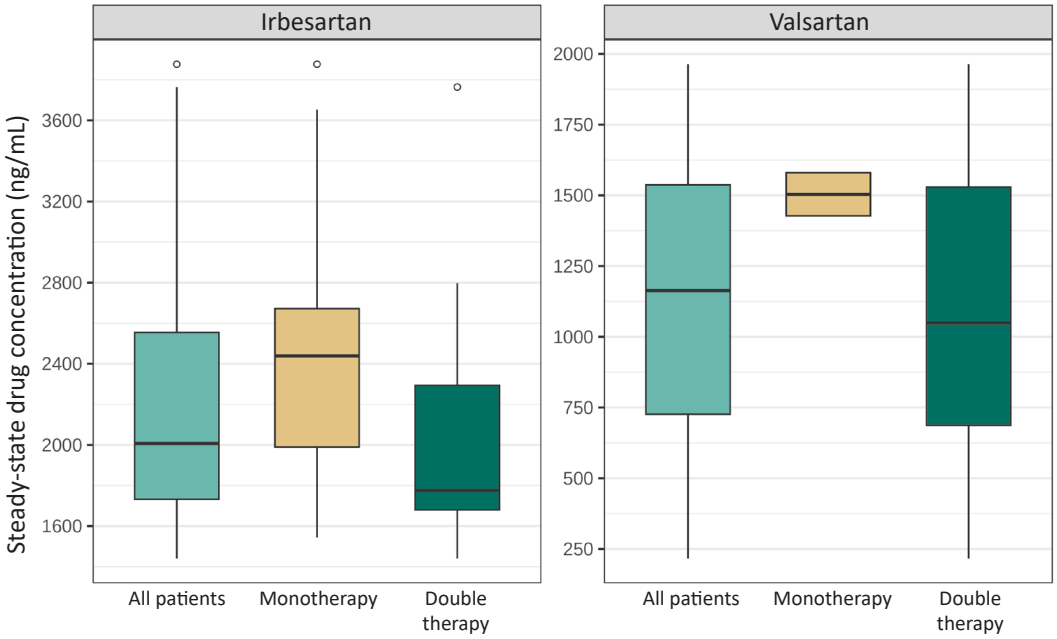


**Figure 1 – Minimum steady-state concentration of irbesartan and valsartan depending on the pharmacotherapy regimen.**

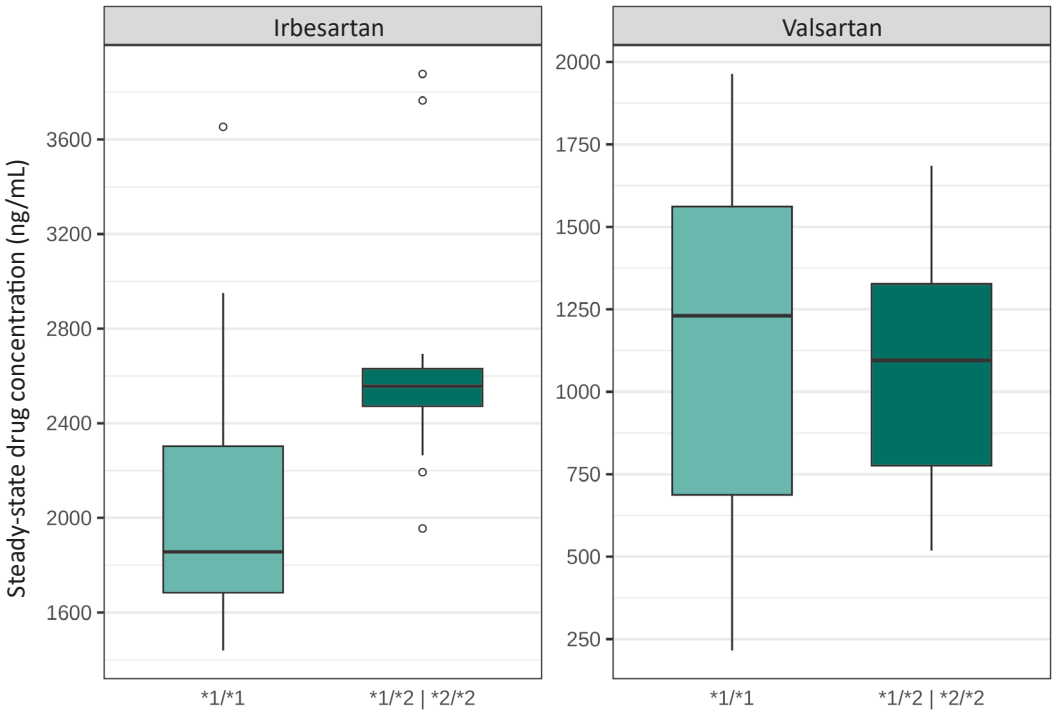


**Figure 2 – Minimum steady-state drugs concentration depending on *CYP2C9* genotype (*Arg144Cys*) in irbesartan and valsartan patient groups.**

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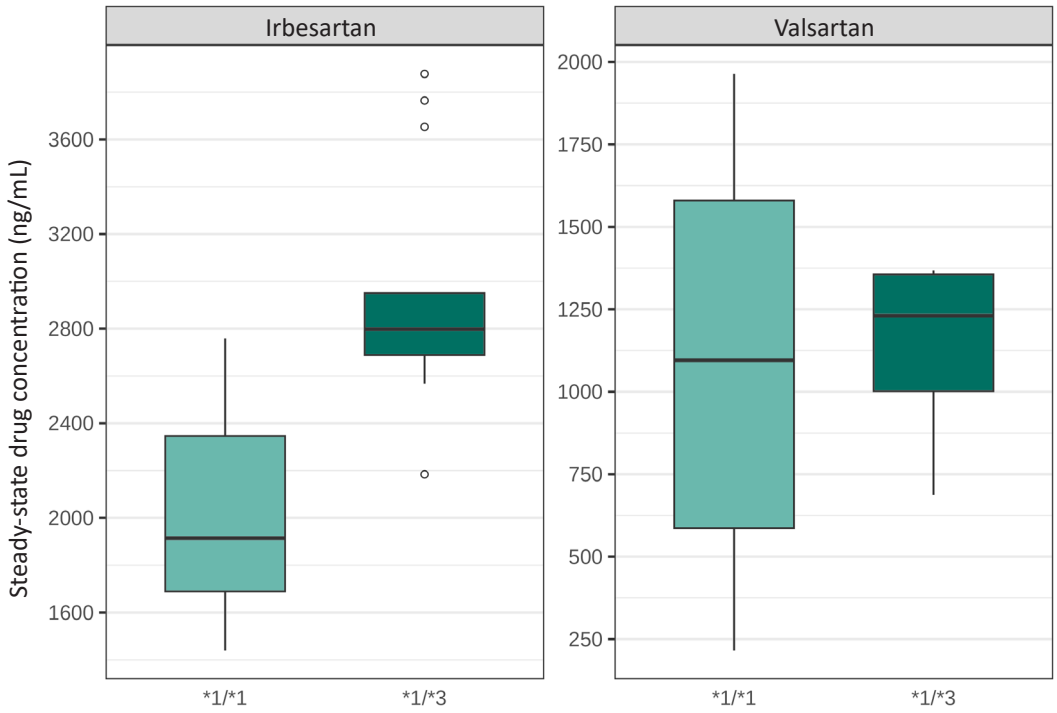


**Figure 3 – Minimum steady-state drugs concentration depending on *CYP2C9* genotype (*Ile359Leu*) in irbesartan and valsartan patient groups.**

