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TRANSLATIONAL MEDICINE: UNTRANSLATED REGIONS AND THE STOP CODONS

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4P medicine is impossible without an efficient transfer of advanced laboratory techniques (such as regenerative cell technology, high-throughput sequencing, genome editing, etc.) into clinical practice. Translational Medicine - a new scientific field, designed to reduce the time of transfer of long-term achievements of fundamental scientific research in the development of innovative product applications. However, on the path of innovation in medicine, there are a number of natural barriers that do not allow to go to practice unsafe and ineffective products. The system of these barriers must be dynamic and up-to-date. As well as the education system for work at the "science–medicine" junction.

Keywords: translational medicine, innovations, preclinical trials, clinical trials

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ТРАНСЛЯЦИОННАЯ МЕДИЦИНА: НЕТРАНСЛИРУЕМЫЕ ОБЛАСТИ И СТОП-КОДОНЫ

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Медицина 4П невозможна без эффективного перевода на медицинские рельсы новейших лабораторных технологий, таких как регенеративные клеточные технологии, высокопроизводительное секвенирование, редактирование генома и др. Трансляционная медицина — новое научное направление, призванное сократить сроки перевода достижений длительных фундаментальных научных исследований в прикладные разработки инновационного продукта. Однако на пути внедрения инноваций в медицину есть ряд естественных барьеров, не позволяющих пройти в практику небезопасным и неэффективным разработкам. Система этих барьеров должна быть динамичной и отвечающей требованиям времени. Так же, как и система подготовки кадров для работы на стыке «наука–медицина».

Ключевые слова: трансляционная медицина, инновации, доклинические исследования, клинические исследования

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> Now, *here*, you see, it takes all the running *you* can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that!
>
> The Red Queen from *Alice Through the Looking Glass* by Lewis Carroll

Let's take a closer look at the list of Nobel Prizes in chemistry and physiology awarded over the past 30 years: a tremendous number of awards celebrate advancements in molecular and cellular biology with respect to their application in medicine (there is no Nobel Prize in biology). More than half of the articles published in *Nature* and *Science* focus on molecular and cellular biology.

After humanity got some insight into the structure of atoms and molecules, it decided to turn to what naturally builds around them, i.e. a living system. We are witnessing a revolutionary shift in the understanding of how living systems are organized, and are coming to realize the connections between the basic structural units of living things, which opens up new opportunities for improving public health and longevity.

Modern medicine is based on P4 principles: prediction, prevention, personalization and patient's participation.

One of the essential components of P4 medicine is breakthrough discoveries and advances in genetics, genomics, transcriptomics, proteomics, molecular biology, and genetic and protein engineering seen in the last few decades. The evolution of medical technologies used in genetics and genomics has made it possible to achieve high accuracy in the prediction of inherited monogenic diseases and multifactorial disorders with an inherited component. Cheaper diagnostic

tests can expedite the launch of population screening programs aimed to reduce the proportion of deleterious mutations in the human population. But transition to P4 medicine is impossible without incorporating such state-of-art lab technologies as regenerative cell technologies, high throughput sequencing, genome editing, etc. into clinical routine.

Translation of a discovery from a scientific lab to the clinical setting requires a comprehensive assessment of the effectiveness and safety of the innovation.

The history of medicine has seen some monstrous outcomes of hasty introduction of new technologies or pharmaceutical agents into clinical practice (remember a thalidomide tragedy [1], Fig. 1). Given that science is evolving rapidly, a thoroughly elaborated and dynamic approach to the adoption of innovations in public healthcare is required, giving birth to translational medicine.

What is translational medicine?

Translational medicine is a new scientific field which aims to narrow or completely eliminate the existing gap between research and clinical practice [2]. According to the basic principles of translational medicine, there are three stages of translational research (Fig. 2) [3]. The first stage is preclinical research that involves monitored translation of scientific discoveries into clinical practice, identifies the need for the diagnostic and treatment methods in question, and also focuses on the effectiveness and safety assessment of innovations. In the second stage, experts determine if innovations can be used in the clinical setting on real patients. In the third stage, clinical research findings make their way into the public health system.

Smooth adoption of an innovation into clinical practice is largely determined by a number of factors: how well research has been scaled up; whether high-tech methods have been applied; whether financial support can be provided by either the state or private investors; whether legislation and ethical standards have been adjusted considering the achievements of translational medicine.

State institutions for translational medicine development started to emerge in the 2000s, and are currently found in the USA, some European countries, China and Russia [4]. The number of research works on translational medicine has been expectedly increasing: the analysis of data stored in the Web of Science database revealed that there were only 5 publications on translational medicine in 1993, while by 2011 their number had reached 1 500 [4].

Lost in translation

As an innovative product is making its way from bench to bedside, it usually bumps into different barriers. Here, we face the need for new technologies that would allow us to make accurate predictions about the effectiveness and safety of novel compounds and medications and to improve the quality of preclinical and clinical trials. Of particular importance are services and projects aimed to ensure broad cooperation between state, commercial and non-profit organizations and to provide better access to and transparency of the new data to all researchers who work in translational medicine.

Over 80 % of substances (depending on the area of research) fail in a preclinical trial; only one of 20 drugs manages to successfully pass through all stages of a clinical trial [5]. Modern safety concepts improve the effectiveness of toxicity tests that new pharmaceutical agents have to undergo and expedite transition from animal testing to *in vitro* experiments. A key to the evolution of preclinical research is development of robotic cell based assay systems for quantitative high throughput screening. Biomodeling *in silico* allows researchers to test dose-response associations and build pharmacokinetic models under simulated conditions. Compared to traditional

Fig. 1. Thalidomide was a sedative prescribed to pregnant women in the late 1950s and 1960s. Among its adverse effects were congenital deformities. The photo shows a child born in 1963 (photo courtesy of *The Age*)

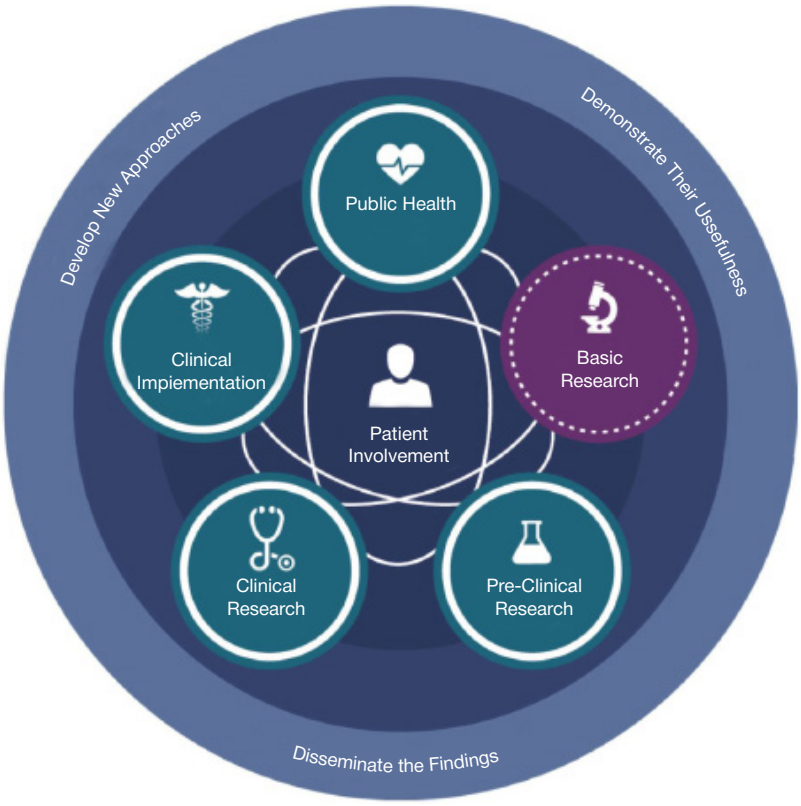

Fig. 2. Translational science spectrum as suggested by the US National Center for Advancing Translational Sciences [3]

toxicological strategies, such computer systems increase predictive capacity of research outcomes as the product moves on to the clinical stage [5, 6].

State-funded research centers and private pharmaceutical companies have always tended to collaborate, because scientists have always been concerned about finding a sponsor for their research and manufacturers have always been interested in developing effective medications in a short time and at little expense. However, recently the nature of this collaboration has changed due to the "customized" approach to research and development and better access to a tremendous volume of digital patient data available to researchers (primarily, in the US) [7]. Digital databases that store large volumes of information on many patients across the world hold promise to improve quality of preclinical and clinical research (Fig. 3); at the same time, there is a need to make this information even more accessible for researchers worldwide to improve its accuracy and quality.

Some barriers that innovations face on their way to practical medicine are quite natural as they prevent dangerous or ineffective products from entering clinical practice. But at the same time, these barriers are a major obstacle [8, 9]. Translational medicine aims to optimize algorithms that serve to incorporate innovations into real life and to make timely amendments to the legislation. Barriers should not be set higher than necessary.

Innovations travel the world at almost light speed, the underlying reason here being globalization. A promising medical technology, no matter where it was created, is bound to evolve and be successfully used in those countries where regulations are more flexible or liberal. Early implementation of innovative approaches gives those countries a head start over others, even if their risks are high. Tough regulations that block scientific achievements from entering medical practice may result in the loss of market.

An example of such competition is a situation in the USA and UK observed some years ago. Regulatory agencies kept banning CRISPR/Cas9 research on human embryos. However, after Chinese researchers published their work on human embryo genome editing that they had carried out using the above mentioned technique, British scientists got permission to use CRISPR/Cas9 and similar designer nucleases in February, 2016 [10]. As technologies find their way into practical application, public opinion starts to change and pushes the local legislation to introduce necessary adjustments.

Ethical factors related to the application of innovations, especially those of human genome editing, are a separate element of the system of translational medicine [11]. If these factors affect clinical implementation of novel technologies, they must be recorded and clarified by ethics committees and authorized agencies.

Structural gap

In Russia, translational technologies do not enjoy much support. Thus, after the Federal Law No. 323 *On fundamental healthcare principles in the Russian Federation* dated November 21, 2011 and the Federal Law No. 532 *On the amendments to individual legislative acts of the Russian Federation on the countermeasures against circulation of faked, counterfeit, improper and unregistered medicines, medical items and faked dietary supplements* dated December 31, 2014 were passed, the majority of innovative medical techniques and equipment created under federal grants or projects were blocked from entering clinical practice (or the procedure was delayed for 3–5 years).

A legislative trap has closed on 1) tests for rare diseases (mass production of medical products used to treat the latter is not profitable and their registration is unreasonable)

and 2) innovative technologies whose effectiveness, safety and clinical significance have been proved, but mass production and registration are still pending (usually the whole procedure takes about 5 years). But sometimes such innovations do not appear on the market because manufacturers do not have any financial reason to register them as medical products.

At the same time in most countries, such as the US, EU member countries and Australia, mass production of commercial medical products and medical laboratory practices are regulated by different laws. The use of unregistered materials or equipment in medical research is permitted if an institution strictly adheres to regulations and requirements established by law.

In these countries, laboratories that meet the established requirements and are closely monitored by authorized agencies are permitted to use in-house tests (or home brew tests, as they are referred to in Europe and Australia; in the USA they are called laboratory developed tests) or equipment/ materials adapted for clinical use (IVD products labeled RUO). Unfortunately, the Russian market may still be unable to offer foreign analogues of medical devices/agents because of their high cost, policies of foreign manufacturers and federal sanctions.

Translational offshore companies

Translation of a discovery from research to medical practice can be more or less difficult depending on a country's legislation; at the same time, administrative regulations within the same jurisdiction can be rather controversial. For example, the Federal Law No. 160 *On the International Medical Cluster and Amending Individual Legislative Acts of the Russian Federation* dated June 29, 2015 created a paradox: neither of Russian federal and national public health institutions now have a right to use unregistered innovative technologies and equipment in their work, while international joint ventures are permitted to do it on the territory of the Russian Federation.

Perhaps, such offshore companies (like Skolkovo) will pioneer translation of research into medical practice in Russia and encourage other public health institutions to adopt those tested and approved technologies. But if some players have everything while others have nothing, there remains a risk that a system cannot keep its balance and we will lose a chance to create a really effective translational space.

Medical education

If we want to expedite the process, we have to update the concept of medical education. A good example is the International School of Personalized and Translational Medicine, a part of Sechenov First Moscow State Medical University, established to develop solutions to the problem of basic research translation into a practical task of creating effective and safe methods of prevention, diagnosis and treatment based on the personalized approach tailored to individual characteristics of every patient. One of the areas the School focuses on is implementation of a new model of medical and pharmaceutical education. As the whole healthcare system is undergoing a unique transformation and the old model is being replaced by personalized and translational medicine, pharmaceutical industry is starting to play a very special role in expediting a pharmaceutical drug lifecycle from its inception to application and promotion. That is why in 2016 the Institute of Pharmacy and Translational Medicine was founded as part of the School structure. The goal of the Institute is to create effective research and learning environment that stimulates the development of innovative biomedical products and ensures excellent research work in all stages of their life cycle with further integration of scientific achievements into the educational

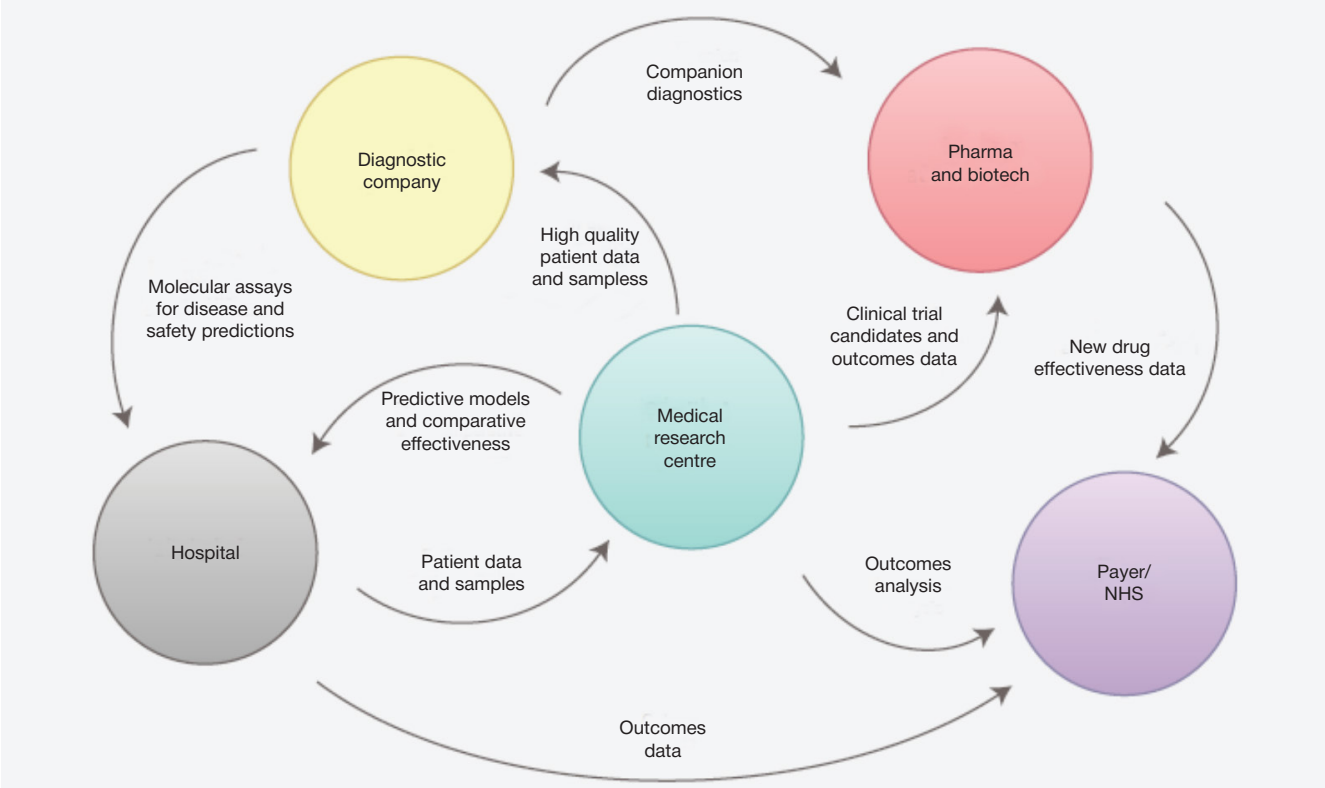

Fig. 3. Digital data on a large number of patients help to expand collaboration of state-funded research centers and private pharmaceutical companies [7]

process. The latter aims to prepare highly qualified specialists and top-class researchers for pharmaceutical industry. The Institute implements the principle of a full product cycle for biomedical products, from a basic idea, through applied and translational research to introduction into clinical practice and postmarketing studies. The Institute develops scientific and methodological principles that can underpin the creation of safe and effective biomedical products.

The Institute provides professional training in the field of translational research of any level. The Institute of Pharmacy and Translational Medicine in cooperation with its key partners was the first educational establishment in Russia to launch an innovative educational project ("Pharmacy") aimed to provide specialized training for the next generation of researchers who will work in various fields of biopharmaceutical industry. The students of the Institute get profound knowledge of molecular and cellular biology, medical genetics, bioinformatics, development of pharmaceutical agents, learn to apply the principles of good practice (laboratory, manufacturing, clinical, etc), study legal aspects of developing and promotion of biomedical products. Students obtain theoretical knowledge and practical skills from top scientists and experts of the pharmaceutical industry. A compulsory component of the educational curriculum is participation in international conferences and symposiums, internship in the world leading universities and international and Russian pharmaceutical companies; grant contests are also a part of the project. All students are required to write a thesis based on the research that they carry out using the facilities of the Institute or partner organizations. Over the course of their research, students must apply knowledge and skills they have obtained into real practice and acquire additional competencies in the specialty they have chosen. The Institute cooperates with private and state organizations and participates in joint projects. It contributes to the development of a system for commercializing innovative products, supports original projects and services that meet the challenges of modern science developed in cooperation with Russian and foreign pharmaceutical companies and other high-tech and research organizations. The Institute collaborates with Chumakov Federal Scientific Center for Research and Development of Immune and Biological Products of the Russian Academy of Sciences, Generium International Biotechnology Center, the Institute of Problems of Chemical Physics of the Russian Academy of Sciences, Federal State Unitary Enterprise "Moscow Endocrine Plant", JSC Rafarma, LLC Rigla, JSC R-Pharm, ITMO University and others.

Another example of integration of medical innovations into the educational system is the Research Institute of Translational Medicine founded in 2014 as part of Pirogov Russian National Medial Research University. Its innovative initiatives gave impetus to the foundation of "Southern" Moscow Medical Technology Cluster in 2015. The Cluster consists of "Slava" Technology Park, 2 higher education institutions, 8 research institutions, 4 clinical and 30 innovative biotech companies. Currently the Cluster supervises over 20 projects on medical product development, hosts a prototyping center, and collaborates with the Foundation for Assistance to Small Innovative Enterprises in Science and Technology. "Southern" MedTech cluster has prepared the environment to implement the full life cycle of innovative products, from a student's idea, through Umnik competition and the prototyping center to mass production at the facilities of small and medium-sized enterprises of "Slava" technology park and Cluster's partners.

CONCLUSION

With regard to the above and in order to deliver the advanced medical care based on the most recent achievements of science and technology to Russian citizens and to bridge a possible gap in medical innovation, we believe it necessary to introduce amendments to the current legislation, specifically, to Art. 38 of the Federal Law No. 323 of November 21, 2011 that would eliminate restrictions on the use of unregistered medical products by medical institutions and establish special requirements to in-house monitoring (similar to the international regulations applied to LDT and IVD-RUO) that may be performed by a local Ethics Committee or Academic Board.

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ANALYSIS OF MILDRONATE EFFECT ON THE CATALYTIC ACTIVITY OF CYTOCHROME P450 3A4

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In this work, we have studied the effect of mildronate on the catalytic properties of cytochrome P450 3A4. The analysis of the catalytic activity was carried out using electrochemical methods, with cytochrome P450 3A4 immobilized on the electrode surface. In the presence of 50 μM mildronate, no increase was observed in the turnover number of cytochrome P450 3A4-dependent N-demethylation of erythromycin. The values of the turnover number k_{cat} calculated from the product formed by the reaction were 6.1 ± 0.6 min^{-1} (P450 3A4 + erythromycin) and 5.5 ± 1.4 min^{-1} (P450 3A4 + erythromycin + mildronate). Thus, electroanalysis of cytochrome P450 3A4 catalytic activity demonstrated the possibility of a safe and effective complex drug therapy with concurrent administration of mildronate and the macrolide (erythromycin).

Keywords: cytochrome P450 3A4, antihypoxic drugs, electroanalysis, enzyme electrodes, drug interference, mildronate

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АНАЛИЗ ВЛИЯНИЯ МЕЛЬДОНИЯ НА КАТАЛИТИЧЕСКУЮ АКТИВНОСТЬ ЦИТОХРОМА P450 3A4

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Исследовано влияние мельдония на каталитические функции цитохрома P450 3A4. Анализ каталитической активности проводили электрохимическими методами с использованием иммобилизованного на электроде цитохрома P450 3A4. В присутствии 50 мкМ мельдония в электрохимической системе не наблюдали увеличения электрокаталитической константы цитохром P450 3A4-зависимого N-деметилирования эритромицина. Электрокаталитические константы k_{cat}, рассчитанные по образованию продукта, составили 6,1 ± 0,6 $мин^{-1}$ (P450 3A4 + эритромицин) и 5,5 ± 1,4 $мин^{-1}$ (P450 3A4 + эритромицин + мельдоний). Таким образом, электроанализ каталитической активности цитохрома P450 3A4 показал возможность проведения безопасной и эффективной комплексной фармакотерапии с использованием мельдония при одновременном приеме макролидного антибиотика эритромицина.

Ключевые слова: цитохром P450 3A4, антигипоксантные средства, электроанализ, ферментные электроды, лекарственная интерференция, мельдоний

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Translational medicine is a developing branch of molecular medicine aimed to translate basic research into clinical practice. Isozymes of cytochrome P450 (CYP) are a superfamily of haem-containing monooxygenases responsible for phase I reactions of biotransformation of xenobiotics, including 75 % of drugs, and for the metabolism of endogenous physiologically active compounds [1, 2].

