The Use of Cryopreserved Autologous Mononuclear Bone Marrow Cells for the Treatment of Non-Healing Ulcers in Diabetic Foot Syndrome

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Abstract

Objective: The diabetic foot syndrome (DFS) is one of the most serious complications of diabetes mellitus significantly reducing life quality of the patients. One of the most promising areas in the treatment of DFS is the use of cell therapy.

Research Design and Methods: In the presented original study the significant improvement in quality of life of 52 patients treated with autologous bone marrow mononuclear cells compared with a control group of 46 people receiving conservative antidiabetic therapy is demonstrated. The main characteristics of the chosen approach is the repeated application of a relatively small dose about 3 × 10⁸ of bone marrow mononuclear cells, the alternation of such a procedure with the addition of even smaller doses of the same cells frozen in liquid nitrogen.

Results: The optimal approach is an alternation of 1 main procedure followed by two replenishments of cells from the freezing with a periodicity of 2 months. With this approach the next basic procedure was performed 8-9 months after the start of treatment which completely avoided the formation of ulcers in the patients. None of the patients have any side effects reported during the whole observation period indicating that the method is safe.

Conclusions: Due to the use of single-point aspiration biopsy under local anesthesia, the standard methods of work with cell material and the use of patient autologous serum the developed method is extremely effective, affordable and safe for the patient way of DFS treatment.

Keywords: Diabetic foot syndrome; Bone marrow; Mononuclear cells; Auto transplantation; Cryopreservation.

Introduction

The diabetic foot syndrome (DFS) is one of the most serious forms of complications of diabetes mellitus. DFS causes a significant decrease in the quality of life, increases the overall morbidity and mortality and causes a huge expenditure of health resources [1,2]. The emergence of DFS contributes to a number of reasons such as ischemia of the lower extremities, neuropathy, traumatic and infectious effects. Diabetic vascular disturbances lead to long-term non-healing ulcers and often the only way to cure them is amputation.

DFS epidemiology appears to be quite complex as there are no single criteria for assessing the condition and diagnostic tests. It is noted that the prevalence of DFS significantly differs in different population groups. It is believed that in Western countries about 2% of the population with diabetes mellitus suffers from long-lasting non-healing ulcers. According to other estimates the risk of the DFS emergence reaches 25% during lifetime in such patients [3].

The early stage of DFS diagnosis by assessing the effectiveness of blood supply is rather difficult due to the specific manifestations of the diabetes effect on peripheral arteries. As a rule, timely diagnosis, preventive procedures and effective treatment by surgical revascularization methods are possible only in modern multi-profiled centers with the involvement of vascular surgeons, interventional radiologists, microbiologists and specialists in a number of other directions.

The classification of DFS is constantly changing due to new data finding on the nature of the syndrome. Considering the impact of various pathophysiological mechanisms of limb injury development according to WHO standards the isolation of neuropathic, ischemic and neuroischemic forms of DFS was accepted since 1991 [4]. Later, depending on the tissue localization of the lesion and taking into account the practically obligate lesion of the peripheral nervous system, the neuropathic form without osteoarthropathy, the neuropathic form with osteoarthropathy, the neuroischemic form and neuroosteoarthropathic form of DFS began to be isolated [5]. Depending on the degree and depth of ulcer damage there are three generally accepted classifications: according to Wagner [6], the classification of S(AD) SAD proposed by English authors [7] and the classification of the Texas University [8]. Despite a number of differences these classifications generally divide DFS at the stages according to the depth and width of the lesion, the involvement of deep tissues, the presence of ischemia, abscess, sepsis, deformity, a combination of factors.

Conservative methods of treatment of DFS include, first of all, stabilization of blood glucose level and blood pressure as the main pathogenesis factors of the syndrome development. The next steps are improvement of neuron trophism, stimulation of lipid and carbohydrate metabolism, strengthening of peripheral blood flow, medicinal vasodilating affection. Further it is possible to use reconstructive vascular surgery and in case of unfavorable course—an expanded surgical intervention to prevent sepsis [5].

Due to the complicated leading-up of patients with DFS and the lack of effectiveness of such conservative methods of treatment the new approaches to the DFS treatment have been searched recently. One of the most promising trends is the use of cell therapy. The effectiveness for the
treatment of DFS of the autologous bone marrow cells [9-11], precursor cells from peripheral blood flow [12,13], activated lymphocytes [14], autologous mesenchymal bone marrow stem cells [15] using has been repeatedly demonstrated in recent years. A significant effect has been shown when applying the combined methods of cell therapy and local gene therapy using a free plasmid construct with the vascular endothelial growth factor (VEGF) gene [16]. The use of allogeneic mesenchymal stem cells, despite clear prospects, remains unacceptable in the clinical practice of DFS treatment because of a number unresolved issues [17,18]. The main problem is the potential conflict with the immune system of the recipient. Numerous studies of application possibilities embryonic pluripotent umbilical cord blood cells which demonstrate the effect on laboratory animals models also do not come into clinical practice [19,20].

The present study demonstrates the advantages of using a relatively simple and safe method of autologous bone marrow mononuclear cell transplantation for the treatment of DFS even in later stages. Long-term results demonstrate the undoubted advantage of the chosen approach for improving the quality of patient’s life in general consistent with earlier elaborations [21]. The described combination of directly surgical manipulations, cell biology technologies and cryogenic biobanking technologies allowed significantly improving the obtained clinical results and making the method itself less traumatic and more convenient for the patient.