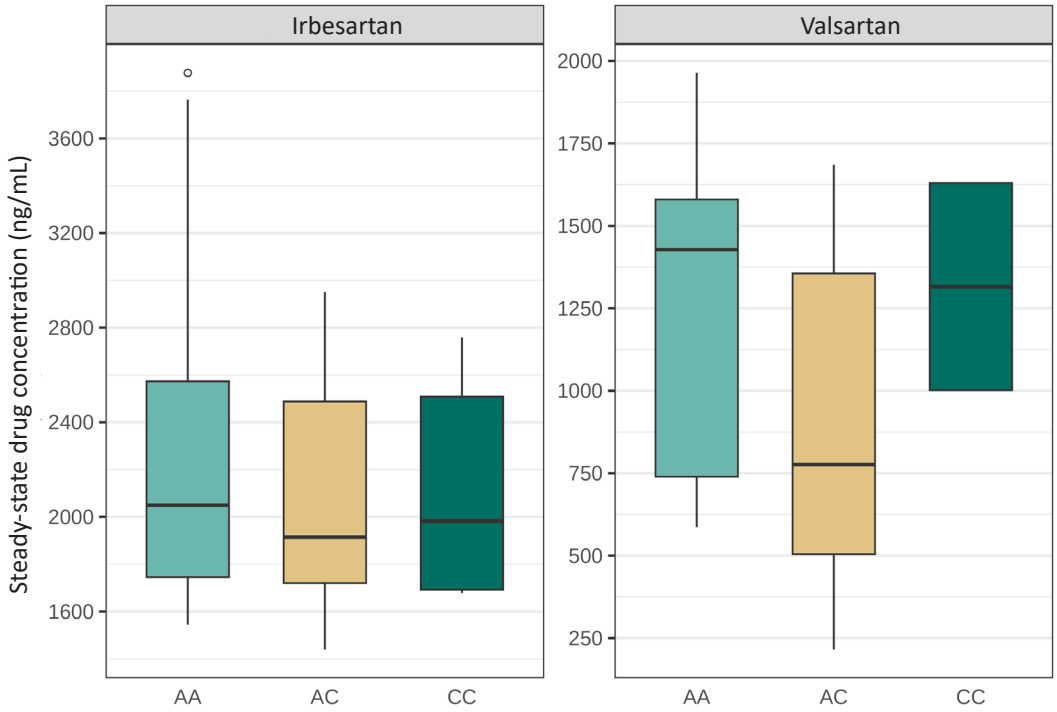


**Figure 4 – Minimum steady-state drugs concentrations depending on *AGTR1* genotype (*A1166C*) in irbesartan and valsartan patient groups.**

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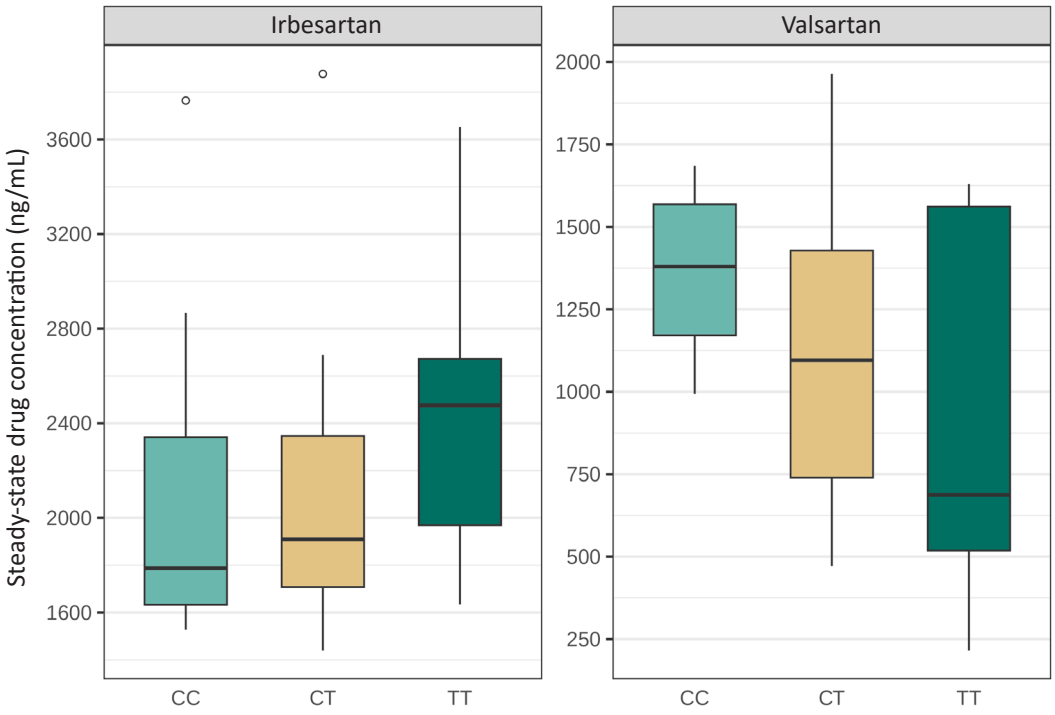