Due to cytochrome P450-dependent metabolism of drugs, drug pharmacokinetics and response to drug therapy vary in individual patients. Among 57 isozymes of human cytochrome P450, 5 basic forms are distinguished (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4/5) responsible for about 95 % of biotransformation reactions [3–5]. Isozymes of cytochrome P450 catalyze a variety of chemical reactions, such as hydroxylation, O-, S-, N-dealkylation, epoxidation, sulfoxidation, deamination, dehalogenation, etc. As cytochromes P450 have broad substrate specificity, research into drug interactions in cytochrome P450-based systems is of particular clinical importance [6–8]. To estimate a risk of drug interference with clinical tests at a preclinical research phase and to predict drug biotransformation rates in *in vitro* systems, methods of electroanalysis have been developed for clinically significant types of cytochrome P450 enzymes [9–12].

Among the participants of a catalytic cycle of cytochromes P450 are their redox partners, namely, cytochrome P450 reductase and cytochrome b5, and nicotinamide adenine dinucleotide phosphate (NADPH), which serves as an electron donor. To trigger catalysis, all components of a complex electron transport chain must make their contribution. Electrochemical analysis of cytochrome P450 catalytic activity does not require the presence of redox partners or NADPH electron donors (Fig. 1). Electroanalysis of cytochrome P450 catalytic activity is a noninvasive tool that can be used to study the mechanism of xenobiotic biotransformation and drug-drug interactions. Due to their high sensitivity, electrochemical methods of analysis may be very efficient in studying enzyme-substrate interactions [13]. During cytochrome P450-dependent catalysis in the electrochemical system, cathodic (reduction) current is registered. Its increase indicates the additional flow of electrons to the organic substrate (drug). Negative cathodic current (unlike positive anodic current) serves to measure the electrocatalytic activity of the enzyme. Study of cytochrome P450 electroanalytical parameters is a crucial step towards the discovery of new substrates/inhibitors of this hemoprotein; it is also important for predicting drug-drug interactions and drug interference with clinical diagnostic tests [14, 15].

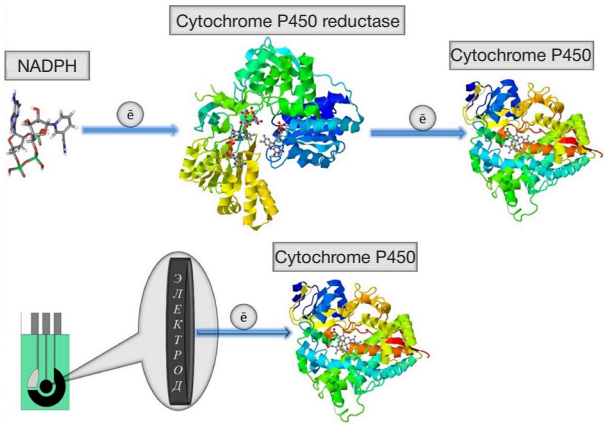

Fig. 1. Electron transfer in a P450-containing monooxygenase system (top) and an electrochemical system (bottom)

Protein structure images — courtesy of PDB database [16], NADPH image — courtesy of PubChem [17].

We have developed a new method of electroanalysis that allows using enzyme-electrode systems as noninvasive tools for the assessment of cytochrome P450 catalytic activity in preclinical research aimed to discover new substrates, inhibitors and modulators for this type of enzymes [18]. In spite of significant advances in diagnostic and treatment approaches, cardiovascular diseases (CVD) remain the leading cause of death and morbidity in the prime working age population in developed countries, including Russia. New strategies for CVD treatment are being elaborated. When an association between free fatty acids (FA) and a risk of death from an atherosclerotic cardiovascular pathology was identified, studies were launched to investigate inhibitors of partial β-oxidation of FA (pFOX, or partial fatty acid oxidation inhibitors) [19]. Metabolic drugs that aid oxygen uptake, ensure more efficient metabolic pathways and protect tissues from oxidative stress at reperfusion are also expected to produce an anti-ischemic effect due to the impact they have on the myocardial metabolism [20].

In the mid 1970s, researchers of the Latvian Institute of Organic Synthesis synthesized trimethylhydrazinium propionate (commonly referred to as mildronate, or meldonium) that inhibits FA transport across membranes [21]. It was shown that mildronate reduces the rate of β-oxidation of FA in mitochondria, which is important in cases of excessive FA accumulation [22]. Mildronate triggers ischemic preconditioning by reducing the rate of FA transmembrane transport, inhibiting accumulation of acyl-CoA and acylcarnitine inside the cell, optimizing oxygen consumption, inhibiting β-oxidation of FA and increasing the rate of γ-butyrobetaine synthesis. It also induces NO synthesis in the vascular endothelium reducing peripheral vascular resistance and platelet aggregation; increases elasticity of red blood cell membranes; minimizes metabolic acidosis caused by anaerobic glycolysis with subsequent accumulation of lactic acid. Mildronate is used in combination therapies of CVD; in patients presenting with fatigue or physical stress; in a post-operative period to expedite recovery; in abstinent patients with chronic alcoholism [23, 24].

Though mildronate is widely used in combination therapies as a metabolic antihypoxant, its effect on the catalytic functions of cytochrome P450 enzymes, i. e. enzymes involved in phase I of biotransformation of xenobiotics, has not been studied. When planning a combination therapy, it is necessary to remember that drug-drug interactions may have both therapeutical and adverse effects on the patient. Therefore, of particular importance is information about substrate properties of pharmaceutical agents used in combination therapies or their capacity to inhibit or induce cytochrome P450 isozymes.

Previously, we used electrochemical methods to study antioxidant vitamins (vitamins C, A and E) and vitamin-like compounds (taurine and coenzyme Q) that were shown to have a positive effect on the electrocatalytical activity of cytochrome P450 3A4 [18, 25]. Therefore, the aim of this work was to investigate the effect of mildronate on catalytic functions of cytochrome P450 3A4, an isozyme that participates in the biotransformation of more than 50 % of existing drugs.

METHODS

Electrochemical experiments were carried out using Autolab PGSTAT 12 potentiostat/galvanostat (Metrohm Autolab, Netherlands) with GPES software (version 4.9.7). All measurements were performed at room temperature. Electrochemical analysis of cytochrome P450 3A4 was carried out in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.05 M NaCl. We used three-pronged screen-

printed electrodes (LLC ColorElectronics, Russia) — graphite working and auxiliary electrodes and a silver chloride reference electrode. The working electrode was 2 mm in diameter. All potentials are referenced to the Ag/AgCl reference electrode. Spectral measurements were done using Cary 100 UV-Vis spectrophotometer (Agilent Technologies, USA) and Cary WinUV software supplied by the vendor.

Cyclic voltammograms were recorded at a scan rate of 10–100 mV/s. Parameters for cathodic square-wave voltammetry were as follows: initial potential of +100 mV, final potential of –600 mV, step potential of 5 mV, amplitude of 20 mV, frequency of 10–100 Hz. The following reagents were used: didodecyldimethylammonium bromide (DDAB) and erythromycin by Sigma-Aldrich (USA); mildronate by Grindeks (Latvia); acetic acid, ammonium acetate and acetylacetone by LLC Spektr-Chim. Recombinant human cytochrome P450 3A4 (182 μM in 550 mM potassium phosphate buffer with pH of 7.2 containing 0.2 % CHAPS, 1 mM dithiothreitol and 20 % glycerol) was engineered, isolated and described at the Institute of Bioorganic Chemistry (Minsk, Belarus). Enzyme concentration was measured spectrophotometrically based on the formation of a reduced enzyme-CO complex using the extinction coefficient $\varepsilon 4_{50-490}$ = 91 $mM^{-1}cm^{-1}$ [26].

The surface of the working graphite electrode was coated with 1 μl of 0.1 M chloroform solution of DDAB. After evaporation of chloroform (10 min) 1 μl of 18.2 μM cytochrome P450 3A4 was applied on the surface of the working electrode. The electrodes were allowed to stay for 12 h at +4 °C in a humid chamber to prevent from total drying. The N-demethylase activity of cytochrome P450 3A4 towards erythromycin was estimated by the accumulation of formaldehyde, that forms a colored compound with Nash reagent (4 M ammonium acetate, 0.1 M glacial acetic acid, 0.04 M acetylacetone); the extinction coefficient ε_{412} was 4 $mM^{-1}cm^{-1}$ [27, 28]. The enzyme-coated electrode was then immersed in 1 ml of electrolytic buffer containing 100 μM erythromycin. Electrolysis was performed at the controlled potential of –0.5 V for 20 min. After the electrolysis Nash reagent was added to the incubation mixture at 1:1 ratio, and the mixture was incubated at +37 °C for 30 min to develop the color. The concentration of formaldehyde that had been produced during electrocatalysis was measured spectrophotometrically. Figures 3, 4 and the table below show mean values and standard deviation obtained in 3-5 individual experiments.

RESULTS

Mildronate belongs to a class of antihypoxant agents and is used in a combination drug treatment of various diseases. Its effect on the catalytic functions of cytochrome P450 3A4 immobilized on a screen-printed graphite electrode (SPE) modified with didodecyldimethylammonium bromide (SPE/DDAB) was studied by registering peak maximum of cathodic current using cyclic voltammetry. As shown in Fig. 2, mildronate does not have any effect on the electrochemical reduction of cytochrome P450 3A4, does not increase or decrease reduction current, i. e., does not exhibit substrate or inhibitor properties towards the enzyme. Besides, the spectral analysis of cytochrome P450 3A4 binding to mildronate showed that the latter does not induce type I (substrate) or type II (inhibitor) changes of cytochrome P450 3A4 difference spectrum, which corresponds to the data obtained using the electrochemical system. Analysis of the dependence of the reduction current on mildronate concentrations at a 10–75 μM concentration range also confirmed the absence of mildronate effect on cytochrome P450 3A4 reduction (Fig. 3).

Effect of mildronate on cytochrome P450 3A4-dependent biotransformation of erythromycin was studied using 50 μM mildronate and 100 μM erythromycin. Erythromycin N-demethylation catalyzed by cytochrome P450 3A4 was registered by the accumulation of formaldehyde. A product of the Hantzsch reaction is a colored formaldehyde derivative, which was registered in our experiment spectrophotometrically at 412 nm [26]. As shown in the table below, catalytic constants k_{cat} of electrocatalytic cytochrome P450-dependent reactions have comparable values.

DISCUSSION

Catalysis and drug-drug interactions were estimated based on the electrochemical activity of cytochrome P450 3A4 enzyme immobilized on the electrode surface. In the course of electroanalysis, we registered voltammetric electrode response by cyclic voltammetry and square-wave voltammetry. Substrates of cytochrome P450 enzymes caused a significant increase in the catalytic current (Fig. 4, experiments 2 and 5) while their inhibitors did not alter or reduce the maximum amplitudes of the currents [13]. Binding of itraconazole, a cytochrome P450 3A4 inhibitor, to the enzyme did not cause increase of the cathodic current, because no additional electrons transfer occurred in the system (Fig. 4, experiment 3).

Previously, we studied a stimulating effect of metabolic antioxidants on phase I of the catalytic cycle of cytochrome P450, which is the reduction of haem iron [18]. A study of interactions between diclofenac, a substrate of cytochrome

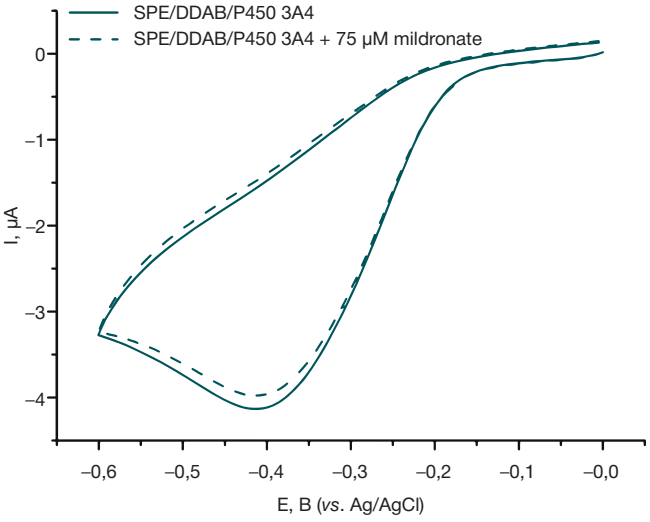

Fig. 2. Cyclic voltammograms of cytochrome P450 3A4 (—) and in the presence of 75 μM mildronate (– –)

The enzyme was immobilized on the electrode modified with DDAB. Scan range: from 0 to –0.6 V (vs. Ag/AgCl); scan rate: 0.05 V/s.

Kinetic parameters of P450 3A4-dependent electrocatalytic N-demethylation of erythromycin

Electrochemical system	k_{cat}, min^{-1}
P450 3A4 + erythromycin	6.1 ± 0.6
P450 3A4 + mildronate (50 μM) + erythromycin	5.5 ± 1.4

Note. Electrolysis was performed at controlled voltage of –0.5 V (*vs*. Ag/AgCl) for 20 min in the presence of 100 μM erythromycin.

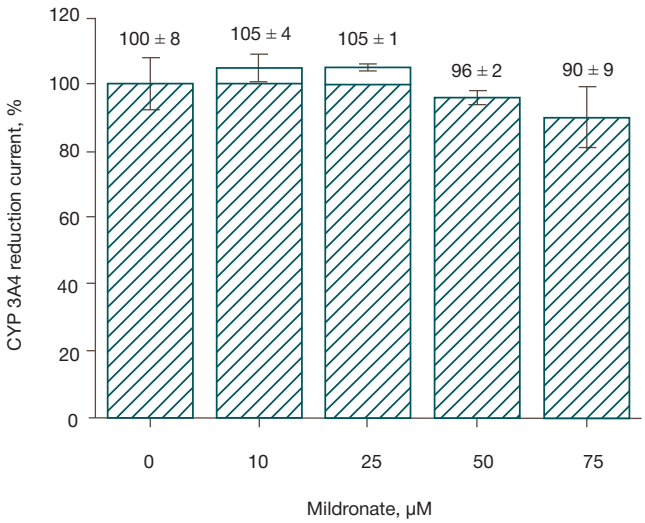

Fig. 3. Dependence of P450 3A4 reduction current (%) on mildronate concentration (μM), obtained from cyclic voltammetry data. Scan range: from 0 to –0.6 V (vs. Ag/AgCl); scan rate: 0.05 V/s

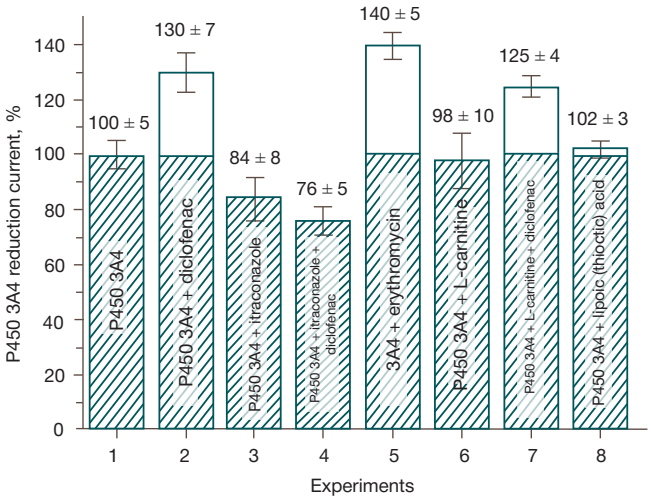

Fig. 4. Peak currents on square-wave voltammograms under aerobic conditions: (1) P450 3A4; (2) P450 3A4 + diclofenac; (3) P450 3A4 + itraconazole; (4) P450 3A4 + itraconazole + diclofenac; (5) P450 3A4 + erythromycin; (6) P450 3A4 + L-carnitine; (7) P450 3A4 + L-carnitine + diclofenac; (8) P450 3A4 + lipoic (thioctic) acid

Values of current amplitudes were baseline-corrected.

P450 3A4, and a number of pharmaceutical agents, such as L-carnitine and a vitamin-like antioxidant thioctic (alpha-lipoic) acid showed that these drugs do not affect the catalytic current registered during diclofenac interaction with the enzyme (Fig. 4, experiments 6–8). All drugs were studied at concentrations of 10–400 μM. Such range of working concentrations was chosen based on Michaelis constant calculations and blood plasma drug concentrations [29]. Directed regulation of the catalytic cycle of cytochrome P450 can both reduce drug metabolism rates and induce substrate biotransformation [30–32].

No increase in the value of the electrochemical constant of P450 3A4-dependent erythromycin N-demethylation was observed in the presence of 50 μM lipoic acid in the electrochemical system. Comparison of kinetic parameters allows us to conclude that the macrolide antibiotic erythromycin and the metabolic antioxidant thioctic acid can be used together in a combination therapy as they do not interact.

Mildronate, as well as L-carnitine and lipoic acid, does not affect the electrocatalytic activity of cytochrome P450 3A4.

CONCLUSIONS

A study of drug-drug interactions between the typical cytochrome P450 3A4 substrates (diclofenac and erythromycin) and metabolic antioxidants and also the antihypoxant drug mildronate was carried out using electroanalytical methods. Mildronate, as well as L-carnitine and lipoic acid, does not affect electrocatalytic activity of cytochrome P450 3A4, which indicates a lower probability of drug-drug interactions with regard to their metabolism in a combination drug therapy. This fact must be considered by physicians when deciding on the optimal antihypoxant and antioxidant in a combination therapy of comorbid patients.

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IODINE QUANTIFICATION WITH COMPUTED TOMOGRAPHY FOR THE PURPOSE OF DOSE ASSESSMENT IN CONTRAST ENHANCED RADIOTHERAPY

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In vivo quantitative determination of high-Z elements such as iodine gadolinium, gold, etc. is an important issue for contrast enhanced radiotherapy (CERT) that aggravates its clinical implementation. X-ray computed tomography (CT) could be a reliable, convenient and universal method for this task. The aim of this study was to demonstrate the feasibility of iodine quantification with CT in a tissue equivalent phantom, meeting the demands for CERT. The results show a linear relationship between iodine concentration and radiopacity on tomographic images expressed in Hounsfield units (HU) over an iodine concentration range of 0.5–50 mg/ml. Furthermore, iodine quantification with CT proofed to be suitable for CERT since the deviation between CT-derived and actual iodine concentration does not exceed 5 % in the concentration range of 10–50 mg/ml. More significant deviations were observed for concentrations below 5 mg/ml with up to 80 %, which is still acceptable for CERT since the corresponding error for the absorbed dose in that range is less than 2.8 %. X-ray beam hardening within the tissue equivalent object does not significantly influence the accuracy of iodine quantification. The placement of iodine water solutions at the surface or in the centre of a visualized object during iodine quantification leads to a less than 2 % change in the determined iodine concentration.

Keywords: contrast enhanced radiotherapy, computed tomography, iodine, quantification, dosimetry

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КОЛИЧЕСТВЕННОЕ ОПРЕДЕЛЕНИЕ ЙОДА ПРИ ПОМОЩИ РЕНТГЕНОВСКОЙ КОМПЬЮТЕРНОЙ ТОМОГРАФИИ ДЛЯ ДОЗИМЕТРИЧЕСКОГО ОБЕСПЕЧЕНИЯ ФОТОН-ЗАХВАТНОЙ ТЕРАПИИ

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Количественное определение *in vivo* дозоповышающих агентов, то есть элементов с Z >52, при фотон-захватной терапии (ФЗТ) необходимо для внедрения метода в клиническую практику. Проведено исследование возможности количественного определения йода (Z = 53) в тканеэквивалентном объекте (полиэтиленовом фантоме) при помощи рентгеновской компьютерной томографии (КТ). Показано, что зависимость значений рентгеноплотности водных растворов йода на томограммах фантома от концентрации йода носит линейный характер в диапазоне концентраций йода от 0,5 до 50 мг/мл. Характеристики предлагаемого метода количественного определения йода при помощи КТ соответствуют потребностям ФЗТ. Отклонение измеренного по томограммам содержания йода в растворах от их истинных значений не превышает 5 % в диапазоне концентраций йода от 10 до 50 мг/мл. Для растворов с концентрацией йода менее 5 мг/мл отклонение достигает 80 %. Однако и этот результат является приемлемым для ФЗТ, так как для концентраций йода менее 5 мг/мл неопределенность величиной в 80 % в измерении концентрации йода приводит к неопределенности определения величины поглощенной дозы не более чем в 2,8 %. Изменение спектра рентгеновского излучения в тканеэквивалентом объекте не оказывает существенного влияния на характеристики предлагаемого метода. Сравнение градуировочных кривых, построенных для растворов, расположенных около поверхности объекта и в его глубине, показало, что разница между определяемыми по ним значениями концентрации йода не превышает 2 %.

Ключевые слова: фотон-захватная терапия, компьютерная томография, йод, количественное определение, дозиметрия

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Effectiveness and clinical potential of contrast enhanced radiation therapy (CERT) in treating malignant tumors, in particular brain tumors, have been demonstrated by many Russian and foreign researchers [1–5]. CERT relies on the absorption of orthovoltage X-rays in the range of 30–300 keV by high-Z elements introduced into the tumor. This method ensures enhancement of the absorbed radiation dose. X-ray absorption is better in high-Z elements, with Z >52 (for the sake of convenience, they will be further referred to as dose enhancers, DE) than in H, C, O, N, or other elements that constitute soft biological tissues [6].

Phase I clinical trials of CERT-based treatment of brain tumors using a modified CT (computed tomography) scanner were first conducted in the USA in the 1990s [2]. Currently, similar studies are carried out in France at the European Synchrotron Radiation Facility [7]. Though they have already yielded some encouraging results, CERT may still be unable to move on to a further research stage: an accurate method for quantification of DE distribution in tumors and surrounding tissues before and after irradiation has not been developed yet. In CERT, the absorbed radiation dose depends on the DE concentration in the target object and can increase 1.5–5 times compared to the dose absorbed by the same object that was not preloaded with DE [8–12]. Therefore, it would be impossible to elaborate a suitable radiation scheme and control a radiation dose delivered to and absorbed by patient's tissues, which is critical for further clinical research, without developing a method for DE quantification in malignant and healthy tissues.

In the studies mentioned above, radiation schemes did not take into account the presence of DE in the target object. Irradiation mode and duration were chosen based on the interaction of X-rays with soft tissues; the energy released from DE atoms was disregarded. Therefore, radiation was delivered in fractionated doses, similar to conventional external beam radiotherapy. The DE-related enhancement of the absorbed dose was analyzed later when study results were processed and data from CT scans performed in the preparatory stage of the research were averaged over all patients. In calculations of the absorbed doses, DE distribution in tumors was perforce considered uniform. No criteria were proposed to estimate DE content in the target object, and their impact on the total radiation dose absorbed by the tumor was not therefore considered. Obviously, accurate quantification of high-Z elements in patient tissues *in vivo* is essential for effective and safe CERT-based treatment of malignant tumors.

CT seems to be the most appropriate method for DE quantification in CERT. CT and CERT rely on the same physical principle, i. e. absorption of X-rays by a substance. CT is widely used in clinical routine and is one of the major medical imaging techniques. The feasibility of CT-based DE quantification is underpinned by the basic physics of CT and has been experimentally proved by a number of researchers [13–15]. However, the accuracy of DE quantification by CT still remains unclear.

The aim of this work was to demonstrate the feasibility of CT-based iodine determination in a tissue equivalent phantom and to assess the accuracy of this method and the effect that X-ray voltage and non-uniform attenuation of various components of X-ray energy spectrum occurring in the deep layers of the phantom have on it.

METHODS

For this study, we fabricated a polyethylene phantom sized 134 × 134 × 63 mm. We made two perpendicular rows of holes in it (superficial and going through deeper layers) to place several 250 µl microtubes filled with aqueous iodine solutions with iodine concentrations ranging from 0.5 to 50 mg/ml (Fig. 1). We used iopromide (marketed as Ultravist 370) by Bayer Schering Pharma AG, Germany. To prepare aqueous iodine solutions, Ultravist 370 that originally contained 370 mg/ml iodine was diluted down using automatic pipette. The phantom with microtubes in it was scanned with the Siemens Biograph 40 CT scanner (Siemens, Germany) operated at different X-ray tube voltages of 80, 100, 120 and 140 kV and 200 mA current. Images were reconstructed using a standard B30f kernel. Quantitative analysis of DICOM images was performed using the ImageJ software (National Institutes of Health, USA). The same software was used to calculate mean radiopacity of iodine solutions expressed in Hounsfield units (HU) and standard deviations. Linear approximation of the obtained mean values was performed using the R environment (R Foundation).

RESULTS

Fig. 2 shows a relationship between radiopacity of aqueous iodine solutions and their iodine concentrations. It was linear (R2 = 0.998) at all studied concentrations ranging from 0.5 to 50 mg/ml. Using the obtained data, calibration curves were constructed. Deviations between iodine concentrations calculated from the calibration curves and their actual values are shown in the table below. They did not exceed 5 % for concentrations between 10 and 50 mg/ml. For a 5 mg/ ml concentration, the deviation was 5–10 %. The biggest deviation (up to 80 %) was observed in solutions with iodine concentrations below 1 mg/ml. A slight change in radiopacity was observed related to the location of the microtube. On the graph, the calibration curves for the solutions placed closer to the phantom's surface appeared below the calibration curves constructed for the solutions placed deeper inside the phantom. However, the difference between the expected concentrations of the solution and the concentrations calculated from the calibration curves did not exceed 2 % at all operating voltages.

DISCUSSION

We have conducted a pioneer study of DE quantification using a CT scanner and assessed the accuracy of the proposed method considering the challenges faced by contrast enhanced radiation therapy. The effect of varying operating X-ray tube

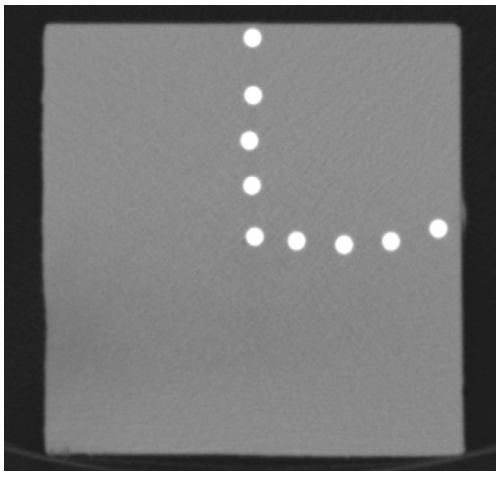

Fig. 1. A CT image of a polyethylene phantom with microtubes in it containing a 35 mg/ml aqueous iodine solution. Operating voltage is 80 kV

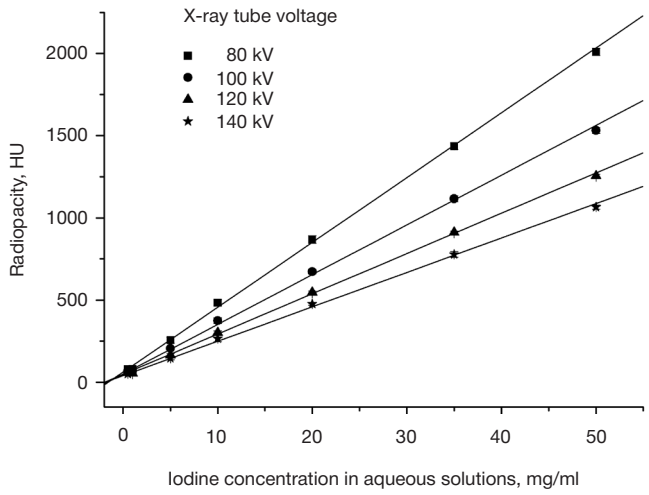

Fig. 2. Calibration curves showing the relationship between radiopacity (expressed in Hounsfield units, HU) and iodine concentrations at X-ray tube voltages of 80, 100, 120 and 140 kV for solutions placed inside the phantom

voltages on the radiopacity of aqueous iodine solutions was studied in a tissue equivalent phantom. We also investigated the relationship between the radiopacity of solutions and the location of the microtubes containing the former (closer to the phantom's surface or in its center). The relationship between radiopacity of aqueous iodine solutions and their iodine concentrations was linear for concentrations ranging from 0.5 to 50 mg/ml at all applied voltages. As we expected, the method exhibited higher sensitivity at 80 kV comparing to 100, 120 and 140 kV voltages. However, this difference in sensitivity is not critical for CERT and does not affect the accuracy of CT-based DE quantification (see the table below); the latter is more influenced by varying HU in the studied area. Thus, the choice of the optimal voltage for DE quantification can be based on other more important criteria [6, 16].

In spite of the considerable deviation between the calculated iodine concentrations and their actual values over the range of 0.5 to 5 mg/ml (up to 80 %), it is acceptable for CERT planning and absorbed dose calculation. As shown previously [16], the increase in the absorbed dose in CERT depends on DE concentration and this relationship can be described linearly (R2 = 0.99764). Thus for the calculation of the absorbed dose, the absolute error of DE quantification is important, and its percentage value is negligible. The observed 80 % uncertainty for a concentration of 1 mg/ml corresponds to the absolute error of 0.8 mg/ml, which in turn leads to a less than 2.8 % change in the absorbed dose value, which is seen as acceptable in radiation therapy.

Thus, the proposed method for CT-based iodine quantification allows the use of the quantitative data on DE distribution for CERT planning and dose control. The accuracy of CT-based DE quantification can be improved by developing special algorithms of image reconstruction aimed to obtain images with lower contrast and sharpness and less varying HU for a homogeneous radiopaque object. Beam hardening in the studied object does not significantly change the accuracy of iodine quantification. CT-based iodine quantification of DE *in vivo* renders it possible to place the reference samples close to a patient and does not require an anthropomorphic phantom for calibration.

CONCLUSIONS

We have experimentally proved the feasibility of CT-based iodine quantification in the tissue equivalent phantom. The method proved to be rather reliable and can be applied for dose assessment and CERT planning. The method is stable in the wide range of X-ray tube voltages and DE concentrations and can be used to study variously shaped objects of different length. CT is also a universal method for DE quantification.

Iodine concentrations in aqueous solutions measured by a CT scanner and their deviations from actual values at various voltages

Actual value of iodine concentrations in aqueous solutions, mg/ml	X-ray tube voltage							
	80 kV		100 kV		120 kV		140 kV	
	Iodine concentration in aqueous solutions (mg/ml) measured by CT and its deviation from the actual value (%)							
	mg/ml	%	mg/ml	%	mg/ml	%	mg/ml	%
0.50 ± 0.01	0.11 ± 0.08	77.1	0.18 ± 0.13	63.3	0.28 ± 0.2	43.9	0.13 ± 0.09	74.8
1.00 ± 0.02	0.18 ± 0.07	81.9	0.39 ± 0.16	60.8	0.10 ± 0.15	89.8	0.63 ± 0.25	37.5
5.0 ± 0.1	4.7 ± 0.2	7.1	4.80 ± 0.2	3.8	4.7 ± 0.2	6.9	4.7 ± 0.2	5.5
10.0 ± 0.2	10.50 ± 0.2	5.0	10.5 ± 0.2	4.7	10.3 ± 0.2	2.5	10.5 ± 0.2	4.9
20.0 ± 0.4	20.4 ± 0.3	1.8	20.4 ± 0.3	2.2	20.3 ± 0.3	1.4	20.7 ± 0.3	3.4
35.0 ± 0.6	34.9 ± 0.4	0.4	35.3 ± 0.4	0.7	35.2 ± 0.4	0.7	35.2 ± 0.4	0.6
50.0 ± 0.9	49.6 ± 0.4	0.8	49.2 ± 0.4	1.6	49.3 ± 0.4	1.3	49.1 ± 0.4	1.7

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CARRIER FREQUENCY OF *GJB2* AND *GALT* MUTATIONS ASSOCIATED WITH SENSORINEURAL HEARING LOSS AND GALACTOSEMIA IN THE RUSSIAN POPULATION

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This article continues a series of works estimating carrier frequencies of mutations associated with the development of common monogenic disorders in the Russian population. The study aimed to establish the frequency of *GJB2* and *GALT* mutations in first-time blood donors. Genotyping of 1000 first-time blood donors who identify themselves as Russians and permanently reside in the Russian Federation detected 37 carriers of *GJB2* mutations associated with sensorineural hearing loss (carrier frequency in the sample was 3.7 %, or 1 : 27) and 6 carriers of *GALT* mutations associated with galactosemia (carrier frequency in the sample was 0.6 %, or 1 : 167). In one carrier, concurrent mutations were detected; thus, in total 42 carriers of *GJB2* and *GALT* mutations were detected (carrier frequency in the sample was 4.2 %, or 1 : 24).

Keywords: sensorineural hearing loss, *GJB2*, galactosemia, *GALT*, genotyping, Russian population

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ЧАСТОТА НОСИТЕЛЬСТВА В РОССИЙСКОЙ ПОПУЛЯЦИИ МУТАЦИЙ В ГЕНАХ *GJB2* И *GALT*, АССОЦИИРОВАННЫХ С РАЗВИТИЕМ НЕЙРОСЕНСОРНОЙ ТУГОУХОСТИ И ГАЛАКТОЗЕМИИ

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Статья продолжает цикл работ, посвященных определению частоты носительства в российской популяции мутаций, ассоциированных с развитием распространенных моногенных заболеваний. Целью исследования было установление частоты распространенных в российской популяции мутаций в генах *GJB2* и *GALT* у доноров первичной кроводачи. При генотипировании 1000 доноров первичной кроводачи, идентифицирующих себя как русских и постоянно проживающих на территории Российской Федерации, обнаружены 37 носителей мутаций в гене *GJB2*, ассоциированных с развитием нейросенсорной тугоухости (частота в выборке составила 3,7 %, или 1 : 27), и 6 носителей мутаций в гене *GALT,* ассоциированных с развитием галактоземии (частота в выборке — 0,6 %, или 1 : 167). Выявлен 1 случай сочетанного носительства мутаций, и, таким образом, всего обнаружены 42 носителя мутаций в генах *GJB2* и *GALT* (частота в выборке — 4,2 %, или 1 : 24).

Ключевые слова: тугоухость, *GJB2*, галактоземия, *GALT*, генотипирование, российская популяция

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Nonsyndromic sensorineural hearing loss is an inherited condition (OMIM #220290) characterized by congenital hearing impairment. The most common type of genetic hearing disorders found in developed nations is autosomal recessive nonsyndromic hearing loss associated with a mutation in the *GJB* gene that encodes a protein known as connexin 26. So far, over 90 *GJB2* mutations have been associated with deafness, 35delG mutation being highly prevalent in European and Russian populations. This mutation results in a premature stop codon. The carrier frequency of *GJB2*:35delG can vary from 1 : 100 to 1 : 30 in European populations and from 1 : 50 to 1 : 25 in some Russian populations [1–5].

Galactosemia is a hereditary disease caused by the deficiency of galactose-metabolizing enzymes. Galactose enters the body in food as a component of disaccharide lactose (milk sugar). It is believed that accumulation of toxic amounts of galactose-1-phosphate in the cells affects cell metabolism and leads to pathology. The most marked changes occur in the liver, kidneys, eye lens and brain. Patients who receive no treatment die within the first months of life from

sepsis or liver failure; all patients develop mental deficiency with typical speech impairment (cluttering). But if a diet is prescribed timely, a child may still develop normally. The disease is linked to the reduced activity of the galactose-1-phosphate uridyl transferase enzyme associated with *GALT* mutations. In healthy individuals, this enzyme catalyzes production of glucose-1-phosphate and uridyl diphosphate-galactose from galactose-1-phosphate and uridyl diphosphate- glucose. Galactosemia follows an autosomal recessive pattern of inheritance; its incidence in the Russian population is 1 in 20 000 births. The most critical mutations for the Russian population are Q188R, K285N, M142K, L358P, IVS3-2A>C [6, 7].

The aim of this study was to determine the frequency of *GJB2* and *GALT* mutations in first-time blood donors who identify themselves as Russians and permanently reside in the Russian Federation.

METHODS

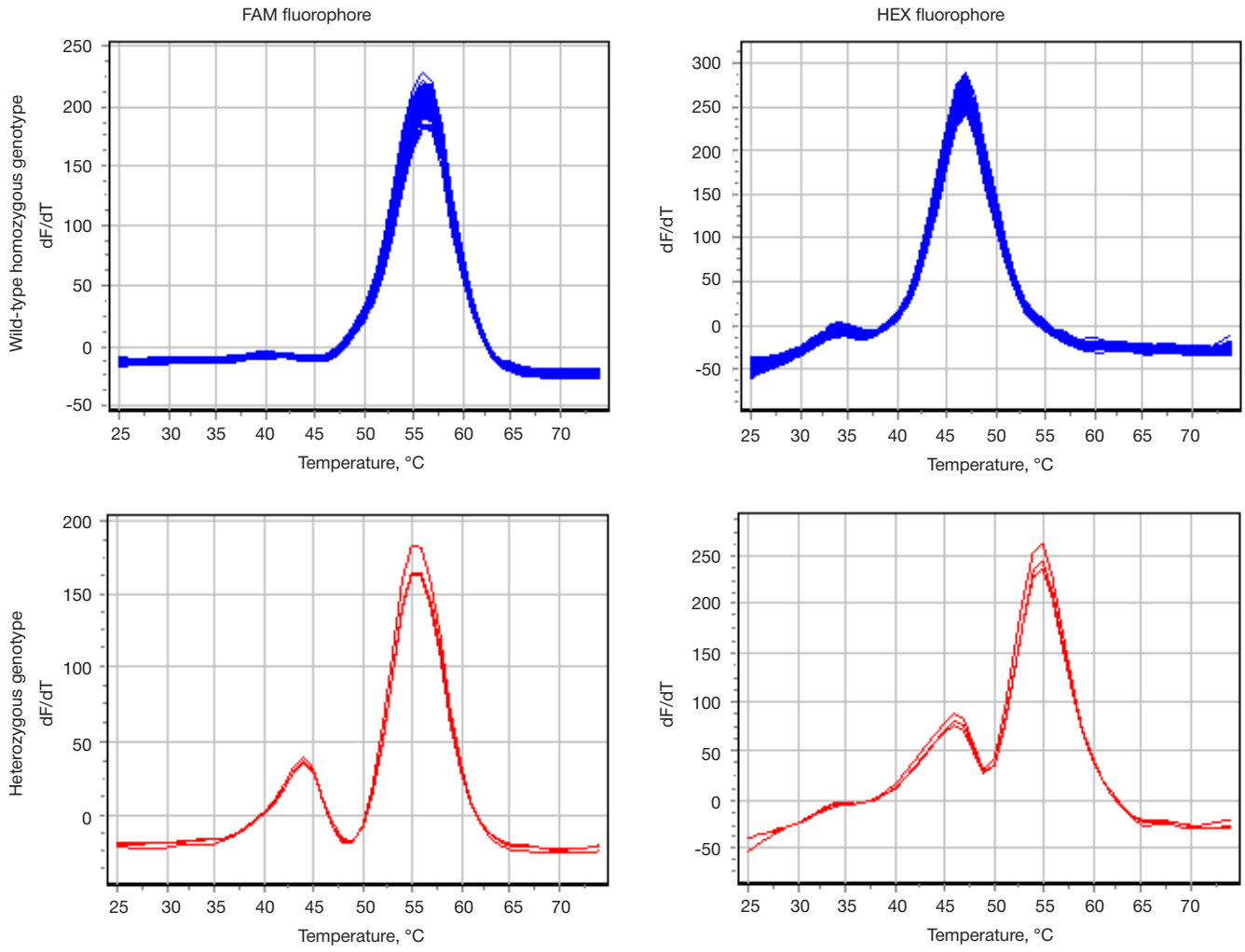
Peripheral blood was collected from 1000 healthy first-time blood donors who identified themselves as Russians.

DNA was extracted from 0.1 ml peripheral blood using the reagent kit "PROBA-GS-Genetics" (DNA-Technology, Russia). The extraction method we used involves lysis of the biomaterial followed by DNA binding to silica support, washing, and DNA elution. Some of the obtained DNA samples were immediately used for genotyping; others were stored under −20 °C. The average DNA concentration measured by the Qubit flourometer (Invitrogen, USA) was 50–100 μg/ml.

To detect single nucleotide substitutions, we used the reagent kit "Screening for monogenic diseases" (DNA-Technology, Russia). The detection principle relies on the method of adjacent (or kissing) probes [8, 9]. The kit can identify 5 *GJB2* mutations associated with nonsydromic sensorineural hearing loss and 1 *GALT* mutation associated with galactosemia.

Each reagent kit includes amplification mixes for identifying an individual mutation. Each mix contains primers complementary to both wild-type and mutant nucleotide sequences, one quencher-labeled oligonucleotide and two sequence-specific fluorophore-labeled oligonucleotide probes. The oligonucleotide probes complementary to wild-type and mutant sequences are labeled with different fluorophores, which makes it possible to simultaneously identify both variants using one test tube.

The first step in the identification of single nucleotide substitutions was PCR. Then the temperature of the reaction mix was brought down to hybridize oligonucleotide probes to the obtained matrices. Genotyping was performed following PCR and hybridization by changing fluorescence intensity during heat-induced denaturation of the oligonucleotide duplexes and the obtained matrices. The measurements were carried out in real time; based on the results, melting curves



Melting curves for two genotype variants based on the data obtained during the detection of Q188R mutation in the *GALT* gene

were constructed (see the image below). If an analyzed sample contained only one variant of a nucleotide sequence, i. e., the studied polymorphism was homozygous, the melting point for a perfect match probe/target duplex was considerably higher than for a mismatched duplex. If a heterozygous sample was analyzed that contained both variants of the nucleotide sequence, then both probes formed a perfect match duplex, which is why their melting points were practically the same.

This approach certainly has advantages over most molecular-genetic techniques for the detection of single nucleotide polymorphisms, including TaqMan assays. Here, genotyping is performed twice and data are obtained independently from two fluorescence channels, which significantly increases the reliability of the genotyping procedure and is highly unlikely to be reproduced using other techniques.

Polymerase chain reaction and measurements of oligonucleotide probe melting points were performed using the detecting amplifier DTprime (DNA-Technology, Russia). The following temperature mode was used for amplification: 94 °C for 10 s, 64 °C for 30 s; number of cycles = 50. After amplification was completed, the reaction mix was cooled down to 25 °C at a rate of 2 °C/s. Melting curves were constructed from the data obtained with the following technique: the temperature of the reaction mix was increased incrementally from 25 to 75 °C (1 °C per heating step); fluorescence was measured at each heating step. We used domestic equipment to automate the main stages of the research, and thus were able to genotype 40 mutations in up to 100 samples a day.

For control, we performed selective automated Sanger sequencing using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA) and reagents by the same manufacturer. Control tests demonstrated the results identical to those obtained in our experiment.

RESULTS

Frequencies of *GJB2* and *GALT* mutations detected in 1000 healthy first-time blood donors who identified themselves as Russians and permanently reside in the Russian Federation are shown in the table below. Genotyping of these individuals identified 37 carriers of *GJB2* mutations associated with sensorineural hearing loss (frequency in the sample was 3.7 %, or 1 : 27) and 6 carriers of *GALT* mutations associated with galactosemia (frequency in the sample was 0.6 %, or 1 : 167). One individual carried both mutations. In total, 42 carriers of *GJB2* and *GALT* mutations were detected (frequency in the sample was 4.2 %, or 1 : 24).

DISCUSSION

Our research identified a total of 42 carriers of *GJB2* and *GALT* mutations (frequency in the sample was 4.2 %, or 1 : 24). Besides, one individual was found to carry both mutations. The results of the present study are in agreement with the published research carried out in the Russian population [1, 3–5]. However, we detected a slightly higher (compared to the average data on the European population) frequency of the *GJB2*:35DELg mutation associated with sensorineural hearing loss.

CONCLUSIONS

In this research study, we determined frequencies of *GJB2* and *GALT* mutations in healthy Russian individuals. In total, 42 carriers of these mutations were detected (frequency in the sample was 4.2 %, or 1 : 24). These data indicate a relatively high prevalence of inherited conditions and provide a rationale for introducing molecular-genetic diagnostic tests into clinical practice (in addition to neonatal screening) that can assist pregnancy planning and be a criterion for the use of reproductive technologies in infertile patients.

The most suitable platform for such research is real time PCR. This approach may open up new horizons for mass high throughput sequencing, facilitate automation of laboratory work and help to achieve highly accurate and reliable results.

Heterozygous variants of *GJB2* and *GALT* mutations and concurrent mutations in 1000 Russian first- time blood donors

Mutation	Number of heterozygous variants detected
In *GJB2*	
35delG	37
167delT	0
235delC	0
313_326del14	0
358_360delGAG	0
In *GALT*	
Q188R	6
Concurrent mutations	
GJB2:35delG + GALT:Q188R	1

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CRITERIA FOR THE SELECTION OF GENETIC MARKERS IN THE ASSESSMENT OF PREDISPOSITION TO MULTIFACTORIAL TRAITS

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The principle of multifactorial traits (MTs) inheritance relies on the presence of a large number of genetic markers, with each marker contributing to the probability of developing those traits. This work proposes an algorithm for the selection of DNA markers that could be used to develope a prognostic test system for the assessment of individual predisposition to MTs. The method is based on the selection of genetic markers that have demonstrated a statistically significant association with an MT under consideration and have been described as functionally significant polymorphisms affecting MT development. If the functional significance of a polymorphism has not been described so far, then to be reliably associated with an MT, this polymorphism is expected to achieve genome-wide significance in one of the studies and such significance must be confirmed in an independent sample. Papers that are used to assess the association of genetic markers with MTs are expected to meet the proposed criteria depending on the study type.