**Figure 5 – Minimum steady-state drugs concentrations depending on *AGT* genotype (*C4072T*) in irbesartan and valsartan patient groups.**

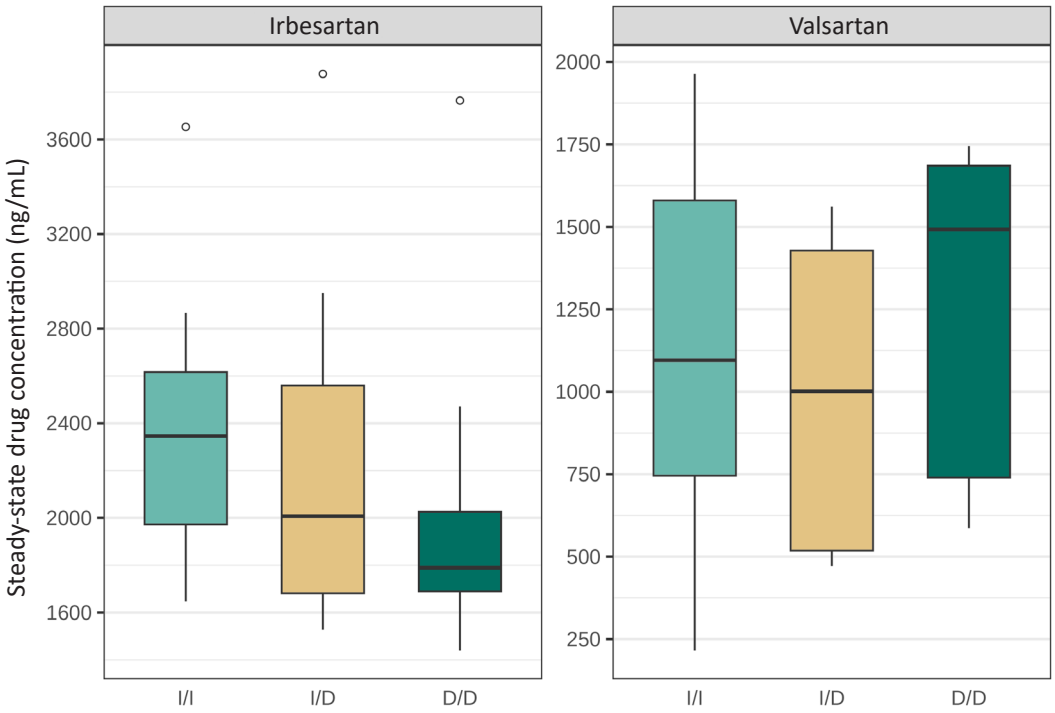


**Figure 6 – Minimum steady-state drugs concentration depending on *ACE* genotype (*I/D* polymorphism) in irbesartan and valsartan patient groups.**

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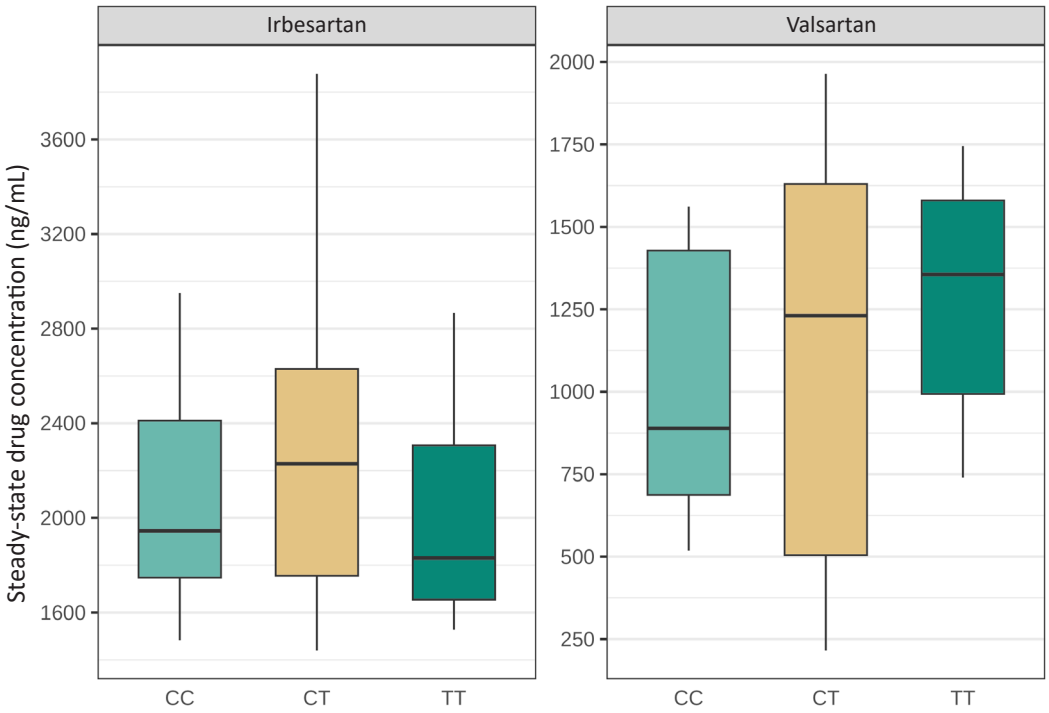