Keywords: genetic marker, multifactorial disease, single nucleotide polymorphism, selection criteria, genome-wide association study, functional significance, meta-analysis, case-control study

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КРИТЕРИИ ОТБОРА ГЕНЕТИЧЕСКИХ МАРКЕРОВ ДЛЯ АНАЛИЗА ПРЕДРАСПОЛОЖЕННОСТИ К МНОГОФАКТОРНЫМ ФЕНОТИПИЧЕСКИМ ОСОБЕННОСТЯМ

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Принцип наследования многофакторных фенотипических особенностей (МФО) заключается в наличии значительного количества генетических маркеров, каждый из которых вносит некоторый вклад в вероятность развития данной особенности. В данной работе предложен алгоритм отбора ДНК-маркеров с целью разработки прогностических тест-систем для определения индивидуальной предрасположенности к МФО. Метод заключается в отборе генетических маркеров, показавших статистически достоверную ассоциацию с данной МФО, а также функционально значимые полиморфизмы, для которых описаны механизмы влияния на развитие МФО. Если функциональная значимость полиморфизма не описана, критерием его статистически достоверной ассоциации с МФО является достижение полногеномной значимости в одном из исследований и подтверждение данной ассоциации на независимой выборке. Научные публикации, используемые для оценки ассоциации генетических маркеров с МФО, в зависимости от типа исследования должны соответствовать приведенным критериям.

Ключевые слова: генетический маркер, мультифакториальная болезнь, однонуклеотидный полиморфизм, критерии отбора, полногеномное исследование, функциональная значимость, метаанализ, исследование случай–контроль

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Genetic testing is an important tool employed by personalized medicine to identify the risks of developing common diseases and to assess patient's predisposition to a certain phenotype. Information about genetic susceptibility to various diseases can be used to personalize preventive measures and develop strategies for the early detection of pathologies, to change lifestyle habits, balance a diet or revise a patient's current physical activity schedule.

Individual susceptibility to cardiovascular disorders can be linked to the abnormalities in different systems of the human body, where defective clotting, dyslipidemia, disorders of the renin–angiotensin system, elevated homocysteine levels, and some congenital conditions (Fabry disease, Moyamoya disease and others) make their own contribution. The majority of these pathologies are genetic; therefore, the risk factors contributing to their development can be eliminated

by personalized preventive care. Information about genetic susceptibility can also assist timely diagnosis or come in handy when a patient is closely monitored for symptoms of a disease. For example, it is known that some mutations in the *MLH1*, *MSH2*, *MSH6*, *PMS2* and *EPCAM* genes can increase the risk of colorectal cancer >10 times [1]. This type of cancer responds well to treatment in early stages, a 5-year survival rate being 94 %; however, it is practically incurable in stage 4. Apart from early disease detection and prevention, information about genetic factors can be used to assist patients in changing their lifestyle. For example, it is known that a polymorphism of the *GC* gene involved in the binding and transport of calciferol and its metabolites can reduce blood concentrations of vitamin D and its metabolites [2]. Adequate intake of vitamin D-containing products can compensate for this genetic trait.

In fitness and sport, genetic factors must also be considered. For example, if a patient is predisposed to varicose veins, it is advisable to exclude intensive straining exercises from their training program.

The successful completion of *The human genome* project has led to a tech boom in personalized genetic testing. Prognostic tests that detect the presence of DNA markers associated with different phenotypes and diseases are finding wide application all over the world. However, there is a significant limitation that impedes the development of such tests, namely a bench-to-bedside issue [3]; normally, the association of genetic markers with certain phenotypes is demonstrated in large population samples; therefore, interpretation of individual patient's data becomes a challenge.

Another significant issue is related to a number of genetic markers used in a test system aimed to detect individual susceptibility to multifactorial phenotypes. If a test relies on all the markers for which an association with the studied phenotype has been shown, its specificity will be low, whereas the costs will be high. But if the markers included in the test are few, it will affect test sensitivity and its prognostic value. Currently, there are a few solutions to this problem.

One of the approaches relies on the use of a small number of markers for which a statistically significant association with a certain phenotype has been previously shown in many studies. For example, to assess an individual risk of myocardial infarction, genetic markers in *Enos* and *CX37* candidate genes may be employed [4], while the presence of other markers, such as a factor V Leiden mutation, may be ignored even if they significantly increase the risk of this acute disorder [5]. Such approach makes it impossible to give a comprehensive assessment of all abnormalities that may trigger a disease and therefore has low sensitivity. On the other hand, statistically significant associations with myocardial infarction have been demonstrated for over 400 genetic markers by some case-control studies so far. A lab test cannot provide data on the presence of all known genetic markers due to technical restrictions. Techniques the majority of the laboratories have now at their disposal (such as real time PCR, restriction fragment length polymorphism analysis and some others) were designed to use a small number of genetic markers (up to several dozens). More markers would mean longer processing times or higher costs due to the use of expensive technologies, such as DNA microarrays.

Besides, the use of a large number of genetic markers entails some training issues: an algorithm may exhibit good prognostic accuracy in a training sample but still be low-sensitive or low- specific in the overall population.

Here, we propose an algorithm for the selection and assessment of genetic markers that can be used as a basis for a good prognostic test aimed to identify susceptibility to multifactorial traits (MTs). The idea behind this method is that selection is performed not only among those genetic markers that have shown a genome wide association with a studied phenotype, but also among those that have not reached multiplicity-corrected statistical significance in genome-wide studies but nevertheless meet other important criteria (such as functional significance). This article describes and discusses these criteria.

Selection of phenotypic traits

Phenotypic traits (PTs) can be divided into 4 types: with low or zero contribution of genetic factors to the development of a particular trait, monogenic, polygenic and multifactorial. Considering these criteria, we propose to develop test systems only for PTs with >30 % heritability. Lower values indicate predominant contribution of environmental factors to PTs; in this case, probability of PT manifestation must be assessed based on patient's lifestyle and the environment.

Monogenic traits are a result of a single-gene mutation; examples of monogenic diseases include cystic fibrosis and phenylketonuria. While designing a test aimed to assess a probability of monogenic trait manifestation, it is important to consider penetrance of known mutations and percentage of their phenotypic manifestations.

Polygenic traits are a summed contribution of a large number of genes. An example of a polygenic trait is eye color; it is almost fully determined by genetic factors [6].

We are not going to talk about these traits here, but instead will focus on multifactorial traits contributed to by both genetic and environmental factors. To design a test aimed to determine a probability of multifactorial trait manifestation in an individual, it is important to assess statistical significance of the association between a studied PT and certain genetic markers and the functional impact of the latter on the manifestation of a studied trait.

Assessment of statistically significant associations of genetic markers

So far, a large number of genetic markers for common MTs have been discovered. To assess a contribution of genetic markers to a multifactorial phenotype, researchers normally calculate a p-value and values of statistical parameters characterizing a degree of association between genetic markers and a given trait separately for each individual marker. The degree of polymorphic associations can be described by various statistical parameters, but in most cases the following ones are used: odds ratio (OR), relative risk, beta coefficient and allele frequencies in affected and healthy individuals.

Statistical significance of differences between individuals with and without MTs is determined by a p-value. Conventionally, to conclude that obtained differences are not due to chance, $p < 0.05$ is required [7]. When testing several hypotheses (investigating several polymorphisms), it is necessary to apply the Bonferroni correction to a p-value threshold. Referring to the statistical significance of a genetic marker, we will further assume that the Bonferroni correction has been already applied.

If a polymorphism bears no functional significance (see the section below), genetic markers must be seen as reliably associated with MTs if their association has been proved in genome-wide studies (GWAS), reached clear genome-wide significance ($p < 5 \cdot 10^{-8}$) and has been verified using an independent sample [8].

Assessment of functional significance of genetic markers

Functional significance of a polymorphism is determined by analyzing its impact on the development of a studied trait. It is highly important that genetic markers involved in pathology should be accounted for when designing tests aimed to assess an individual's risk of developing a disease. Some rare genetic markers do not reach genome wide significance and therefore are sifted out in GWAS. A functionally significant marker must meet one of the following criteria.

1) The exact mechanism is known by which a genetic polymorphism influences MT development

Such polymorphic variants occur in candidate genes for which an association with a particular multifactorial phenotype has been established. For example, the methylenetetrahydrofolate reductase enzyme, a *MTHFR* gene product, plays an important role in the metabolism of vitamin B2: it catalyzes production of folic acid that participates in converting homocysteine to methionine. The *rs1801133* polymorphism results in the amino acid substitution in the MTHFR protein, which impairs its affinity to the substrate leading to defective homocysteine metabolism [9]. Poor homocysteine metabolism is a risk factor for hyperhomocysteinemia. It should be noted though that this polymorphism alone does not guarantee that a person will develop hyperhomocysteinemia, as it is not the only risk factor for this condition.

2) The indirect mechanism is known by which a genetic polymorphism influences MT development

For example, the *rs1799983* polymorphism in the endothelial nitric oxide synthase gene is a missense mutation that ultimately affects protein processing and inhibits enzymatic activity. The changed protein synthesizes smaller amounts of nitrogen(II) oxide required for vasodilation. This leads to increased blood pressure and may cause hypertension [10]. Since hypertension causes luminal narrowing and endothelial dysfunction, it is, in turn, a risk factor for coronary artery disease. The *rs1799983* polymorphism can thus be seen as a genetic marker associated with the risk of ischemia.

Associations of all functionally important markers with phenotypic traits must be experimentally confirmed in case-control studies.

Selection criteria for scientific publications

Scientific publications that analyze associations of genetic polymorphisms with phenotypic traits can be divided into three types: case-control and quantitative studies, meta-analysis and reviews.

Since reviews do not aim to conduct a statistical analysis of the association of genetic markers with studied MTs, they must be disregarded when assessing the feasibility of using specific genetic markers in prognostic tests. However, such publications can be used to draw up an initial list of genetic markers to which our criteria can be further applied.

In case-control studies, associations between genetic markers and pathologies or certain physiological traits are analyzed by comparing allele frequencies in individuals with MTs and controls. These studies can be divided into two types: genome wide association studies (GWAS) and candidate gene association studies.

Genome wide association studies are a type of biological research in which genomes of people with different phenotypes for a particular trait are compared. These studies analyze associations of genetic markers distributed across the genome using high density DNA microarrays.

Studies of associations between individual genes and MTs employ a limited number of genetic markers and focus on the genes with a known or hypothetic mechanism by which they influence MT development.

A meta-analysis is a type of analysis that summarizes data provided by a large number of research works. All studies included in the meta-analysis must test the same hypothesis.

Because each of the study types is quite specific, the criteria used for the selection of scientific publications are also different.

To minimize the number of shortlisted genetic markers that demonstrated false-positive associations in GWAS, the following criteria must be applied [11]:

1. The original genome wide association study must include no less than 750 patients. Smaller samples undermine the accuracy of statistical analysis and yield a large number of false- positive and false-negative results.
2. Only genetic markers with $p < 0.01$ must be considered.
3. Revealed associations must be replicated in at least one independent study (there may be no replication study available for a rare disease). P-value must be <0.01; 95 % confidence intervals for OR must overlap in all analyzed studies; articles selected for the meta-analysis must be published in scientific journals with a >2 impact factor.

Studies involving a small number of genes must meet the following criteria:

1. Data must be obtained from biological tissues (biopsy or autopsy material, tissue obtained during surgery) or biological fluids.
2. Associations must be obtained through the experiment carried out by the authors of the publication. Publications in which authors cite conclusions drawn by other researchers must be ruled out.
3. $p < 0.05$.
4. Sample sizes must be sufficient to detect associations of genetic markers with certain phenotype frequencies [12].
5. If the association between genetic markers and a risk of a disease was investigated in a few publications, then it is advisable to select a) an article that was published earlier (an article published in 2009 should be preferred over the one published in 2015); b) an article in which a studied sample was larger.

If the association between genetic markers and MTs was studied by meta-analysis, the data obtained from it have a higher priority than the data from other studies. Only a high-quality meta-analysis must be taken into account that satisfies the following criteria [13]:

1. No clear mechanisms are currently known by which genetic markers studied through meta-analysis shape the pathology. If such mechanisms are known, then the functional significance of the polymorphism in question should be analyzed.
2. The work focuses on literature search. A meta-analysis must include those publications in which the association of a polymorphism with a disease was confirmed AND those publications in which such association was disproved.
3. Information sources and keywords used to implement the search must be specified.
4. An automatically generated list of publications must be manually checked for relevance prior to meta-analysis.

5. Publication inclusion and exclusion criteria must be specified and explained (such as sample sizes, the language of the article, demographic characteristics of participants, etc).

6. Research data must be combinable.

7. A risk of publication bias must be assessed using a funnel plot or sensitivity analysis.

8. If a meta-analysis contains data obtained from various populations and demonstrates a statistically significant association for Caucasian populations only, then a studied genetic marker should be seen as a DNA marker associated with a particular phenotype, given that Caucasian populations were analyzed in a number of works selected for meta-analysis.

9. In the studies that reveal statistically significant associations, 95 % confidence intervals for OR (or other statistical parameters describing the association) must overlap.

Assessing eligibility of genetic markers for a prognostic test

The algorithm aimed to assess if a genetic marker is eligible for using in a prognostic test is shown in the Figure below.

If a genetic marker association was studied in the course of GWAS that demonstrated its statistical significance and the study itself met the criteria described above, this marker should be used in a prognostic test. If a corresponding p-value was above 0.01 but below 0.05, then the analysis of functional significance of the marker should be carried out.

If a genetic marker was never studied in the course of GWAS or was sifted out in the first research stage but a high-quality meta-analysis showed its significant association with MTs, this genetic marker must be considered when designing a prognostic test. It is good to have a training sample to make sure that introduction of a new marker into a test system does not increase the empirical risk.

If a genetic marker was never studied in the course of GWAS or subject to meta-analysis but still is functionally significant, given that there are published candidate gene association studies confirming its association with a certain phenotype, it can be included into a prognostic test system.

Once a list of genetic markers eligible for a prognostic test has been prepared, the analysis of linkage disequilibrium must be carried out.

An example of a list of genetic markers

So far, 6 genetic markers have been discovered that have a significant genome-wide association with ischemic stroke confirmed in independent samples [14–16]. This list does not include polymorphic variants of *F5*, *F2*, *F7*, *F13B*, *MTHFR*, *ACE*, *APOE*, *GPIIIa*, *eNOS*, *PAI*, *GP1BA*, *ITGA2*, *ITGA2B*, *LPL*, *IL6* and *PON1* genes, whose association with stroke was shown previously in the studies of individual candidate genes [17]. These polymorphisms must be viewed from the perspective of their functional significance considering the results of a high-quality meta-analysis of their associations with ischemic stroke.

Coagulation factor V (gene *F5*) is an important component of blood coagulation system. It is involved in the conversion of prothrombin to thrombin. The *rs6025* polymorphism of *F5* known as Leiden mutation leads to increased resistance of the enzyme to inhibitors and thus causes excessive blood clotting. A meta-analysis was conducted in which the association of this mutation with a risk of stroke was confirmed [17]. Hypercoagulation is a risk factor for cardiovascular diseases including stroke; therefore, this polymorphism can also be considered functionally significant.

The *rs1799963* polymorphism (*G20210A*) is located in the 3'-untranslated region of the *F2* prothrombin gene and causes hypercoagulation. The meta-analysis [17] demonstrated that this polymorphism is associated with a risk of ischemic stroke.

The polymorphic variant *rs1801133* of the *MTHFR* gene was shown to be associated with a risk of developing hyperhomocysteinemia. Increased levels of homocysteine are a risk factor for vascular disease [19]. This polymorphism was shown to be associated with a risk of ischemic stroke by a high-quality meta-analysis [17].

The angiotensin-converting enzyme plays an important role in the regulation of blood pressure by converting angyotensin I to angiotensin II. The *rs1799752* polymorphism was previously shown to cause disturbances in the activity of this enzyme [20], which in turn results in the increased vascular tone and leads to atherosclerosis. According to meta-analysis results [17], this polymorphism is associated with a risk of ischemic stroke.

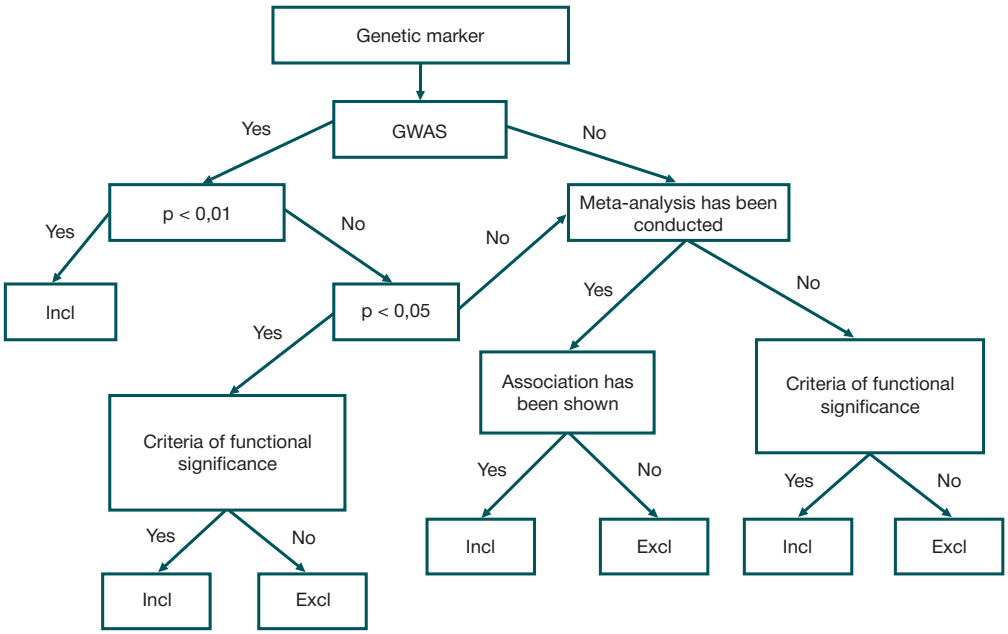
Polymorphic variants of *F7 F5*, *F2*, *MTHFR* and *ACE* genes must be considered when developing test systems for detecting individual risks of ischemic stroke because their association with this disease has been shown by a high-quality meta-analysis.

Associations of *F7*, *F13B*, *APOE*, *GPIIIa*, *eNOS*, *PAI*, *GP1BA*, *ITGA2B* and *LPL* polymorphisms with ischemic stroke were also studied in the course of a high-quality meta-analysis [17]; however, no significant association was detected. Therefore, polymorphic variants of these genes must not be considered when developing test systems for detecting individual risks of ischemic stroke.

Although no association between a polymorphic variant of the *APOE* gene (apolipoprotein E-encoding gene) and ischemic stroke in the overall population has been revealed, its association with the disease has been shown in individuals under 45 years of age [21]. The polymorphism of this gene is functionally important and has an essential role in neurological pathologies and lipid-related disorders. Allele e4 of *APOE* is associated with increased levels of total blood cholesterol and intima-media thickness in the carotid. Besides, allele e4 shows a significant association with a risk of some neurological conditions, such as Alzheimer's, brain concussion, prolonged rehabilitation period after head injury, etc. [22]. Damage to individual neurons (traumas, hematomas) may trigger formation of beta amyloids that exhibit toxicity towards healthy cells. The product of *APOE* expression facilitates clearance of beta amyloids across the blood-brain barrier. Allele e4 reduces APOE affinity to beta amyloids stimulating their deposition and thus causing neuronal death. This polymorphism can be seen as a functionally significant; however, it should be used in the tests sensitive to early ischemic changes.

The *ITGA2* gene encodes the alpha 2 subunit of integrins, i.e. proteins that mediate platelet adhesion to tissues when vascular damage occurs. Formation of a platelet monolayer in the lesion area launches a coagulation cascade. The *rs1126643* polymorphism (*c.759C>T*) accelerates platelet adhesion and is associated with a risk of thrombophilia [23]. This polymorphism directly affects the rate of pathological processes seen as risk factors for ischemic stroke and can be considered functionally significant.

The *IL6* gene encodes interleukin 6 and is actively expressed in atherosclerotic plaques. IL6 and other mediators of inflammation significantly affect arterial stiffness even if an artery is not in the vicinity of the ischemic lesion [24]. In spite of the effect *IL6* has on stroke severity and progression,



The algorithm for the selection of genetic markers to be used in prognostic tests aimed to assess individual risks of multifactorial phenotypic traits

rs1800795 functional significance is not obvious here. This polymorphism is located in a promoter region of the gene and affects the levels of IL6 and C-reactive protein. A meta-analysis also did not reveal any association of this polymorphism with a risk of stroke [25], therefore it should not be considered indicative of a risk of ischemic stroke.

Paraoxonase (the *PON1* gene) is an enzyme that has a crucial role in atherosclerosis prevention; it protects LDL (low density lipoproteins) from oxidation and hydrolizes lipids derived from LDL, inhibits monocyte-to-macrophage differentiation, macrophage foam cell formation and uptake of oxidized LDL by macrophages [26]. The *rs854560* polymorphism results in reduced paraoxonase levels, which can be viewed as a risk factor for atherosclerosis and stroke. However, the conducted meta-analysis did not confirm the association of this polymorphism with a risk of stroke [27], therefore this polymorphism should not be used in prognostic tests aimed to assess individual risks of developing ischemic stroke.

CONCLUSIONS

According to the criteria proposed above, prognostic tests based on the analysis of genetic polymorphisms should employ only those DNA markers that have shown statistically significant associations with studied MTs or are functionally significant in terms of manifestation of these phenotypic traits.

Present day approaches to the development of prognostic tests imply that these tests either employ those genetic markers that have shown a statistically significant association with a phenotype in question or rely on a few functionally important polymorphisms. Both approaches have their own drawbacks that affect the prognostic value of a test. If genetic markers are selected based on their statistically significant associations [11], some functionally important polymorphisms may be ignored due to their relatively low frequency or once a multiplicity correction has been applied.

At the same time, mechanisms of MT development are still unclear in many cases, which means that mechanisms by which genetic markers associated with pathology have their effect on MTs are also unknown. If a prognostic test relies only on those polymorphisms for which functional significance has been demonstrated and associations have been confirmed in a number of candidate gene studies, its sensitivity and specificity may be quite low. For example, if the assessment of an individual risk of ischemic stroke relies on the *PDE4D* polymorphism only [28], the number of false-negative results is likely to be quite large because there are a lot of genes whose polymorphic variants are associated with stroke. This approach will also yield a lot of false-positive results because the meta-analysis [29] has not confirmed the association of the *PDE4D* polymorphism with a risk of stroke in Caucasian population. Functional significance of genetic markers in this gene has not been established as well.

These drawbacks can be eliminated if both marker types are checked for eligibility. If functional significance of a genetic marker has not been established so far, its genome wide association can be considered statistically significant given that it has been confirmed in the independent sample. This approach helps to minimize the number of shortlisted genetic markers whose association with a studied phenotype is false-positive. Meta-analysis can provide a solution to the eligibility issue for those markers whose association has not reached genome-wide significance. If no GWAS or meta-analysis have been conducted, a genetic marker may be selected only if its effect on MTs has been established.

For further validation of our method, we plan to prepare a few lists of genetic markers associated with MTs using the criteria described above and the criteria proposed by other authors. These lists may be used to build a few prognostic models depending on the criteria applied. By comparing the obtained models using real genotype data, we will be able to assess the feasibility of these criteria.

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NUCLEAR MEDICINE IN THE DIAGNOSIS OF RENAL AND CORONARY ANGIOPATHIES IN PATIENTS WITH TYPE 2 DIABETES AND IMPAIRED GLUCOSE TOLERANCE

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The number of patients suffering from diabetes mellitus (DM) is increasing necessitating the development of new strategies for early detection of the disease. Here, radionuclide imaging may be a promising diagnostic technique. We have conducted a retrospective analysis of medical records and scintigrams of patients with type 2 diabetes (n = 83) and impaired glucose tolerance (n = 52) to evaluate the effectiveness of dynamic renal scintigraphy and myocardial perfusion scintigraphy at rest (single-photon emission computed tomography, SPECT) in detecting coronary and renal angiopathies. The control group consisted of patients with normal levels of blood sugar. To evaluate the functional state of the renal parenchyma, we conducted a qualitative analysis of patients' scintigrams and renographic curves; the glomerular filtration rate (GFR) was evaluated using Gates and Cockroft-Gault methods; myocardial scarring was evaluated using perfusion SPECT images synchronized with ECG. The functional activity of the renal parenchyma was shown to decrease significantly in patients with type 2 DM (Pearson's chi-squared test was applied, p-value was 0.03). With Gates method applied, GFR in both experimental groups was significantly lower than in the controls (Mann-Whitney U was calculated; p-value was 0.0004 and 0.0002, respectively). In patients with type 2 DM, GFR was lower than in patients with impaired glucose tolerance (p = 0.0004). With Cockroft–Gault method applied, we observed the same GFR pattern; however, the difference between patients with impaired glucose tolerance and the controls was insignificant (p = 0.08). The correlation between GFR values obtained using different methods was moderate in all groups (Spearman's rank correlation coefficient rs = 0.53, with p = 0.038).

Keywords: diabetes mellitus, impaired glucose tolerance, dynamic renal scintigraphy, myocardial perfusion scintigraphy

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ВОЗМОЖНОСТИ ЯДЕРНОЙ МЕДИЦИНЫ В ДИАГНОСТИКЕ АНГИОПАТИЙ ПОЧЕК И СЕРДЦА У ПАЦИЕНТОВ С САХАРНЫМ ДИАБЕТОМ 2 ТИПА И НАРУШЕНИЕМ ТОЛЕРАНТНОСТИ К ГЛЮКОЗЕ

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Число больных сахарным диабетом (СД) растет, и требуется разработка эффективных подходов к ранней диагностике заболевания. Перспективными являются радионуклидные методы диагностики. Проведен ретроспективный анализ историй болезни и сцинтиграмм пациентов с СД 2 типа (n = 83) и нарушением толерантности к глюкозе (n = 52) для оценки эффективности динамической нефросцинтиграфии и перфузионной томосцинтиграфии (однофотонной эмиссионной компьютерной томографии, ОФЭКТ) миокарда в покое при выявлении ангиопатий почек и сердца. В группу сравнения (n = 45) включили пациентов с нормальным содержанием глюкозы в крови. Оценивали функциональное состояние паренхимы почек путем качественного анализа сцинтиграмм и ренографических кривых, скорость клубочковой фильтрации (СКФ) — по методам Gates и Кокрофта–Голта, наличие и площадь повреждений миокарда — по данным перфузионной ОФЭКТ, синхронизированной с эхокардиографией. Функциональная активность почечной паренхимы значимо снизилась только у пациентов с СД 2 типа (критерий χ^2 Пирсона, p = 0,03 при сопоставлении с группой сравнения). СКФ по Gates в обеих опытных группах была значимо ниже, чем в группе сравнения (U-критерий Манна–Уитни, p = 0,0004 и p = 0,0002 соответственно), а в группе пациентов с СД 2 типа — ниже, чем в группе пациентов с нарушением толерантности к глюкозе (p = 0,0004). Для показателя СКФ по Кокрофту–Голту наблюдали те же закономерности, но различия при сопоставлении группы пациентов с нарушением гликемии и группы сравнения были недостоверными (p = 0,08). Корреляция между значениями СКФ, полученными разными методами, во всех группах была средней силы (коэффициент корреляции Спирмена r = 0,53 при p = 0,038). Рубцовые повреждения миокарда в обеих опытных группах выявлялись достоверно чаще, чем в группе сравнения, но различия по показателю между опытными группами были незначимыми.

Ключевые слова: сахарный диабет, нарушение толерантности к глюкозе, динамическая нефросцинтиграфия, перфузионная томосцинтиграфия миокарда

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Diabetes mellitus (DB) is a common chronic disease. Its main symptom is hyperglycemia induced by defective insulin synthesis/secretion, insulin deficiency or a combination of both [1–3]. DB is a serious medical and social issue due to its high prevalence, increasing incidence, chronicity and worrying disability rates. According to World Health Organization reports, there are currently about 250 million diabetics worldwide. This number is expected to reach 380 million by the year 2025 [4].

DB can be classified based on various criteria, including its complications, such as diabetic micro- and macroangiopathy, arthro-, polyneuro-, ophthalmo-, retino-, nephro,- and encephalopathy [5]. Microvascular and macrovascular pathologies are among the most common DB complications; the former occur in smaller blood vessels and induce nephropathy, the latter occur in medium and large vessels and are equivalent to coronary artery disease [6–9].

Early detection of diabetic complications requires a complex approach. Renal damage is most often discovered by clinical laboratory tests (manifesting through microalbuminuria) or by ultrasound imaging (altered parenchymal echogenicity or enlarged kidneys). Macroangiopathy (affected coronary arteries) can be detected using standard diagnostic techniques normally employed to detect coronary artery disease, such as cardiac markers, functional techniques (electrocardiography, echocardiography, exercise tolerance tests) and radiation techniques (coronary angiography, or in other words imaging of coronary arteries). These methods are used to assess structural and morphological changes in the organs and tissues in symptomatic patients. To verify angiopathies in asymptomatic patients with inconspicuous structural changes, state-of-art imaging techniques are used, such as single-photon emission computed tomography (SPECT) and positron emission tomography [10, 11].

Patients with impaired glucose tolerance constitute a separate group. A 2-hour glucose tolerance test reveals elevated glucose blood levels (from 7.8 to 11.1 mmol/L). Temporary increase in blood sugar leads to micro- and macroangiopathy occurring even before patients can be diagnosed with diabetes [12]. Therefore, individuals with impaired glucose tolerance should be monitored very closely as they are at risk of renal pathology: as the disease progresses and type 2 diabetes develops, the vessels of the kidneys will be affected in the first place [13]. The left ventricular myocardium may also be affected even if macroangiopathy has not yet been detected by routine tests [14]. Besides, in patients with impaired glucose tolerance, temporary increase in blood sugar can be sufficient to cause damage to the endothelium of small vessels [15]. Also, there is a risk of late cardiac events, such as silent myocardial ischemia, early infarction, etc. [16]. In patients with impaired glucose tolerance, results of radionuclide scans such as myocardial perfusion imaging (scintigraphy) can influence the choice of treatment. It is known that radionuclide diagnostic techniques are assistive in detecting functional damage to the organs in the early stages of the disease [17].

In light of the above, the aim of our study was to detect the diagnostic effectiveness of radionuclide technique, namely dynamic renal scintigraphy and myocardial perfusion SPECT synchronized with ECG at rest, for the detection of micro- and macroangiopathy of the kidneys and the heart in patients with impaired glucose tolerance and type 2 diabetes.

METHODS

We conducted a retrospective analysis of medical records and scintigrams of 180 patients who had been admitted to the Central Clinical Hospital of RAS in 2011–2016. The study included patients diagnosed with type 2 diabetes mellitus or with impaired glucose tolerance who had undergone dynamic renal scintigraphy (DRS) and myocardial perfusion imaging (SPECT). Exclusion criteria applied were as follows: previously diagnosed myocardial infarction, signs of myocarditis, chronic renal insufficiency, the absence of one kidney, congenital renal anomalies (horseshoe kidney, L- and S-shaped kidney), tumors and severe co-morbidities (multiple metastases, stage 4–5 chronic kidney disease).

Of 180 patients (mean age of 69.0 ± 11.6 years, male to female ratio of 1 : 0.8), 83 were diagnosed with type 2 diabetes mellitus (group 1) and 52 had impaired glucose tolerance and were not diagnosed with diabetes (group 2); 45 patients with normal blood sugar referred to hospital for other reasons (pain associated with angina, arrhythmias of unclear etiology, difficulty urinating, girdling pain in the loin) were included in the control group. The participants were divided into subgroups depending on the type of radionuclide imaging technique applied; their demographic characteristics are presented in Table 1. Scans were performed using the Infinia 4 Hawkeye SPECT/CT scanners (General Electric, USA). Blood chemistry tests were performed 3 days before and after the scan to measure troponin, creatinine and glycated hemoglobin levels. Also, echocardiography (ECG) records were analyzed to obtain information about the ejection fraction (EF).

Dynamic renal scintigraphy can be used to assess individual renal function and to study the concentrating ability and excretory function of the kidneys impaired by defective microcirculation. We used DRS to measure relative renal uptake of a radiotracer (RT), glomerular filtration rate (GFR) by Gates method [18], the maximum amplitude of the time-activity curves for both kidneys normalized to the administered RP dose, and its mean value. To estimate the volume of the functional parenchymal tissue, scintigrams and renogram curves were analyzed; the curve peak indicated maximum RT accumulation [19]. Based on the obtained data, the volume of the functional renal tissue was considered normal or reduced.

Prior to DRS, patients received an i. v. bolus injection of 200 MBq technetium-labeled diethylenetriamine pentaacetate, or ^{99m}Tc-DTPA (Pentatech, ^{99m}Tc by Diamed, Russia). DRS was recorded for 30 minutes at 1 frame per minute; renal angiography was performed in parallel. In total, 60 frames (1 second long each) of the vascular phase were recorded immediately after RT administration [18]. To calculate GFR, pre- and post injection syringe activity was recorded for 10 seconds (a 128 x 128 matrix was used); the difference in the activity was calculated from the obtained scintigrams. The total accumulation of RT was estimated based on the renogram curve between minutes 2 and 3 after the injection [18, 19]. Dynamic kidney images were reconstructed using Xeleris 2.1 workstation (General Electric) and Renal Analysis software (Emory University Hospital, USA). An example of the reconstruction process is shown in Fig. 1.

In addition, GFR was calculated based on serum creatinine using the Cockroft–Gault equation:

$$\text{GFR} = K \times \frac{(140\text{-age}) \times \text{weight}}{\text{serum creatinine}},$$

where K is 1.23 for men and 1.05 for women [20].

Patients who were ordered a myocardial perfusion scan received 750 MBq of methoxyisobutyl isonitrile (Technetril, ^{99m}Tc by Diamed) 40 min before the scan. ECG-gated SPECT images were acquired from 120 projections (60 per detector); acquisition time per projection was 32 seconds. Images were

Table 1. Demographic characteristics of patient subgroups based on the radionuclide diagnostic technique applied

Parameter	Dynamic renal scintigraphy			Myocardial perfusion SPECT		
	Group 1 (type 2 DM)	Group 2 (IGT)	Controls	Group 1 (type 2 DM)	Group 2 (IGT)	Controls
Number of patients n	25	17	20	58	35	25
Mean age. years (M ± SD)	71.7 ± 9.0	67.0 ± 14.4	67.0 ± 11.9	69.0 ± 9.8	67.9 ± 11.9	70.7 ± 12.3
Male to female ratio	1 : 0.4	1 : 0.7	1 : 0.6	1 : 0.9	1 : 0.4	1 : 1.1

Abbreviations: type 2 DM — type 2 diabetes mellitus; IGT — impaired glucose tolerance; M — arithmetic mean; SD — standard deviation.

reconstructed using Quantitative Gated SPECT/Quantitative Perfusion SPECT software (Cedar-Sinai Hospital, USA). Myocardial pathologies were indicated by perfusion defects on polar maps, reduced regional contractility in the same area and myocardial damage >5 %. An example of the reconstruction process is shown in Fig. 2. The same software was used to evaluate blood flow in the coronary arteries by measuring end systolic (ESV) and end diastolic (EDV) volumes (ml) and ejection fraction calculated as (EDV-ESV)/ESV.

Data were statistically processed using STATA-13 MP (StatCorp LP, USA). Due to a small size of the samples and the absence of normal distribution in them evaluated by the Shapiro–Wilk test, we used nonparametric statistical methods (Mann–Whitney U, Wilcoxon matched-pairs test, Spearmen's correlation, Pearson's chi-squared test; see RESULTS for further details). Difference was considered significant at p <0.05.

RESULTS

The analysis of scintigrams revealed reduced amount of functional renal parenchyma in almost all patients with type 2 DB (group 1) in contrast to the controls (Pearson's chi squared test was applied; p = 0.03); while patients with impaired glucose tolerance (group 2) demonstrated no significant reduction of the renal parenchymal volume (Pearson's chi squared test was applied; p = 0.23).

The Gates GFR was lower in groups 1 and 2 than in the controls (Mann–Whitney U-test was applied; p was 0.0004 and 0.0002, respectively). It was also lower in patients from group 1 compared to patients from group 2 (42.7 ± 15.9 vs. 50.4 ± 16.5 at p = 0.0004) (Table 2). The Cockroft–Gault GFR was lower in patients from group 1 than in the controls (Mann–Whitney U-test was applied; p = 0.003). Group 2 also demonstrated lower values but the difference between this group and the controls was insignificant (p = 0.08). The Cockroft–Gault GFR was lower in patients with type 2 DB in comparison with group 2 patients (58.2 ± 23.3 vs. 73.1 ± 25.9 at p = 0.005).

In group 1, the Cockroft–Gault GFR was significantly higher than the Gates GFR (Wilcoxon test was applied; p = 0.023). The same pattern was observed in group 2 (p = 0.02). Inside the control group, the difference was statistically insignificant (p = 0.243). The correlation between GFR values obtained by different techniques was moderate and statistically significant in all groups (Spearmen's r = 0.53 at p = 0.038).

The Mann-Whitney U-test showed that group 1 patients had higher levels of glycated hemoglobin and troponin than the controls (p was 0.01 and 0.0007, respectively) (Table 3). The same pattern was observed when comparing group 2 and the controls (p was 0.027 and 0.0069, respectively). When comparing groups 1 and 2, slightly higher levels of glycated hemoglobin were observed in patients with type 2 diabetes (6.96 ± 2.03 vs. 6.10 ± 0.80 at p = 0.025). Within these groups, the difference in troponin levels was unreliable (p = 0.38).

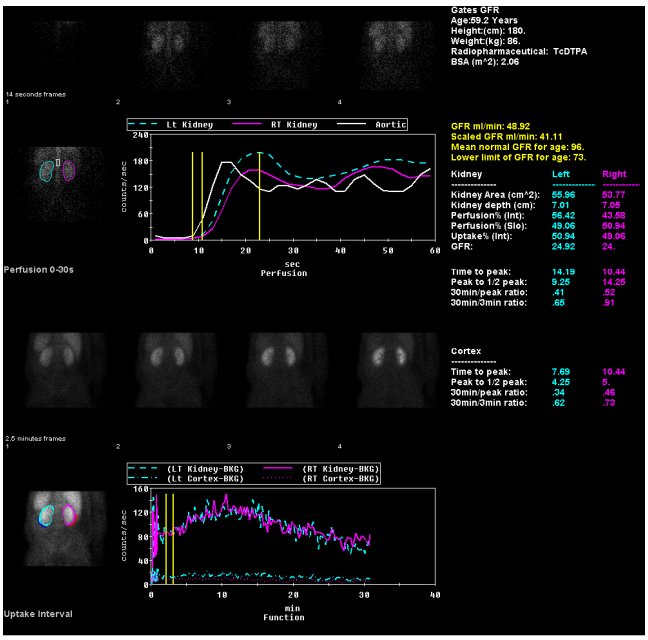

Fig. 1. Reconstruction of kidney images obtained by dynamic renal scintigraphy using Xeleris 2.1 working station (General Electric, USA) and Renal Analysis software (Emory University Hospital, USA)

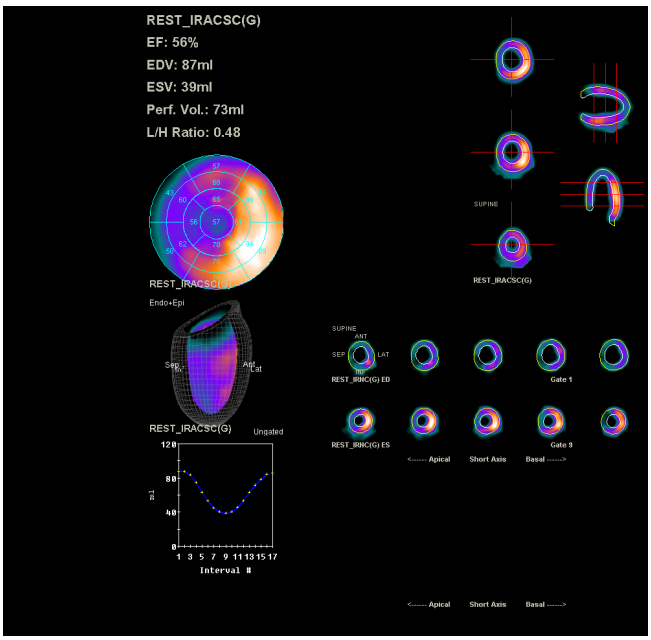

Fig. 2. Reconstruction of myocardium images obtained by perfusion SPECT using Quantitative Gated SPECT/Quantitative Perfusion SPECT software (Cedars-Sinai Hospital, USA)

Myocardial perfusion SPECT and ECG revealed no significant difference between patients with normal glucose levels and patients with impaired glucose tolerance with respect to the ejection fraction used to assess the myocardial contractile function (Table 3). ECG revealed a statistically significant EF reduction in patients with type 2 DM compared to the controls (Mann-Whitney U was applied; p = 0.01). Myocardial perfusion SPECT did not reveal any significant difference between the groups in this respect. Also, no significant difference in EF was observed when comparing groups 1 and 2 using the data from both scan types.

The results of myocardial perfusion SPECT showed that patients with type 2 DM had a higher prevalence of myocardial scarring (30 %) and greater lesion extent (6.4 ± 10.8 %) than the controls (0 and 0.06 ± 1.1 % p = 0.02 with Pearson's chi-squared test applied and at p = 0.02 with Mann–Whitney U-test applied, respectively) (Table 3). The same pattern was observed when comparing patients with impaired glucose tolerance and the controls (p = 0.001 for both statistical methods). When comparing groups 1 and 2, no significant difference was observed.

DISCUSSION

Similar patterns of changing glomerular filtration rates in patients with impaired glycemia and patients with type 2 diabetes prove our hypothesis about the renal endothelial dysfunction in the early stages of the disease, which is also confirmed by other works [9, 13, 15, 16].

The assessment of the renal function by means of dynamic renal scintigraphy in patients with impaired glucose tolerance revealed a tendency for reduced GFR (based on the analysis of time-activity curves), while their creatinine levels remained normal. It means that the Cockroft–Gault equation for GFR calculation in such patients is of no informative value. We believe that such patients should undergo DRS (fasting is required) for the primary assessment of the renal dysfunction and deciding on the adequate protective therapy. It is advisable to consider other renal conditions besides microangiopathy that may be present in a patient and lead to reduced functional activity of the kidneys, which in our case was a limitation. It is also known that in cases of severe renal dysfunction with GFR <15 ml/min, the outcome of the Gates measurement is unreliable due to decreasing count rates [18, 21].

In our study, myocardial perfusion SPECT detected even minimal damage to the left ventricular myocardium and helped to assess lesion sizes in the earliest stages of the disease in patients with impaired glucose tolerance; note that in those patients troponin levels were normal. However, ECG-gated perfusion SPECT can also detect defective perfusion, lesions and scars unrelated to impaired glycemia, which means that additional diagnostic techniques will be required to verify the diagnosis and medical records will have to be considered. This restricts the use of myocardial perfusion SPECT.

Due to the exposure to radiation during radionuclide scans, it is important to make sure they are really necessary. Here, the criteria may be such risk factors for cardiovascular disease as elevated cholesterol levels, smoking, arterial hypertension, obesity, hypersthenic habitus.

CONCLUSIONS

In the course of our study that employed dynamic scintigraphy and myocardial perfusion SPECT, we discovered changes in renal and cardiac tissues in patients with type 2 diabetes, as well as in patients with impaired glucose tolerance. We recommend that dynamic renal scintigraphy should be ordered for patients with impaired glucose tolerance for early detection of renal tissue dysfunction and vascular pathologies. Myocardial perfusion SPECT may be recommended for patients at risk of cardiovascular disease.

A limitation to this study was strict inclusion criteria resulting in small patient samples. Our research continues. So far, the prognostic role of radionuclide diagnostic techniques and their application as a treatment monitoring tool remain unclear. A multicenter prospective study of sufficient duration would be a solution.