**Figure 7 – Minimum steady-state drugs concentration depending on *CYP11B2* genotype (*C-344T*) in irbesartan and valsartan patient groups.**

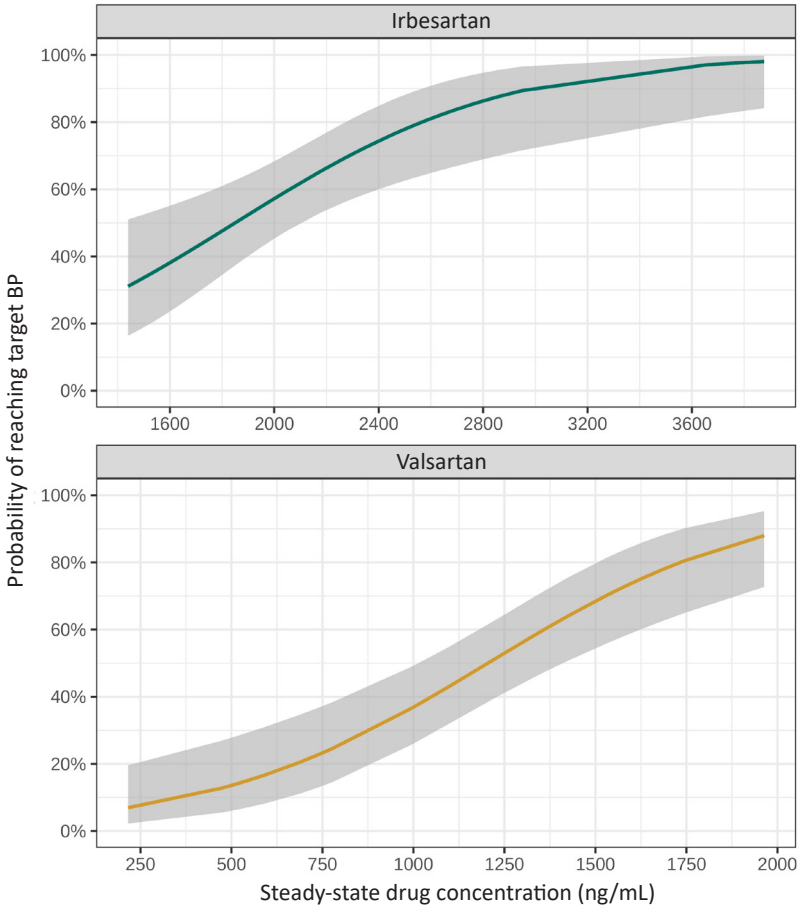


**Figure 8 – Probability of achieving the target blood presure at the intermediate stage as a function of the minimum steady-state drugs concentration.**

Note: BP — blood pressure.