Table 2. Glomerular filtration rates calculated using Gates method (dynamic renal scintigraphy) and Cockroft–Gault equation

Method	Group 1 (type 2 DM, n = 25)	Group 2 (IGT, n = 17)	Controls (n = 20)
Glomerular filtration rate calculated using Cockroft–Gault equation, ml/min	58.2 ± 23.3 (56.9)	73.1 ± 25.9 (71.4)	80.1 ± 23.7 (79.0)
Glomerular filtration rate calculated using Gates method (dynamic renal scintigraphy), ml/min	42.7 ± 15.9 (44.0)	50.4 ± 16.5 (57.3)	71.9 ± 25.7 (69.8)

Abbreviations: type 2 DM — type 2 diabetes mellitus; IGT — impaired glucose tolerance. Data are presented as M ± SD (μ), where M is arithmetic mean, SD is standard deviation, μ is median.

Таблица 3. Результаты биохимического анализа крови и перфузионной ОФЭКТ миокарда

Group	Parameter					
	Glycated hemoglobin (HbA1C), %	Troponin (Ths), pg/ml	Ejection fraction measured by echocardiography, %	Ejection fraction measured by myocardial perfusion SPECT, %	Scarring prevalence, %	Lesion extent, %
Group 1 (type 2 DM)	6.96 ± 2.03 (6.90)	30.9 ± 104.8 (8.8)	50.2 ± 8.8 (52.0)	56.5 ± 14.2 (59.0)	30	6.4 ± 10.8 (1.0)
Group 2 (IGT)	6.10 ± 0.80 (6.10)	29.6 ± 82.5 (10.0)	52.9 ± 9.2 (56.0)	59.9 ± 15.9 (66.0)	36.3	6.2 ± 10.5 (1.0)
Controls	5.07 ± 0.60 (5.10)	6.6 ± 3.6 (5.5)	56.7 ± 4.4 (58.0)	64.2 ± 10.6 (61.0)	0	0.6 ± 1.1 (0)

Abbreviations: type 2 DM — type 2 diabetes mellitus; IGT — impaired glucose tolerance. Data are presented as M ± SD (μ), where M is arithmetic mean, SD is standard deviation, μ is median.

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DYNAMICS OF FUNCTIONAL ACTIVITY OF THYROCYTES IN THE SETTING OF CHANGING MORPHOFUNCTIONAL ACTIVITY OF MAST CELLS OF THE THYROID GLAND UPON INFRARED LASER THERAPY

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Exposure to laser radiation is an interesting method of treating hypothyroidism and autoimmune thyroiditis. Its effect depends on the applied radiation dose. Degranulation of stromal mast cells of the thyroid gland (mastocytes) is dose dependent; release of granular contents into the surrounding tissues may affect microcirculation, result in the increased activity of the thyroid epithelium or stimulate the thyroid. Our study aimed to investigate the effect of moderate-intensity infrared laser radiation at total surface doses of 112 and 450 J/cm^2 on the functional state of mast cells and blood serum levels of thyroid gland hormones in healthy and hypothyroid subjects. The experiment was carried out in 78 random-bred mature male rats. Hypothyroidism was modeled by *per os* administration of 25 mg/kg Mercazolil (Akrikhin, Russia) for 21 days. Radiation therapy was performed using a IRE-Polus laser with a wavelength of 970 nm (NTO IRE-Polus, Russia). The animals received radiation therapy for 5 days and were sacrificed 1, 7, and 30 days after the experiment. Subsequently, we calculated the total number of mast cells, degranulated mastocytes, mastocytes with degree 1, 2 and 3 degranulation, degranulation coefficient, and levels of TSH in blood serum. Mast cells of the intact thyroid gland demonstrated low sensitivity to laser radiation, but hormone levels changed soon after radiation was discontinued. In the animals with hypothyroidism induced by thiamazole (the active component of Mercazolil), activation of mast cells was observed in the recovery period after the drug was discontinued. Hypothyroidism was accompanied by changes in TSH, T_4 and T_3 levels in blood serum. Comparison of the effects of two laser therapy modes in the animals with induced hypothyroidism revealed increased functional activity of mastocytes and normalized levels of secreted hormones at a total dose of 112 J/cm^2 and reduced mast cell activity at a total dose of 450 J/cm^2.

Keywords: hypothyroidism, thyroid gland, mast cells, mastocytes, degranulation, thyroid stimulating hormone, laser radiation

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ДИНАМИКА ФУНКЦИОНАЛЬНОЙ АКТИВНОСТИ ТИРЕОЦИТОВ ПРИ ИЗМЕНЕНИИ МОРФОФУНКЦИОНАЛЬНОГО СОСТОЯНИЯ ТУЧНЫХ КЛЕТОК ЩИТОВИДНОЙ ЖЕЛЕЗЫ ПОД ВОЗДЕЙСТВИЕМ ИНФРАКРАСНОГО ЛАЗЕРНОГО ИЗЛУЧЕНИЯ

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Лазерное воздействие представляет интерес в качестве метода терапии гипотиреоза и аутоиммунных тиреоидитов. Его эффективность зависит от применяемой дозы излучения. Дегрануляция стромальных тучных клеток щитовидной железы (мастоцитов) является дозозависимым процессом, а попадание содержимого клеточных гранул в окружающие ткани может привести к изменению микроциркуляции, повышению активности тиреоидного эпителия и усилению эндокринной функции железы. Целью исследования являлось изучение влияния инфракрасного лазерного облучения средней интенсивности при суммарной плотности дозы с поверхности кожи 112 и 450 Дж/см^2 на функциональное состояние тучных клеток и содержание гормонов щитовидной железы в сыворотке крови в норме и при гипотиреозе. Эксперимент провели на 78 беспородных половозрелых самцах крысы. Гипотиреоз моделировали пероральным введением «Мерказолила» («Акрихин», Россия) в дозе 25 мг/кг в течение 21 дня. Облучение производили с использованием аппарата «ИРЭ-Полюс» с длиной волны 970 нм (НТО «ИРЭ-Полюс», Россия) ежедневно в течение 5 дней. Животных выводили из эксперимента на 1, 7 и 30 сутки. Подсчитывали общее количество тучных клеток, количество дегранулированных мастоцитов, количество мастоцитов I, II и III степени дегрануляции, коэффициент дегрануляции, а также содержание в сыворотке крови тиреотропных гормонов. Показано, что тучные клетки интактной щитовидной железы малочувствительны к лазерному воздействию, но гормональный профиль изменяется в ранние сроки после облучения. При моделировании гипотиреоза тиамазолом (действующее вещество «Мерказолила») происходит активация тучных клеток в восстановительном периоде после прекращения дачи препарата. Моделирование гипотиреоза сопровождается изменением содержания в сыворотке крови гормонов ТТГ, T_4 и T_3. При сравнении результатов воздействия двух режимов лазерного излучения на железу животных с экспериментальным гипотиреозом отмечается повышение функциональной активности мастоцитов и нормализация уровня гормональной секреции при суммарной плотности дозы 112 Дж/см^2 и снижение активности тучных клеток при суммарной плотности дозы 450 Дж/см^2.

Ключевые слова: гипотиреоз, щитовидная железа, тучные клетки, мастоциты, дегрануляция, тиреотропный гормон, лазерное излучение

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Over the last decade, new laser-based technologies have become increasingly integrated into clinical routine. In addition to popular low-level lasers [1], high-power lasers are also employed by new treatment techniques to achieve therapeutic effects in deep tissues [2, 3].

Because the thyroid gland is a superficial organ, laser beams can easily reach it percutaneously. Laser radiation modulates thyroid function, stimulates secretion of thyroid hormones, improves microcirculation in the thyroid and produces a positive effect on tissue repair; it is widely used to treat hypothyroidism and autoimmune thyroiditis [4–7]. Some researchers associate structural changes in the thyroid epithelium with the effect of light photons on the thyroid stroma and its immune cells in particular. It is known that low-level laser irradiation of the thyroid stimulates secretion of thyroid hormones [8].

Thyroid hormones work by binding to the receptors in the cell nucleus; they regulate metabolism, control enzyme activity, and stimulate tissue growth, development and differentiation. Unquestionable is the effect of the thyroid hormones on the central and peripheral nervous systems, higher nervous activity and other endocrine glands.

The major function of the thyroid stimulating hormone (TSH) produced by the anterior pituitary is to exert a feedback control over synthesis and release of thyroid hormones. Thyroid function can be evaluated by thyroxine (T_4) and triiodothyronine (T_3) levels in blood serum, especially by measuring their unbound fractions which are a reliable indicator.

Stromal mast cells produce a variety of mediators, cytokines and enzymes, which determines the wide range of their activities. They were shown to participate in microcirculation and angiogenesis, tissue response to extreme conditions, inflammation and allergic reactions [9]. Bioactive substances released by degranulating mast cells affect the microvasculature and follicular epithelium, play an important role in the reparative regeneration of the thyroid and can change its functional activity [10]. Exposed to a laser, mast cells degranulate [11, 12].

The effect of varying doses of medium-level laser radiation on the functional activity of thyroid mast cells is still understudied. The aim of this work was to investigate the effect of different doses of medium-level infrared laser radiation on the functional activity of stromal mast cells by the assessment of their degranulation degree and on the functional activity of thyrocytes by the assessment of blood serum levels of thyroid hormones in healthy and hypothyroid subjects.

METHODS

The experiment was carried out in 78 random-bred adult male rats (body weight of 200–220 g) who were housed in cages, 2–3 animals per cage, ad libitum, under standard lighting conditions (day/night) and received a balanced diet. The experiment complied with the *Rules for Carrying out Activities involving Experimental Animals* (Addendum to Order No. 755 of the Ministry of Healthcare of the USSR dated September 12, 1977) and the revisions of the years 1975, 1983 and 1989 of The Declaration of Helsinki (1964).

The animals were divided into 6 groups: 1) intact animals; 2) animals with induced hypothyroidism; 3) intact animals who received a total radiation dose of 112 J/cm^2 (exposure time of 45 s); 4) intact animals who received a total radiation dose of 450 J/cm^2 (exposure time of 60 s) 5) animals with induced hypothyroidism who received a total radiation dose of 112 J/cm^2 (exposure time of 45 s); 6) animals with induced hypothyroidism who received a total radiation dose of 450 J/cm^2 (exposure time of 60 s). The animals were irradiated 5 days in a row; irradiation started one day after the induction of hypothyroidism. We used the IRE-Polus laser with a wavelength of 970 nm (IRE-Polus, Society for Research and Technology, Russia). The chosen irradiation mode with a total dose of 450 J/cm^2 did not cause any thermal damage to thyroid tissues.

Hypothyroidism was induced by a 21-day oral administration of 25 mg/kg Mercazolil (thiamazole by Akrikhin, Russia) by gavage [13]. Intact animals received the same dose of 0.9 % sodium chloride per os. Hypothyroidism progression was assessed by its clinical signs: changing body weight, appetite, hair condition, and temperature, considering thyroid morphology and the levels of thyroid hormones in blood serum. The animals were anesthetized with ether and sacrificed by cervical dislocation 1, 7 and 30 days after laser treatment was over.

Tissue samples were placed in 10 % neutral buffered formalin, and standard paraffin sections were prepared. The sections were then stained with hematoxylin-eosin and toluidine blue (pH = 2.0). Microscopy was performed using the DMRXA microscope (Leica, Germany). Images were transmitted to and analyzed by the DiaMorph Cito-W software (Russia). The morphometric analysis determined the total number of mast cells and the number of mast cells at different degranulation stages; the value of the degranulation coefficient was calculated. Mast cells were counted in 10 fields of view in each paraffin section; their number was then expressed as cells per 1 mm^2. Mast cells were subgrouped depending on their degranulation degree: 1) 1st degree — 1–2 granules outside the cytoplasm; 2) 2nd degree — 3–10 granules outside the cytoplasm; 3) 3rd degree— over 10 granules outside the cytoplasm. The degranulation coefficient was calculated as a ratio of degranulated cells to the total number of mast cells.

The levels of thyroid hormones in blood serum were measured by enzyme immunoassay using the fully automated Personal LAB analyzer (Adaltis, Italy) and two reagent kits: one kit by Cusabio Biotech (China) was used to determine TSH levels, and the other by Vector-Best (Russia) was used to measure the levels of bound and unbound thyroxine and triiodothyronine.

Nonparametric statistical analysis was performed using Microsoft Excel and SPSS Statistics 20 (IBM, USA). The median, the upper and lower quartiles were calculated. To assess the significance of difference, Mann–Whitney U-test was used. Difference was considered significant at $p < 0.05$.

RESULTS

The total number of thyroid mast cells did not differ significantly in the untreated hypothyroid animals and the intact animals (Table 1). The number of mast cells of the 1st degree of degranulation was lower in the animals who were sacrificed 1 day after hypothyroidism induction was completed compared to the intact animals (Table 2). The same experimental groups demonstrated a statistically significant increase in the number of mast cells of the 2nd and 3rd degrees of degranulation and the increased value of the degranulation coefficient in the animals sacrificed 1 month after irradiation.

Unbound T_4 was significantly reduced and TSH was significantly elevated in the hypothyroid animals compared to the intact animals regardless of the time of sacrifice (Table 3).

No changes in the number of mast cells and their activity were observed in the thyroid of the irradiated intact animals sacrificed in the early stages of the experiment (24 hours and 7 days after irradiation) regardless of the total radiation dose

Table 1. Comparison of animal groups by the total number of mast cells, including degranulated cells, and the degranulation coefficient

Group		Total number of mast cells (per mm²)	Number of degranulated mast cells (per mm²)	Degranulation coefficient
Group 1: intact animals		15.4 (7.48;21.78)	5.06 (4.07;10.56)#,1	0.53 (0.35;0.58)#,1
Group 2: intact animals; a total radiation dose of 112 J/cm²	24 hours after the experiment	8.58 (7.32;15.02)	4.07 (3.52;6.44)	0.45 (0.38;0.55)
	1 week after the experiment	10.78 (7.59;16.61)	6.27 (4.84;11.66)	0.66 (0.53;0.73)
	1 month after the experiment	18.15 (15.90;23.05)	12.87 (10.45;15.62)#,°	0.71 (0.59;0.80)#,°
Group 3: intact animals; a total radiation dose of 450 J/cm²	24 hours after the experiment	11.65 (9.96;11.88)	3.30 (2.64;6.11)	0.40 (0.23;0.57)
	1 week after the experiment	13.20 (7.87;16.34)	3.96 (3.69;11.72)	0.46 (0.30;0.72)
	1 month after the experiment	12.54 (8.36;19.03)	4.84 (2.42;8.36)°	0.36 (0.30;0.49)°
Group 4: animals with induced hypothyroidism	24 hours after the experiment	13.86 (8.75;14.19)*	5.83 (5.34;6.82)*	0.47 (0.40;0.71)*
	1 week after the experiment	12.87 (8.47;16.23)	6.60 (5.17;11.61)	0.67 (0.39;0.79)
	1 month after the experiment	17.60 (16.28;20.68)*	14.74 (11.66;15.62)1,*	0.84 (0.73;0.92)1,*
Group 5: animals with induced hypothyroidism; a total radiation dose of 112 J/cm²	24 hours after the experiment	18.81 (16.78;20.85)*	17.05 (15.24;18.87)*,°	0.91 (0.90;0.91)*,°
	1 week after the experiment	15.51 (13.59;18.59)*	12.21 (11.06;16.17)°	0.82 (0.78;0.87)°
	1 month after the experiment	12.32 (10.34;15.18)*	10.78 (9.57;12.87)*,°	0.86 (0.83;0.95)°
Group 6: animals with induced hypothyroidism; a total radiation dose of 450 J/cm²	24 hours after the experiment	10.34 (8.58;14.41)*	6.27 (4.57;8.31)°	0.55 (0.52;0.66)°
	1 week after the experiment	9.35 (6.44;10.29)*	5.28 (3.80;6.60)°	0.60 (0.55;0.65)°
	1 month after the experiment	15.18 (14.63;16.06)	5.17 (4.57;5.94)*,°	0.35 (0.29;0.40)*,°

Notice. Data are presented as a median (lower quartile; upper quartile)

1 — p <0.05 when comparing groups 4 and 1;
— p <0.05 when comparing groups 2 and 3 with group 1;
* — p <0.05 when comparing groups 5 and 6 with group 4;
° — p <0.05 when comparing group 2 with group 3 and group 5 with group 6.

Table 2. Comparison of animal groups by the number of mast cells depending on their degree of degranulation

Group		1st degree of degranulation. number of mast cells	2nd degree of degranulation. number of mast cells	3rd degree of degranulation. number of mast cells
Group 1: intact animals		2.64 (2.31;4.40)#,1	1.54 (0.88;2.53)#,1	0.44 (0.33;4.40)1
Group 2: intact animals; a total radiation dose of 112 J/cm²	24 hours after the experiment	1.21 (0.44;3.47)#	0.99 (0.44;1.38)	2.64 (1.49;3.41)°
	1 week after the experiment	3.96 (1.93;6.16)	2.64 (1.60;4.35)	1.87 (1.21;3.52)
	1 month after the experiment	5.06 (4.40;6.05)#,°	4.51 (2.86;5.45)#,0	3.19 (1.87;5.39)
Group 3: intact animals; a total radiation dose of 450 J/cm²	24 hours after the experiment	1.76 (1.54;2.64)	2.42 (1.43;4.07)	0.22 (0;0.94)°
	1 week after the experiment	3.30 (2.15;4.68)	2.09 (1.27;3.08)	0.22 (0.17;4.24)
	1 month after the experiment	0.88 (0.55;1.43)#,°	1.98 (0.66;2.64)°	1.54 (1.21;4.51)
Group 4: animals with induced hypothyroidism	24 hours after the experiment	1.54 (1.16;1.93)1,*	2.42 (1.60;3.74)*	1.87 (0.99;2.75)*
	1 week after the experiment	3.63 (2.09;6.60)	2.42 (1.71;3.91)*	1.65 (1.21;3.30)*
	1 month after the experiment	3.52 (1.98;3.96)	4.84 (1.98;5.50)1,*	5.94 (5.06;10.12)1,*
Group 5: animals with induced hypothyroidism; a total radiation dose of 112 J/cm²	24 hours after the experiment	4.07 (3.14;5.01)*,°	4.73 (4.46;5.01)*,°	8.25 (7.32;9.19)*,°
	1 week after the experiment	4.29 (2.53;4.90)°	3.96 (2.75;6.82)°	4.07 (3.30;6.82)*
	1 month after the experiment	3.74 (3.41;4.18)°	3.30 (2.75;3.63)°	3.74 (2.31;6.16)*
Group 6: animals with induced hypothyroidism; a total radiation dose of 450 J/cm²	24 hours after the experiment	1.10 (0.94;1.76)°	1.98 (1.16;5.45)°	2.75 (2.64;5.83)°
	1 week after the experiment	1.87 (1.21;5.50)°	1.21 (0.88;1.71)*,°	2.86 (1.21;3.69)
	1 month after the experiment	1.65 (0.66;1.98)°	1.32 (0.88;5.39)*,°	2.75 (1.54;3.96)*

Notice. Data are presented as a median (lower quartile; upper quartile)

1 — p <0.05 when comparing groups 4 and 1;
— p <0.05 when comparing groups 2 and 3 with group 1;
* — p <0.05 when comparing groups 5 and 6 with group 4;
° — p <0.05 when comparing group 2 with group 3 and group 5 with group 6.

applied. However, there were fewer mast cells of the 1st degree of degranulation in the thyroid of the animals sacrificed 1 day after receiving a total dose of 112 J/cm² (Table 2). On day 30 after irradiation with a total dose of 112 J/cm², the total number of degranulated mast cells and mast cells of the 2nd and 3rd degrees of degranulation, as well as the value of the degranulation coefficient, were increased, but the number of the cells of the 1st degree of degranulation was low. The total number of mast cells in the thyroid of the irradiated intact animals did not change significantly on day 30 after irradiation.

Blood samples collected 24 hours after irradiation showed that laser treatment of the thyroid of the intact animals with

a total dose of 112 J/cm^2 resulted in reduced TSH levels in blood serum, increased levels of unbound T_4 and increased levels of total and unbound T_3 (Table 3).

The subgroup of the hypothyroid animals who received 112 J/cm^2 irradiation and were sacrificed 24 hours after demonstrated a statistically significant increase in the total number of mast cells, the total number of degranulated mast cells of all degranulation degrees and the value of the degranulation coefficient (compared to the untreated hypothyroid rats) (Tables 1, 2) A month after laser treatment, we observed a statistically significant decrease in the total numbers of mastocytes, degranulated mast cells and mastocytes of the 3rd degree of degranulation. We also observed a statistically significant change in hormone levels: lower TSH and elevated unbound and bound T_4 and T_3 in the animals sacrificed a week and a month after irradiation.

Hypothyroid animals treated with a total dose of 450 J/cm^2 were found to have decreased levels of mast cells of the 2nd degree of degranulation a week after treatment; a month after irradiation, the number of total degranulated mast cells and mast cells of the 2nd and 3rd degrees of degranulation, the value of the degranulation coefficient and hormone levels in those animals were also low compared to the untreated hypothyroid rats (Tables 1, 2).

Comparison of different groups of animals with induced hypothyroidism who received the identical laser dose revealed that 450 J/cm^2 radiation led to the reduced number of degranulated mast cells, lower value of the degranulation coefficient and lower levels of hormones regardless of the time of sacrifice.

DISCUSSION

Thiamazole inhibits production of hormones by thyroid cells by blocking the activity of the peroxidase enzyme that mediates thyronine iodination necessary for the biosynthesis of T_3 and T_4, and thus causes hypothyroidism. Mast cells can participate in the paracrine regulation in healthy and hypothyroid subjects due to a large amount of bioactive substances contained in their granules. Degranulation of mast cells can be activated by cytokines, hormones and neuropeptides. This leads us to hypothesize that there may be an association between the functional status of stromal mast cells and the functional status of thyrocytes. This hypothesis needs to be tested.