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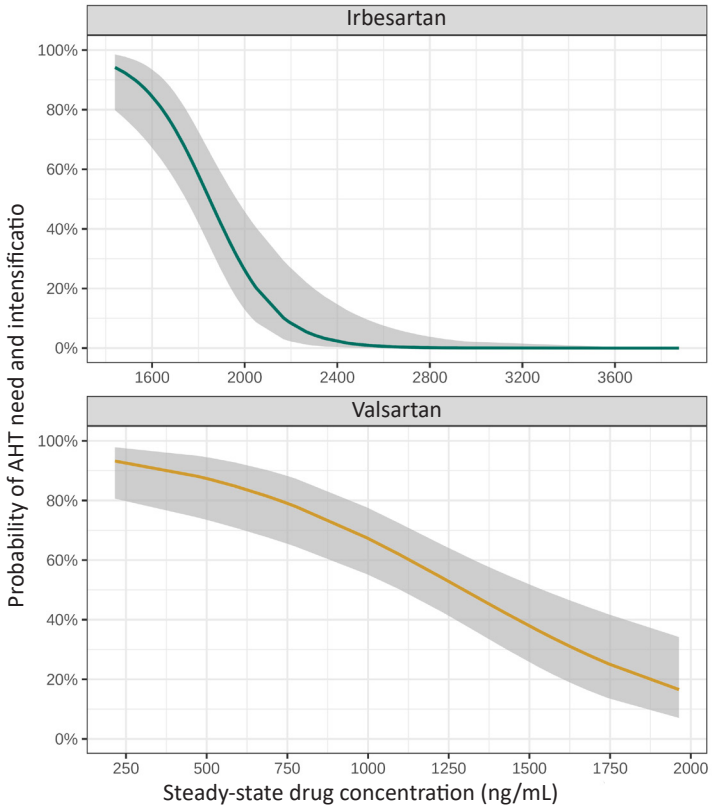


**Figure 9 – Probability of need and intensification of antihypertensive therapy depending on the minimum steady-state drugs concentration.**

Note: AHT — antihypertensive therapy.

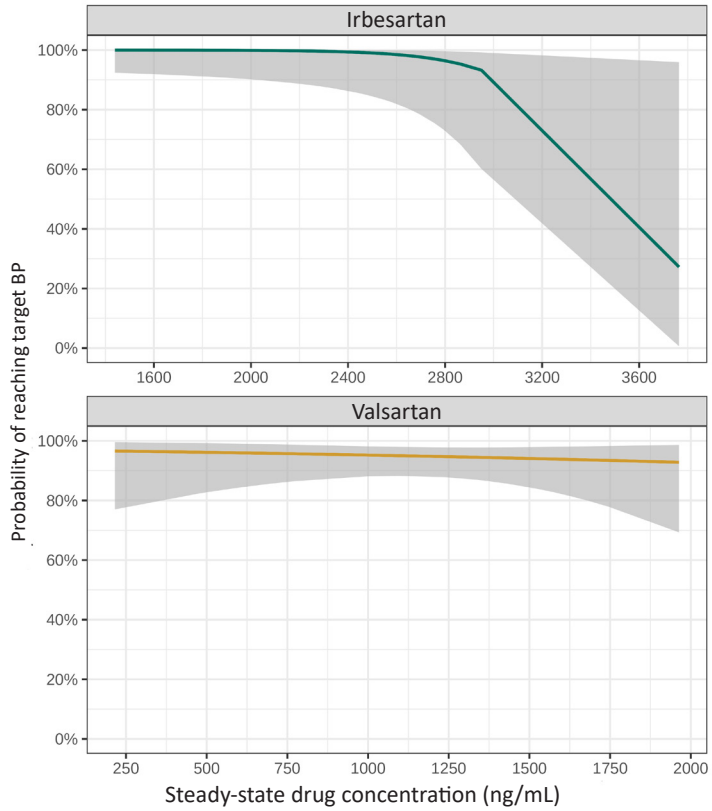


**Figure 10 –Probability of achieving the target blood pressure at the end of the study depending on the minimum steady-state drugs concentration.**

Note: BP — blood pressure.