Table 3. Levels of TSH, total (tot) and unbound (unb) thyroxine (T4) and triiodothyronine (T3) in the blood serum of experimental animals

Group		TSH (mU/l)	T4 (unb) (pmol/l)	T4 (tot) (nmol/l)	T3 (unb) (pmol/l)	T3 (tot) (nmol/l)
Group 1: intact animals		0.28 (0.21;0.75)$^{\#,1}$	18.87 (14.32;20.93)$^{\#,1}$	64.29 (39.29;138.27)$^{\#,1}$	2.09 (1.64;2.59)$^{\#,1}$	1.30 (0.96;1.39)$^{\#,1}$
Group 2: intact animals; a total radiation dose of 112 J/cm^2	24 hours after the experiment	0.19 (0.12;0.25)$^{\#,\circ}$	27.07 (22.60;27.67)$^{\#}$	72.45 (69.90;78.06)°	4.63 (4.52;6.59)$^{\#,\circ}$	1.89 (1.51;2.02)$^{\#}$
	1 week after the experiment	0.21 (0.09;0.26)°	19.55 (18.87;21.67)	90.82 (76.53;91.33)°	4.48 (4.35;4.82)$^{\#,\circ}$	2.05 (1.81;2.47)$^{\#}$
	1 month after the experiment	0.42 (0.31;0.45)°	23.87 (23.40;24.80)$^{\#,\circ}$	77.36 (75.00;78.06)	4.21 (3.59;4.77)$^{\#}$	2.10 (2.05;2.46)$^{\#}$
Group 3: intact animals; a total radiation dose of 450 J/cm^2	24 hours after the experiment	0.72 (0.49;0.83)°	15.85 (12.60;24.54)	22.40 (19.09;44.41)$^{\#,\circ}$	2.53 (1.96;2.74)°	1.21 (0.94;1.74)
	1 week after the experiment	1.49 (1.22;1.57)$^{\#,\circ}$	20.40 (15.59;26.66)	58.02 (46.99;62.76)°	2.32 (2.21;2.48)°	2.03 (1.88;2.17)$^{\#}$
	1 month after the experiment	0.10 (0.06;0.20)$^{\#,\circ}$	22.00 (18.11;23.07)°	86.74 (75.00;93.37)	4.72 (4.62;4.95)$^{\#}$	2.10 (1.93;2.40)$^{\#}$
Group 4: animals with induced hypothyroidism	24 hours after the experiment	1.52 (1.27;1.75)^{1,*}	10.39 (7.61;14.87)^{1,*}	25.96 (19.59;35.04)^{1}	3.23 (3.08;3.50)^{1,*}	1.53 (1.21;1.78)^{1,*}
	1 week after the experiment	1.24 (1.17;1.39)^{1,*}	5.64 (4.62;7.78)^{1,*}	23.83 (22.32;42.35)^{1,*}	2.98 (2.39;3.14)^{1,*}	1.44 (1.32;1.95)^{1,*}
	1 month after the experiment	2.36 (2.32;2.44)^{1,*}	5.41 (5.07;8.45)^{1,*}	57.30 (41.51;77.48)*	2.74 (2.64;3.12)^{1,*}	0.99 (0.81;1.00)*
Group 5: animals with induced hypothyroidism; a total radiation dose of 112 J/cm^2	24 hours after the experiment	0.38 (0.20;0.63)*	6.92 (5.16;8.65)	30.24 (24.03;59.13)	2.96 (2.64;3.83)	1.56 (1.06;1.95)°
	1 week after the experiment	0.34 (0.30;0.45)$^{*,\circ}$	10.41 (8.91;11.29)$^{*,\circ}$	51.22 (43.83;60.20)$^{\circ,*}$	3.89 (3.72;4.44)$^{*,\circ}$	2.68 (2.49;2.71)$^{*,\circ}$
	1 month after the experiment	0.19 (0.10;0.57)*	12.21 (11.80;12.97)$^{\circ,*}$	30.42 (28.74;41.43)*	3.57 (3.40;4.07)$^{*,\circ}$	1.77 (1.37;1.96)*
Group 6: animals with induced hypothyroidism; a total radiation dose of 450 J/cm^2	24 hours after the experiment	0.61 (0.36;0.71)*	6.76 (6.46;7.68)*	38.33 (32.40;48.19)	3.76 (3.51;4.20)*	2.94 (2.65;3.44)$^{*,\circ}$
	1 week after the experiment	0.51 (0.43;0.57)$^{*,\circ}$	6.52 (4.57;8.33)°	13.92 (12.78;14.75)$^{\circ,*}$	2.55 (2.49;2.71)°	2.14 (1.79;2.22)°
	1 month after the experiment	0.42 (0.31;0.70)*	9.26 (8.65;11.56)°	30.10 (29.14;34.64)*	2.96 (2.74;3.04)°	1.83 (1.77;2.10)*

Notice. Data are presented as a median (lower quartile; upper quartile)

[1] — p <0.05 when comparing groups 4 and 1;
[#] — p <0.05 when comparing groups 2 and 3 with group 1;
[*] — p <0.05 when comparing groups 5 and 6 with group 4;
[°] — p <0.05 when comparing group 2 with group 3 and group 5 with group 6.

Based on the obtained data, we can assume that the absence of significant changes in the degranulation degree of mast cells observed in the hypothyroid animals sacrificed 24 hours after hypothyroidism induction, compared to the intact irradiated animals, is an indirect evidence of the inhibiting effect of thiamazole on the functional activity of both thyrocytes and mast cells. The increased number of mast cells of the 2nd and 3rd degrees of degranulation and the high value of the degranultation coefficient observed a month after irradiation may reflect the regenerative process in the thyroid triggered in response to the damaging effect of the thyrostatic agent and may be considered an indicator of increasing thyroid activity [10].

Lower levels of unbound T_4 and elevated levels of TSH in the hypothyroid animals (in comparison with the intact rats) regardless of the time of sacrifice indicate disease progression and suppression of the compensatory mechanisms. Elevated levels of T_3 may indicate increased T_4 deiodination in peripheral tissues aimed to restore their regulatory functions.

The observed activation of mast cells in the thyroid of the animals with induced hypothyroidism treated with a radiation dose of 112 J/cm^2 and sacrificed 24 hours after treatment, low TSH levels and increased levels of bound and unbound T_4 and T_3 a week and a month after irradiation prove that this dose stimulates mast cells and has a positive effect on the functional activity of thyrocytes in hypothyroid subjects. In contrast, at a higher radiation dose of 450 J/cm^2, mast cell degranulation was less intense and hormone levels were decreased, which leads us to conclude the activity of mast cells, as well as the activity of the entire thyroid, was inhibited.

The literature reports the stimulating effect of low-level and high-level lasers on the degranulation of mast cells in different tissues [11–13]; however, in those experiments high-level laser radiation induced thermal damage to tissues. Heating the tissue up to a temperature at which protein coagulation was observed (or to higher temperatures) activated mast cell degranulation. Tissue precooling was shown to decrease the level of mast cell degranulation [14]. The laser irradiation mode selected for our experiment did not cause any thermal damage to thyroid tissues; therefore, slower mast cell degranulation was probably due to critical laser energy doses inside the tissue. It is known that laser radiation has stimulating effects at doses below 5 J/cm^2, while higher doses have an inhibiting effect. This is however true only for superficial wounds and tissue cultures [15]. As the laser penetrates deeper tissue layers, photons are attenuated and absorbed, and the ultimate energy dose that cells receive cannot be compared to the energy dose on the skin surface [2].

Microcirculatory changes in the laser-irradiated thyroid may be associated with nitric oxide production in the irradiated area [1, 4]. Vigorous blood flow to the thyroid can be explained by the effect of laser irradiation on the mast cell degranulation which is accompanied by the release of cytokines and mediators, such as heparin, histamine, enzymes, and growth factors; in turn, sufficient blood supply is a prerequisite for the biosynthesis of iodine-containing hormones of the thyroid epithelium [8, 12, 14].

Effects of laser radiation can be explained by the absorption of photons by mitochondrial and membrane chromophores matching the wavelength of the laser, changes in the redox cell potential and processes involving secondary messengers that trigger signaling cascades inside the cell [15, 16]. In particular, mast cell degranulation after laser irradiation is traditionally associated with increasing intracellular concentrations of calcium ions [11]; therefore, decreased functional activity of mast cells may be associated with poor permeability of cell membranes to calcium resulting from the inhibiting effect of high radiation doses on calcium channels.

CONCLUSIONS

Thiamazole-induced hypothyroidism changes the levels of TSH, T_3 and T_4 in blood serum, but does not affect the functional activity of stromal mast cells of the thyroid: their response is observed only in the regeneration period when the drug is not administered any longer.

The obtained data prove that the effect of the laser on the thyroid is dose-dependent and is manifested through stimulated functional activity of mast cells and hormone-producing tissue of the thyroid. Increased functional activity of thyroid mast cells and normalized levels of hormones in blood serum in the hypothyroid animals are observed after infrared laser treatment with a total dose of 112 J/cm^2; inhibiting effects were observed at 450 J/cm^2.

Considering the above, infrared laser irradiation with a superficial dose of 112 J/cm^2 should be studied further as an antihypothyroid therapeutical technique.

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CLINICAL AND LABORATORY FEATURES OF HEMOSTATIC DISORDERS IN PATIENTS WITH RETINAL VEIN OCCLUSION

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The number of patients with retinal venous occlusions is increasing, especially among young people. Often, they have revealed a genetic predisposition to thrombosis. Risk factors for thrombosis are genetic resistance to activated protein C (RAPC), genetic defect in factor V (FV Leiden) and the presence of lupus anticoagulant (LA). In this study we analyze the dependence of the various parameters of hemostasis in patients with retinal vein occlusion (RVO) on the background of FV Leiden mutation and LA. A total of 150 patients (150 eyes) with RVO (mean age — 42 ± 10 years) were examined and divided into three groups. Group 1: patients with RVO, FV Leiden and LA (n = 12); group 2: patients with RVO and FV Leiden (n = 11) without LA; group 3: patients with RVO without FV Leiden and LA, selected from remaining 107 people for a comparable number of groups (n = 30). The control group was 50 people without RVO, but with hypertension. It was shown that RAPC index in patients with FV Leiden mutation and the LA has the less value (0,6 ± 0,01) on comparison to patients with RVO (1,50 ± 0,18) (p <0,05). They also have enhanced V, VIII and von Willebrand factors and intravascular platelet activity. LA exacerbates endotheliosis in the microvasculature of the retina and in combination with FV Leiden mutation increases the thrombogenesis, participating in the pathogenesis of ischemic thrombosis of central retinal vein and its branches, which clinically manifested as retinal thrombo-hemorrhagic syndrome. The hemostasis regulation genes polymorphisms detection (as well as lupus anticoagulant detection) is recommended to clarify the diagnosis and selection of adequate therapy.

Keywords: retinal vein occlusion, activated protein C, resistance, FV Leiden mutation, lupus anticoagulant

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ОСОБЕННОСТИ КЛИНИКО-ЛАБОРАТОРНОЙ ДИАГНОСТИКИ НАРУШЕНИЙ В СИСТЕМЕ ГЕМОСТАЗА У ПАЦИЕНТОВ С ОККЛЮЗИЕЙ ВЕН СЕТЧАТКИ

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Растет число пациентов с венозными ретинальными окклюзиями, особенно среди среди молодых людей. Зачастую у них выявляется генетическая предрасположенность к тромбозу. Факторами риска возникновения тромбоза являются наследственная резистентность к активированному протеину С (РАПС), генетически обусловленный дефект фактора V (FV Leiden) и присутствие в организме волчаночного антикоагулянта (ВА). В исследовании была изучена зависимость различных параметров гемостаза у пациентов с окклюзией вен сетчатки (ОВС) при наличии у них мутации FV Leiden и ВА. Обследовали 150 пациентов (150 глаз) с ОВС (средний возраст — 42 ± 10 года), затем разделили их на три группы. Группа 1: пациенты с ОВС, FV Leiden и ВА (n = 12); группа 2: пациенты с ОВС и FV Leiden (n = 11); группа 3: пациенты с ОВС без FV Leiden и ВА, отобранные из 107 человек для сопоставимой численности групп (n = 30). В контрольную группу включили 50 человек без ОВС, но с гипертонической болезнью. Показано, что индекс РАПС у пациентов с мутацией FV Leiden и ВА имеет наименьшее значение (0,6 ± 0,01) при сравнении с пациентами только с ОВС (1,50 ± 0,18) (p <0,05). У них также повышена активность факторов V, VIII и Виллебранда и внутрисосудистая активность тромбоцитов. ВА усугубляет эндотелиоз в микроциркуляторном русле сетчатки, а в сочетании с мутацией FV Leiden усиливает тромбогенез, участвуя в патогенезе ишемического тромбоза центральной вены сетчатки и ее ветвей, что клинически проявляется ретинальным тромбогеморрагическим синдромом. Для уточнения диагноза и выбора адекватной терапии рекомендуется определение полиморфизмов генов, участвующих в регуляции гемостаза, и присутствия волчаночного антикоагулянта в организме пациента.

Ключевые слова: окклюзия вен сетчатки, активированный протеин С, резистентность, мутация FV Leiden, волчаночный антикоагулянт

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Retinal vein occlusion (RVO) accounts for up to 60 % of acute vascular disorders of the eye; it ranks second to diabetic retinopathy in terms of severity of damage to the retina and carries a poor prognosis [1, 2]. Retinal vein thrombosis often precedes the onset of such life threatening conditions as acute myocardial infarction and stroke. RVO is traditionally seen as imbalance between thrombogenic and antithrombogenic factors. In the recent years, the number of patients with retinal vein occlusion has been increasing, especially among young and working-age individuals with genetic predisposition to thrombosis [3].

Thrombophilia is susceptibility to thrombosis associated with inherited or acquired defects of procoagulant and anticoagulant pathways. Among the risk factors for venous thrombosis are inherited resistance to activated protein C (activated protein C resistance, APCR) and a mutant variant of factor V (factor V Leiden, FV Leiden). Activated protein C breaks down the mutant factor V more slowly than it occurs in healthy individuals, which increases the rate of thrombin production and under certain conditions may lead to thrombosis at any age. The nature of APCR can be genetic associated with FV Leiden or acquired associated with the presence of antiphospholipid antibodies and the effects of oral contraceptives [4–6].

Antiphospholipid antibodies (APA) alter the homeostatic regulation of blood coagulation. The exact mechanism of thrombosis involving APA (in particular, the lupus anticoagulant, LA) has not been identified yet. The prothrombotic mechanism of APA action is putatively based on the inhibition of endogenous anticoagulant pathways: reduced antithrombotic potential of the vessel wall and impaired activity of natural coagulants cause hypercoagulation [7–11].

Hemostatic defects and their clinical manifestation in patients with RVO who also have FV Leiden and LA are understudied. The aim of this work was to study some aspects of clinical laboratory testing for these disorders in patients with RVO who have FV Leiden alone or a combination of FV Leiden and LA.

METHODS

The study included 63 male and 87 female patients (a total of 150 individuals or 150 eyes) with retinal vein occlusion. Mean age was 42 ± 10 years. Branch retinal vein occlusion was detected in 78 patients (52 %), central retinal vein thrombosis was detected in 72 patients (48 %); 56 patients had hypertensive disease; 30 patients were diagnosed with coronary artery disease; 15 had varicose veins of the lower extremities. The length of the observation period varied from 2 weeks to 2 years.

The patients were divided into 3 groups. Group 1 included 12 patients with both FV Leiden and LA; group 2 included 11 individuals with FV Leiden; group 3 included 107 patients with RVO and without activated protein C resistance. In group 3, we analyzed data from 30 patients to make sample sizes comparable. The control group included 50 individuals without RVO who had hypertensive disease, no signs of systemic or autoimmune damage to the connective tissue, no coronary artery disease, cancer, or severe chronic infections. Male to female ratio in the control group was 20 : 30.

The clinical diagnosis was made using standard ophthalmic techniques: a visual acuity test, ocular tonometry, perimetry, direct ophthalmoscopy, and a number of specific techniques, such as the examination of the ocular fundus using a Goldmann lens, fundus fluorescein angiography, optical coherence tomography of the retina, computed perimetry.

To study the hemostatic system, automated coagulation screening was performed. A number of measurements were taken, including determination of von Willebrand factor (vWF), antithrombin III, activated protein C, and coagulation factor VIII activities; determination of factor V levels in blood plasma, levels of soluble fibrin monomer complexes (SFMC) and fibrinogen (the Clauss assay); resistance of factor V to activated protein C (APCR index).

The presence of the lupus antigen was detected by venom tests and confirmed by donor plasma and phospholipid tests using reagent kits by Technology-Standard (Russia) and Instrumentation Laboratory (Italy). Six weeks later, the tests were repeated.

Factor V Leiden and other polymorphisms responsible for susceptibility to thrombophilia were detected using real time PCR.

For this study we used the CL4 homeostasis analyzer (Behnk Elektronik, Germany) and a platelet aggregometer (Research and Production Company Biola, Russia). All tests were carried out at the facilities of the Laboratories for Hemostasis and Genetics (Kemerovo Regional Clinical Hospital).

Statistical analysis was performed using Statistica 6.0 software by StatSoft, USA. To describe the groups, we used the mean and the standard error of the mean. The groups were compared using Student's T-test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Of 150 patients with RVO, the lupus anticoagulant was found in 32 (12 %) individuals; 20 of them did not have FV Leiden, while 12 did. There were 11 (7.3 %) patients with RVO who had only FV Leiden. The hemostatic analysis (see the Table) demonstrated that SFMC levels in group 2 (mutant FV) and group 1 (FV Leiden + LA) were increased by 20 and 15 %, respectively, compared to group 3 (no FV Leiden or LA). Fibrinogen levels in groups 1 and 2 were by 37.5 and 20.0 % higher than in group 3. In group 1, intravascular platelet activation was more marked than in group 2: the total sum of activated platelets was increased by 16.2 % and the number of platelets involved in aggregation was by 26.3 % higher. Intravascular platelet activity was also increased in group 3, but it was less conspicuous than in patients with FV Leiden and no LA: 10.7 % vs. 16.4 %, respectively. Factor V activity was increased by 25 % in groups 1 and 2, compared to the controls. Groups 1 and 2 demonstrated a statistically significant 2.3- and 2.6-time increase in the activity of coagulation factor VIII, respectively, compared to the controls. Compared to group 3, this activity was by 27 and 30 % higher, respectively. Von Willebrand factor also exhibited increased activity in all three groups of patients with RCO, in contrast to the controls: by 55, 70 and 30 % in groups 1, 2 and 3, respectively. It proves that in retinal vein occlusion, the main role is played by the venous endothelium. However, increased activity of vWF in groups 1 and 2 was more conspicuous compared to group 3: by 16.0 and 23.5 %, respectively.

Protein C activity was more conspicuous in patients from groups 2 and 3 who had RVO in comparison with the controls: by 36.5 and 39.4 %, respectively. APCR was reduced in groups 1 and 2 by 64.0 and 58.9 %, respectively, in comparison with healthy individuals. In group 3 APCR was by 53.6 and 46.4 % higher than in groups 1 and 2, respectively. In patients with FV Leiden, the APCR index value was lower if LA was present.

In all groups, patients with RVO had elevated fibrinogen (inflammation protein) levels in comparison with healthy

individuals: they were increased by 56.3, 43.3 and 25.0 % for groups 1, 2 and 3, respectively. In groups 1 and 2 this index was 1.5- and 1.2-times higher, respectively, than in group 3.

Clinical test revealed that patients from groups 1 and 2 had microcirculatory disorders accompanied by vaso-occlusive processes in the ocular fundus. Fundus fluorescein angiography proved the presence of retinal leakage and local ischemia (both in the retinal center and on the periphery) and the absence of capillary perfusion (Fig. 1, 2). All patients had ischemic occlusion of the central retinal vein or its branches accompanied with marked cystoid macular edema (mean macular thickness measured by OCT was 790 ± 20 μm). Persistent cystoid macular edema was observed in 8 % of patients.

DISCUSSION

Increased activity of factor V demonstrated by groups 1 and 2 can be explained by the presence of FV Leiden in patients' blood. Activated protein C breaks it down more slowly, so the mutant factor V accumulates in blood. Factor V participates in the activation of prothrombinase complexes (XIa, VIIIa, vWF) mediating prothrombin conversion to thrombin. Protein C inactivates the mutant factor V slowly, which results in the prolonged factor VIII activity and can explain its elevated levels.

Antiphospholipid antibodies of the lupus type interact with the components of the vascular wall and endothelium; this stimulates the synthesis of vWF and factor VIII. LA activates platelets through membrane receptors, mediates the release of histamine, serotonin and platelet factors 3 and 4 from platelets, inhibits the synthesis of prostacyclin (a powerful inhibitor of platelet aggregation and a vasodilator), inhibits thrombomodulin and proteins C and S activities and thus provokes hypercoagulation. LA disrupts normal hemostatis and increases consumption of procoagulants and natural anrticoagulants. It also maintains aseptic inflammation in the venous wall and triggers thrombus formation in the retina.

CONCLUSIONS

Clinical and laboratory hemostatic tests performed in patients with retinal vein occlusion revealed that in the presence of FV Leiden, the activity of factors V, VIII and von Willebrand factor is increased and the resistance to activated protein C is reduced; in the presence of lupus anticoagulant significant aggravation of endotheliosis, thrombinemia and thrombus formation in the retinal microvasculature is observed manifested through thrombohemorrhagic complications. In patients with FV Leiden, the APCR index reduction is more marked in the presence of LA.

APC-resistance, both inherited (FV Leiden) and acquired (LA), disrupts the regulation of hemostasis in patients with RVO and therefore one of the clues to the understanding of the ischemic occlusion of the central retinal vein.

The obtained results prove that detection of polymorphisms participating in the hemostatic regulation, as well as detection of LA, is essential in patients with RVO, as it helps to clarify the nature of retinal thrombosis and can be used in the continuous monitoring of hemostasis in such patients aiming to deliver timely diagnosis and plan an adequate combined anticoagulation, platelet antiaggregation, antioxidant and afferent therapy.

Homeostasis in patients with retinal vein occlusion depending on the presence of FV Leiden and lupus anticoagulant (LA) in patients' blood

Parameter	Controls (n = 50)	Group 1: patients with RVO, FV Leiden and LA (n = 12)	Group 2: patients with RVO, FV Leiden and no LA (n = 11)	Group 1: patients with RVO, no FV Leiden and no LA (n = 30)
Factor V, %	100.0 ± 8.7	125.0 ± 4.3[a,c]	128.0 ± 4.3[a,c]	132.0 ± 3.9[a,c]
Factor VIII, %	102.0 ± 9.6	235.0 ± 2.2[a,b,c]	265.0 ± 4.3[a,b,c]	185.0 ± 6.6[a,b,c]
von Willebrand factor, %	100.0 ± 7.5	155.0 ± 5.8[a,b,c]	170.0 ± 9.0[a,b,c]	130.0 ± 12.3[a,c]
ACPR index	1.8 ± 0.02	0.65 ± 0.01[a,b,c]	0.74 ± 0.01[a,b,c]	1.40 ± 0.02[a,c]
C protein, %	104.0 ± 7.8	118.0 ± 4.7[a,b,c]	142.0 ± 5.1[a,b,c]	145.0 ± 6.7[a,c]
Antithrombin III, %	116.0 ± 11.7	155.0 ± 5.8[a,b,c]	118.0 ± 3.8[a,b,c]	122.0 ± 10.4[a,c]
Fibrinogen, %	3.2 ± 0.2	5.0 ± 0.3[a,c]	4.6 ± 0.2[a,c]	4.0 ± 0.2[a,c]
SFMC. mg%	4.6 ± 0.2	11.0 ± 0.5[a,c]	10.0 ± 0.5[a,c]	8.0 ± 0.5[a,c]
Total active platelet forms, %	12.8 ± 0.5	37.3 ± 1.7[a,b,c]	31.0 ± 1.1[a,b,c]	28.0 ± 2.1[a,c]
Number of platelets involved in aggregation, %	6.8 ± 0.4	9.9 ± 0.3[a,b,c]	7.1 ± 0.6[a,b,c]	6.1 ± 0.5[a,c]

a — p <0.05; comparison of groups 1, 2 and 3 with the controls;
b — p <0.05; group 1 compared to group 2;
c — p <0.05 groups 1 and 2 compared to group 3.