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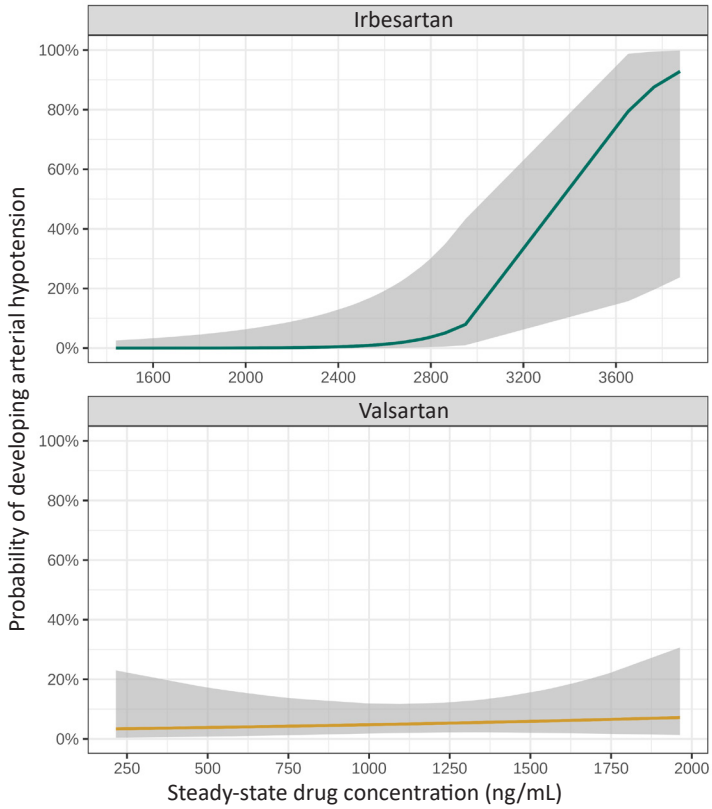


**Figure 11 – Probability of developing arterial hypotension depending on the minimum steady-state drugs concentration.**

In the study by S. Zhang et al. [25], the association between the *ANP Val7Met* polymorphism (a single nucleotide polymorphism database identifier — rs5063) and a baseline BP, the minimum steady-state plasma concentration of irbesartan and the antihypertensive efficacy of irbesartan, were studied in the AH patients in the Chinese population. A total of 756 patients were included in the study. The authors found no significant association between the antihypertensive efficacy and the *Val7Met* polymorphism in the overall population. But when analyzed by the baseline DBP level, the patients with the Val / Met+Met / Met genotype and the baseline DBP greater than or equal to 100 mmHg, had a significantly smaller reduction in DBP (the adjusted regression coefficient -5.7 [1.4] mmHg; $p < 0.001$) and SBP compared with the patients with the Val / Val genotype and the baseline DBP greater than or equal to 100 mmHg (the adjusted regression coefficient -9.8 [2.9] mmHg; $p<0.001$). Thus, the authors concluded that in the AH patients living in China, the *ANP Val7Met* polymorphism could be a genetic marker of the baseline DBP, the plasma irbesartan concentration and the antihypertensive efficacy of a short-term irbesartan therapy.

In the study by S. Hu et al. [26], the relationship of the kininogen gene (*KNG1*) polymorphism *Ile197Met* and the gender was investigated in the hypertension patients' plasma with the concentration of irbesartan. The study enrolled 1 100 patients. The authors determined that the male patients carrying the G allele had significantly lower plasma concentrations (GG — $p$=0.015; TG — $p$=0.015, respectively) compared with the TT genotype, and concluded that the interaction of the gender and the *KNG1 Ile197Met* polymorphism could influence the plasma concentration of irbesartan, which can contribute to a better development of a personalized hypertension treatment in Chinese patients.

According to the results of the study, in the course of a comparative analysis, the differences were established with regard to the influence of a genotype according to the studied genetic polymorphisms, both in the group of patients taking irbesartan and valsartan, on the values of the minimum steady-state ARBs concentration.

Thus, among the patients taking irbesartan, the figures of the minimum steady-state concentration were significantly higher in the carriers of allele *2 for the genetic polymorphism of the *CYP2C9* gene (*Arg144Cys*), the carriers of allele *3 for the genetic polymorphism of the *CYP2C9* gene (*Ile359Leu*), in T/T homozygotes for the genetic polymorphism of the *AGT* gene (*Met235Thr*, *C4072T*), in the homozygotes I/I by the I/D polymorphism

of the *ACE* gene, and there was also a tendency for an association between the minimum steady-state concentration and the genotype by the genetic polymorphisms of the *AGTR1 (A1166C)* and *CYP11B2 (C-344T)* genes — higher values of the minimum steady-state concentration were observed in the homozygotes A/A and heterozygotes C/T, respectively. Herewith, the patients in the irbesartan group showed a significantly more pronounced decrease in the office SBP and DBP at an increase in the concentration for every 100 ng/mL after three weeks of the drug administration compared to the group of the valsartan patients, and after 3 months of therapy with irbesartan, there was a statistically significant less pronounced decrease in the office DBP for every 100 ng/mL. Increasing the plasma concentration of irbesartan for every 100 ng/mL was associated with a mean of 1.21 [95% CI: 1.08; 1.37] fold increase in the odds of achieving the target BP numbers after 3 weeks of therapy ($p$=0.001) and a corresponding decrease in the need for the intensification of therapy (OR=0.51 [95% CI: 0.36; 0.7] ($p$<0.001).

Among the patients taking valsartan, the figures of the minimum steady-state concentration were significantly higher in the A/A homozygotes for the *AGTR1* gene polymorphism (*A1166C*), in the D/D homozygotes for the I/D polymorphism of the *ACE* gene, and there was also a tendency to the presence of a relationship between the minimum steady-state concentration of valsartan and the genotype of the genetic polymorphism of the *AGT* gene (*Met235Thr*, *C4072T*) — higher values were determined in the C/C homozygotes. Herewith, no statistically significant associations with the office SBP and DBP were found when increasing the minimum steady-state concentration by every 100 ng/mL when assessed after 3 weeks of pharmacotherapy. When analyzed after 3 months of the valsartan therapy, an increase in the concentration for every 100 ng/mL was significantly associated with a smaller decrease in the office SBP and a less pronounced decrease in the night SBP variability. Increasing the valsartan plasma concentration by every 100 ng/mL was associated with a mean of 1.3 [95% CI: 1.16; 1.46] fold increase in the odds of achieving the target BP after 3 weeks of therapy ($p$ <0.001) and a corresponding decrease in the need for the intensification of therapy (OR=0.78 [95% CI: 0.7; 0.88] ($p$ <0.001).

### Study limitations

One of these study limitations is a relatively small sample size and a separate region of the study. A promising avenue for further research is to identify predictors of response to the antihypertensive therapy, including age, renin levels, and genetic polymorphisms affecting the pharmacodynamics and pharmacokinetics of the antihypertensive drugs, as these factors may have a significant impact on the efficacy and safety of the treatment.

A detailed analysis of an individual sensitivity of patients to different classes of antihypertensive drugs will allow the development of personalized approaches to the choice of starting therapy, which may improve the effectiveness of a BP control and reduce the risk of adverse reactions.

## CONCLUSION

Patients with newly diagnosed hypertension of stages 1–2, of the Moscow region, the carriers of alleles *2 and *3 of the *CYP2C9* gene, the genotype T/T of the *AGT* gene, the genotype I/I by the I/D-polymorphism of the *ACE* gene achieved significantly higher values of the minimum steady-state irbesartan concentration after 3 weeks of pharmacotherapy. The patients with newly diagnosed newly diagnosed hypertension of stages 1–2 of the Moscow region homozygotes A/A for the genetic polymorphism of the *AGTR1* gene (*A1166C*), the homozygotes D/D for the I/D polymorphism of the *ACE* gene achieved significantly higher values of the minimum steady-state valsartan concentration after 3 weeks of pharmacotherapy.

In the patients on the ARBs monotherapy, the values of the minimum steady-state irbesartan and valsartan concentrations were significantly higher compared to the patients on the combination therapy with hydrochlorthiazide.

The obtained irbesartan and valsartan effects indicate a maximal modulation of pharmacodynamic effects during 3 weeks of pharmacotherapy with a subsequent consolidation in the therapeutic range and stopping in the increase of the efficacy with a further increase of the steady-state concentration, which can be used for a pharmacokinetic therapy prediction, its personalization, a better control and a high safety profile.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## AUTHORS' CONTRIBUTION

Ekaterina V. Rebrova — concept development, conducting the study, preparation and editing the text, approval of the final version; Evgenia V. Shikh — concept development, conducting the study, preparation and editing the text, approval of the final version; George S. Anikin — conducting the study; Valery V. Smirnov — conducting the study, resource support of the study; Maxim M. Bogdanov — conducting the study; Ludmila M. Ignatova — conducting the study.

All the authors confirm that their authorship meets the international ICMJE criteria (all the authors have made a significant contribution to the development of the concept, research and preparation of the article, read and approved the final version before the publication).

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