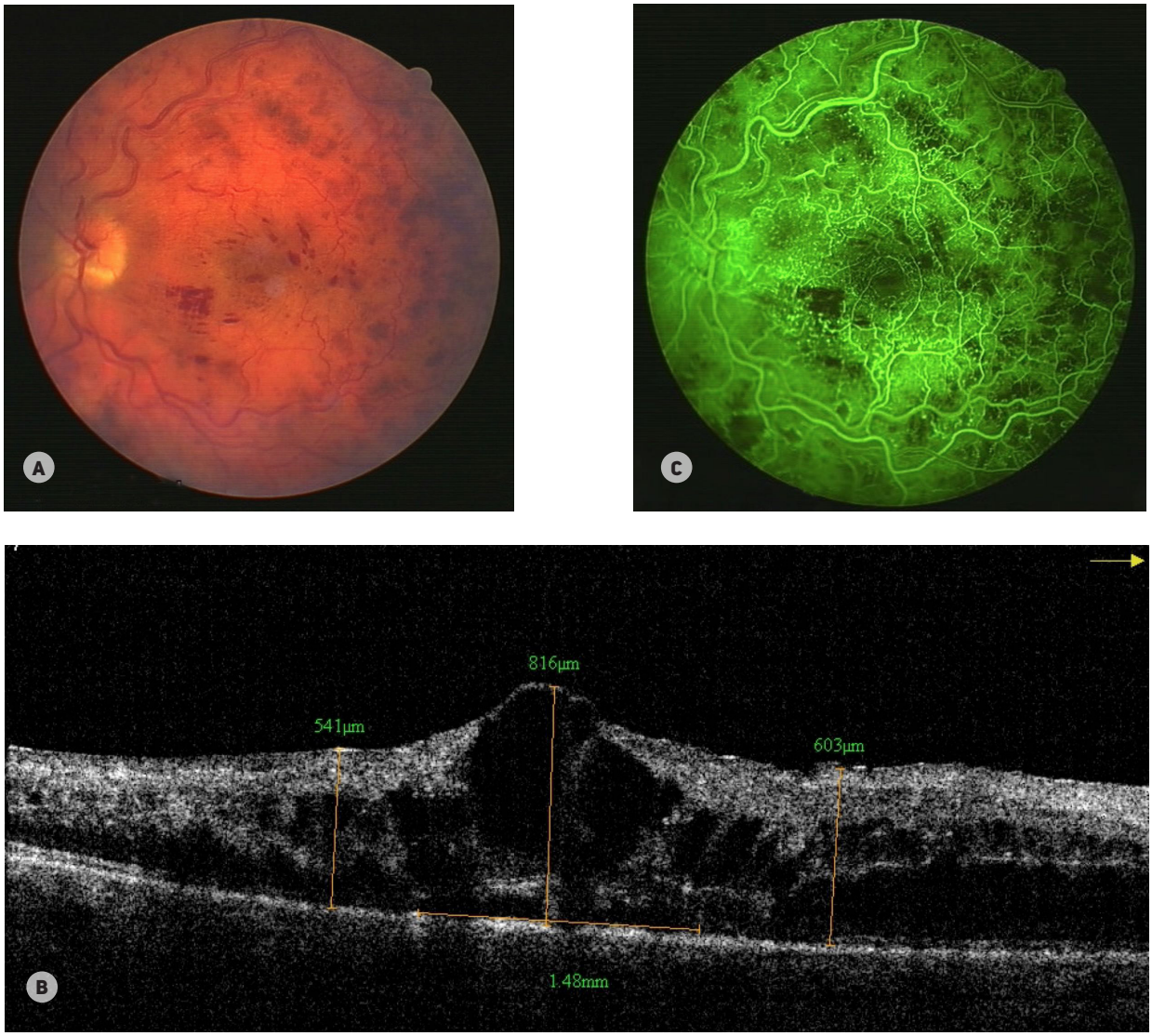

Fig. 1. Patient S., 38 years old. Diagnosed with central retinal vein occlusion; lupus anticoagulant and FV Leiden detected. **(A)** Conspicuous retinal hemorrhage syndrome. **(B)** Results of optical cogerence tomography. Diffuse cystoid macular edema; foveal thickness of 816 µm, peri- and parafoveal thickness of 541–603 µm; destroyed pigment epithelium and photoreceptor layer, d = 148 mm. **(C)** Fundus fluorescein angiography. Leakage from retinal vessels, cystoid macular edema, retinal ischemia

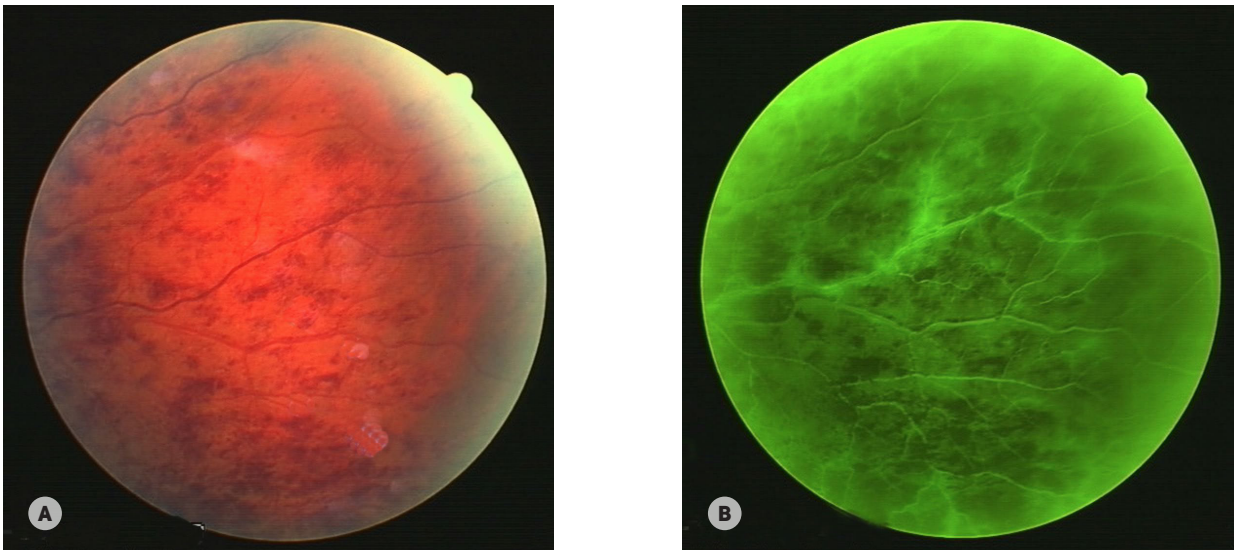

Fig. 2. Patient S., 38 years old. Diagnosed with central retinal vein occlusion; lupus anticoagulant and FV Leiden detected. **(A)** Medium periphery. Conspicuous retinal hemorrhage syndrome. **(B)** Fundus fluorescein angiography. Leakage from retinal vessels, cystoid macular edema, retinal ischemia

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STOLEN SCIENCE: WHY PLAGIARISM AND SELF-PLAGIARISM ARE UNACCEPTABLE

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Plagiarism is appropriation of someone else's ideas, texts, images and other materials without acknowledging their author. It is a serious violation of publication ethics that once detected results in the retraction of the submitted article. It has a disastrous impact on the author's reputation, because the publication is not removed from online databases, but stored there with a retracted publication tag. Plagiarism comes in different forms many of which still cannot be detected even by a special software; Plagiarism comes in different forms; the originality of an article is still assessed by peer reviewers and readers in the first place. Plagiarism can be unintentional. Most often, poor citation and reference style is typical of young researchers. To avoid unpleasant situations, authors are advised to use paraphrasing instead of merely copying and pasting fragments of texts. A verbatim use of a source requires quotation marks, references are expected to come right after the fragment borrowed from the original source; with multiple references (from 5 to10) pointing to a single idea are bad style. Authors are advised to always double check basic information about the publication they specify in a reference. The first author and a corresponding author are expected to monitor the quality of their co-authors' work. Full or partial copying of a previously published article by the same author is considered self-plagiarism and does not comply with the guidelines of the majority of academic journals.

Keywords: publication ethics, plagiarism, self-plagiarism, retraction of publication

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КРАДЕНАЯ НАУКА: ПОЧЕМУ ПЛАГИАТ И САМОПЛАГИАТ НЕПРИЕМЛЕМЫ

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Плагиат, то есть заимствование чужих идей, текстов, рисунков и других материалов без указания того, кто является их автором, — это грубое нарушение публикационной этики, которое при обнаружении факта заимствований в публикации влечет за собой отказ (retraction) журнала от нее. Для авторов это непоправимый удар по профессиональной репутации, так как публикация не изымается из многочисленных электронных архивов, а присутствует там с пометкой retracted publication. Плагиат принимает различные формы, многие из которых не могут быть выявлены с помощью специализированного программного обеспечения, и по-прежнему важную роль в оценке оригинальности работ играют рецензенты и читатели. Плагиат может быть непредумышленным. Чаще всего неправильное цитирование и оформление ссылок свойственно работам молодых исследователей. Во избежание неприятных ситуаций следует отказаться от копирования фрагментов в пользу их перефразирования, дословные цитаты брать в кавычки, расставлять ссылки непосредственно после заимствованных фрагментов, избегая «кустовых» ссылок (5–10 ссылок на один тезис), тщательно проверять выходные данные всех ссылок. Первому автору или автору-корреспонденту следует также проверять качество работы соавторов. Полное или частичное копирование авторами собственных ранее опубликованных работ считается самоплагиатом и недопустимо по правилам большинства научных журналов.

Ключевые слова: этика публикаций, плагиат, самоплагиат, отказ от публикации

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> Your work is both good and original. Unfortunately, the parts that are good are not original, and the parts that are original are not good.
>
> Samuel Johnson

"Publish or perish" — these three words convey the tragedy of a modern researcher. To derive maximum pleasure from scientific research, one has no choice but to compete for resources, such as position and money, or, in simpler terms, to make a career. The number and quality of scientific papers are the criteria commonly used to motivate personnel and to assess their performance efficiency. As a result, hundreds of manuscripts are published worldwide every day, and this creates a nourishing environment for unscrupulous researchers who steal ideas and texts or publish their own work multiple times to get to the top. Unfortunately, it is difficult to catch them red-handed. We would like to warn young researchers against silly mistakes and to remind experienced scientists of the responsibility they bear for their work. The following article

explains the essence of plagiarism and self-plagiarism and contains recommendations on how to avoid ethical issues that can bury your professional reputation.

Scientific misconduct is a serious social and economic issue, especially when it comes to medicine. According to some sources, about 90% of biomedical research cannot be reproduced; therefore the data obtained from it cannot be used for further studies. The reasons behind it are publication bias and sloppy statistical analysis [1]. Falsification and fabrication of data are plagiarism's companions that help to mask content matches. That is why, in compliance with the guidelines of the International Committee of Medical Journal Editors, retraction policies are currently applied to those articles that have been proved to be falsified, fabricated or stolen [2]. Once plagiarism has been detected, a notice of retraction is issued by the editorial board informing the audience that research findings cannot be considered trustworthy.

Both absolute and relative numbers of retracted publications are pretty low: according to the research by Amos [3], in 2008–2012 PubMed contained 835 articles tagged as retracted, which amounted to only 0.02 % of the total publications submitted to this database over the specified time period. However, Wager and Williams [4] demonstrate that the number of retractions has been increasing. According to some sources, 9.8 to 17.0 % of retractions are due to plagiarism [3]. Retractions are also tracked by Retraction Watch, a blog on the Internet [5].

Plagiarism: ctrl + c, 404 error and salami

But what exactly is plagiarism in science? In short, it is appropriation of someone else's ideas and texts without giving credit to their author. Some researchers [6] refer to the definition of plagiarism provided by the World Association of Medical Editors (we failed to find this definition on WAME website, though) and insist that the term *plagiarism* should be applied to a sequence of 6 or more words (no less than 30 letters in total) copied from an unnamed source. Some of Russian (Antiplagiarism) and foreign (iThenticate, CrossCheck) software used to detect matches in the submitted texts relies on calculating the percentage of copied content. However, a quantitative approach is ineffective, as plagiarism can take different shapes [7, 8].

Appropriation of someone else's publication with almost no changes introduced to the text is the most blatant form of plagiarism. It is commonly found in the work of those researchers who write in a language that an English speaking community does not understand or who steal research works from non-English speaking authors. For example, an unauthorized English translation of an article by Olga Baydik that was first published in Russian in a non-indexed journal was later found in a Pakistani journal [7]. A study by Amos [3] shows that plagiarism is very common in the works by Chinese, Indian, Italian, Turkish or Tunisian researchers.

The most common type of plagiarism relies on plain copying and pasting pieces of information with no reference to the original source. There is a similar form of plagiarism based on the "find-replace" idea: the author replaces a few words in a borrowed fragment with his own expressions thus masking scientific misconduct. A combination of properly cited text fragments with those that have no reference to the original source represents a hybrid form of plagiarism. It creates and illusion that the author adheres to ethical guidelines.

404 Error and aggregator types of plagiarism are quite peculiar [8]. In the former case, authors copy someone else's text and accompany it with a reference to a non-existent source or provide an inaccurate reference. In the latter case, citation is styled properly, but the source the fragment refers to does not contain the borrowed portion of the text. Usually, peer reviewers and editors make sure that original sources contain the fragments cited by the authors. Therefore, these types of plagiarism are easy to detect.

The most controversial form of plagiarism is self-plagiarism. Many researchers find it perplexing that one can steal his/her own work. On the one hand, you really cannot steal your own ideas from yourself (the only exception here is a situation when copyrights belong to the publisher, and by copying your own work you actually steal it from the latter). On the other hand, self-plagiarism is a form of scientific misconduct when seen from the ethical perspective. First, any publication aims to convey new knowledge; recycling of a previously published text or redundant publications are frustrating to your colleagues and fail their expectations. Second, the number and quality of scientific publications determine whether a researcher will be promoted or sponsored in the future; to multiply scientific articles beyond necessity means to lie to your employer and sponsors. Third, after the research has been published, it becomes part of the public domain and may be referred to by other researchers. If you have your article re-published by another journal, its citation index will drop, and the person who will benefit from it least is you.

Often, authors seek to publish one and the same work in many languages, for example, in their native language and in English. However, the guidelines of the International Committee of Medical Journal Editors clearly stipulate that duplicate publication is possible only for those manuscripts that contain extremely important information concerning public health that must be promoted as widely as possible. Such work must contain a clear reference to the original source, and the journal that decides to re-publish it must obtain permission from a previous publisher.

Another trick that unscrupulous researchers resort to is salami slicing, i.e., disclosing results of one study in a series of articles in small batches, with each article discussing one or several aspects of the same study. Though such approach can be justified if the obtained data are quite extensive, most often researchers are simply driven by a desire to increase the number of publications under their names forgetting that such form of results presentation deprives readers of a chance to thoroughly and comprehensively evaluate the published work.

It may be appropriate to build your manuscript around a report delivered at a conference or to include into it a portion of data borrowed from a dissertation. However, one should be very careful here too. Style requirements for a conference report may vary depending on a conference type, and sometimes propositions and concepts must be described in such great detail that a report starts to resemble a full-fledged publication. The editors of *Bulletin of RSMU* have agreed to reject such manuscripts as the information they contain cannot be considered new. Very often researchers want to publish parts of their dissertation after it has been defended. It usually happens when researchers do not publish preliminary results of the research a dissertation is built around prior to dissertation defense because they already have a sufficient number of publications (though unrelated) under their name, but they still believe that the results of their work must become part of the public domain. We accept such manuscripts only if a dissertation was defended no earlier than a year ago. Since the abstract is normally available to the public and the work itself can be accessed through different online libraries, indexed and

therefore referred to by other authors, we believe that it does not exhibit any scientific novelty.

Crime and punishment

Plagiarism and self-plagiarism can be both intentional and unintentional, the latter resulting from the lack of experience in writing scientific papers. Ethical misconduct can be fueled by professional competition, personal ambitions, especially if a researcher is not well-educated, or the burden of responsibility to sponsors. Some non-English speaking researchers engage in plagiarism when they write in English simply because they want to improve the style of their work [9]. However, you cannot be sure that linguistic forms you have borrowed from other articles are always correct. Besides, any serious international journal will refuse to publish such work once the copied fragments are identified. Poorly formatted citations and references may not be critical, and sometimes the authors are given a chance to make corrections to their manuscript [10], but this is not a universal rule. The higher impact the journal has (meaning more serious competition between authors), the less time the editor has to analyze each individual situation: the manuscript may be rejected on formal grounds.

If plagiarism is detected after the article has been published, the journal must retract it. The editorial board issues a notice providing full information on the article, naming the initiator of the retraction procedure and specifying the reasons for retraction. After that, all online copies of the article must be tagged as retracted [2]. A retracted publication will not be removed from online databases or withdrawn from circulation, but it will be permanently tagged as retracted. Spotless reputation of the authors is not an argument to mitigate the punishment. Moreover, the editor has a right to report ethical misconduct to the institution the authors are affiliated with and suggest initiating an investigation of their previously published work. If a person accountable for plagiarism is one of the co-authors, every author who signed the author agreement required by the publisher will share full responsibility. It ensues from the internationally accepted definition of authorship [2].

How can plagiarism be detected? Once a manuscript is submitted, it is run through specialized software, which, unfortunately, is not perfect. First, it is impossible to compare texts written in different languages; second, access to a number of journals and books can be restricted or completely blocked, which hinders the analysis; third, even if data or ideas have been stolen but the text itself has been reworded, plagiarism will not be identified; fourth, such software cannot differentiate between blunders and unintentional mistakes. This last circumstance is most unfavorable for those authors who submit their manuscripts to journals that totally rely on a software-based analysis. Another way to detect appropriated work is peer review. If experts invited to evaluate the quality of manuscripts are qualified enough, they can easily recognize the pieces seen somewhere else previously. The third anti-plagiarism filter is audience who can always report their findings to the editor.

How to avoid plagiarism

A good publication is a result of a good research work. If you are truly fascinated by the subject of your research, adhere to the international standards for research planning and implementation, are not afraid to face a frustrating outcome and are brave enough to report your mistakes to colleagues, you will most likely feel no need to falsify, fabricate or plagiarize data. Unintentional breaches of ethical code are not rare though, so in order to reduce the risk of unpleasant situations, it might be a good idea to use the following hints.

Hint 1. Familiarize yourself with the ethical regulations the journal adheres to. They normally contain definitions of plagiarism and self-plagiarism and stipulate responsibilities ensuing from scientific misconduct.

Hint 2. Avoid copying bits of scientific publications by other researchers. Carefully read the articles written by your colleagues and search for your own words and expressions to articulate ideas or patterns you have discovered. One and the same idea can be worded differently even if a text is technical. Paraphrasing also helps to better understand the original text.

Hint 3. Always provide information about the original source when quoting or paraphrasing it in your draft; specify the original source in brackets as follows: (Lastname et al., 2016). Later on, you can format your work as required by the journal you plan to submit your work to [9]. When quoting someone else's publication, use quotation marks, even if a citation is only a few words long.

Hint 4. Provide information on the original source of the citation right after the borrowed fragment (thesis – reference) and avoid multiple (5 to 10) references when communicating a single idea. First, a multiple-reference style can indicate that you have not analyzed the publications you refer to. Second, readers will not know where exactly to search for the information they find interesting. Third, you complicate editor's work: remember that the editor has to make sure that the facts you list match the content of every original source you refer to. The more time the editor spends on your manuscript, the less patience he/she is left with to scrupulously revise the paper.

Hint 5. Double check the title of the publication you refer to, the year it was published and other relevant information.

Hint 6. If you plan to use an image or a graph borrowed from some other source, find out who owns a copyright to it and what are the procedures (if any) to use these materials. In some cases you will need to obtain permission from a copyright owner.

Hint 7. If you are the first author or a corresponding author, monitor the work done by your co-authors. Ensure your colleagues are familiar with publication ethics. After a draft is ready, see if it contains fragments different from the rest of the work in style and containing zero references: usually such pieces are indicative of plagiarism [9].

Hint 8. Avoid copying fragments of your own previous publications; paraphrase them instead. Remember that an honest researcher does not tread water: although a subject of your research may still be the same, every new article on this subject requires a refreshed introduction. If you need to use previously published data, clearly specify it in the manuscript body and also warn the editor.

Hint 9. If the editor has detected unintentional copyright infringement, be honest when explaining the situation. Honesty is your chance to be allowed to make corrections to your manuscript.

CONCLUSION

Plagiarism is a serious breach of publication ethics which discredit science and scientists. In the age of digital technologies, it has become easier to present and share research data; at the same time protecting your research from unscrupulous colleagues

has become harder. Current algorithms of plagiarism detection are far from being perfect, and the role of peer review and vigilant audience is still important.

Plagiarism can be unintentional, especially in the works of young scientists who do not know yet the nuances of proper citation styling. Experienced researchers should be more attentive to their students and younger colleagues and not only share scientific knowledge with them, but also teach them to adhere to ethical standards. Unfortunately, copyright issues receive little attention in Russian schools and universities, and many unscrupulous authors experience plagiarism for the first time when preparing their thesis.

To avoid ethical issues, we recommend authors should try paraphrasing instead of quoting, use quotation marks when citing works by other researchers, style references appropriately, and submit accurate information about the original publication. Remember that all co-authors of the work share full responsibility, and do not let your colleagues down.

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