Research Design and Methods

Patients

The study participants were residents of the North-West region of Russia, who were treated at the University Hospital of St. Petersburg State University (Federal State Budget Institution St. Petersburg Multiprofile Centre of Russian Ministry of Healthcare) in 2008-2017 years. The experimental sample consisted of 52 patients with severe DFS, 36 men and 16 women. The average age was 63.5 years (from 55 till 84 y.). The control group of 46 patients included patients with DFS who for whatever reasons did not agree to treatment with the use of cell biology technologies and received conservative treatment only. This conservative therapy consisted in stabilizing the level of glucose, insulin therapy according to indications, hypoglycemic drugs, cardiotoxic drugs, stabilization of blood pressure, for example, beta-blockers, diuretics, blood pressure, for example, beta-blockers, and diuretics.

Ethical agreement consisted of three stages

All patients have filled informed consent to participate in the research, to provide their biomaterials for research, and personal data for statistical processing. The study itself was approved by the Ethical board of University Hospital of Saint-Petersburg State University. In addition the procedure itself received the status of an approved methodology from Russian Ministry of Healthcare.

The parameters of inclusion of patients in the experimental groups

Absence of psychotic reactions, absence of acute infectious processes controlled by ESR and C-reactive protein, absence of neutrophilic leukocytosis, controlled leukocyte index of intoxication, absence of anemia with a hemoglobin level less 90 g/l, absence of thrombocytopenia less than 100 x 109/l.

In the experimental sample the patients were divided into three groups

1) WHO received bone marrow cells autotransplantation once (25 people), 2) those who underwent this procedure from 2 to 5 times (10 people), and 3) who underwent the procedure with subsequent use the cells that underwent cryopreservation and storage in liquid nitrogen as a material for autotransplantation (17 people).

Obtaining bone marrow cells

Bone marrow cells were obtained by single-point ileum bone crest of the pelvis aspiration biopsy. The procedure was carried out under sterile conditions in a manipulative (operating) room under local nococaine anesthesia. The aspiration needle MIELO-CAN 15G (Sterylab, Italy) was used. The bone marrow was placed in pre-prepared sterile 50 ml tubes containing 20 ml of a NaCl solution supplemented with 50 IU/ml heparin. Immediately after filling the tubes were closed and neatly mixed to dilute the bone marrow.

For the preparation of autologous blood serum, a standard venous puncture with Lind-Vac vacuum systems (Corway, Estonia) was used.

Isolation of mononuclear cell fraction

Mononuclear fraction cells from patient’s bone marrow aspiration biopsies were obtained by the original method, which is based on [22]. The tubes with diluted bone marrow obtained from a manipulation were transferred to the cell biology laboratory. All procedures with biological material were carried out in a laminar box of class II protection in a sterile room. The diluted bone marrow was layered on an equal volume of density gradient Histopack 1077 (Sigma-Aldrich, Germany). The fractionation was carried out by centrifugation at 400G for 35 minutes. The interphase containing the mononuclear cells fraction was transferred to a clean test tubes and subjected to a two-stage washing in RPMI 1640 medium (Biolot, Russia). During the first wash 25 IU/ml of heparin was added to the medium in order to prevent coagulation of the residual platelets fraction. Purified mononuclear cells were placed in Petri dishes for cell cultures (Thermo Scientific Nunc, Denmark) in RPMI 1640 medium supplemented with 5% autologous blood serum of the patient and incubated for 1 hour in an atmosphere of 5% CO₂. Subsequently the medium with non-adherent to the substrate cells was centrifuged, the cells were made into suspension in 5-8 ml of NaCl solution and divided into several aliquots: for immediate administration to the patient, for 2-3 portions of cryopreservation, for counting. The count was performed either in the Goryaev chamber or using the Sysmex XT 2000i hematology analyzer (Sysmex, Germany). In the case of using the analyzer the cells were counted with separation into fractions by differentiation and maturity.

Cryopreservation

2 to 3 isolated aliquots of bone marrow mononuclear cells suspended from 20 to 30 x 10⁶ cells each were placed in 2 ml cryovials (Thermo Scientific Nunc, Denmark) in the following medium: 80% RPMI 1640, 10% DMSO (Sigma-Aldrich), 10% autologous serum of the patient. The cryopreservation was performed using a device for programmable freezing Cryologic CL 8800i (CryoLogic Pty. Ltd, Austria) using the Cryogenesis software (CryoLogic). The cooling was carried out at a rate of 1°C/min with a pause of 20 minutes at 0°C. Subsequently cryovials with cells were transferred to liquid nitrogen.

For defrosting cryovials with calls were removed from liquid nitrogen and heated in a water bath at 37 °C for 20 minutes. Further it was centrifugation washing performed in a pure PRMI medium, counting the cells number with viability evaluation, preparing a cell suspension in 3-5 ml of a NaCl solution for administration to the patient.

The introduction of cells to the patient

The cell suspension obtained from the cell biology laboratory was transferred to the manipulation (operating), thoroughly but neatly mixed. 

and taken into several 1 ml insulin syringes. Injections were performed intramuscularly to a depth of 3-8 mm in relatively healthy tissues as close as possible to the area of the affected limb along the perimeter of the ulcer. The total injection points were 15-20 depending on the size of the ulcer and the number of cells obtained. Depending on the localization of ulcers, the injection was attempted considering the anatomy of the supplying blood vessels. A sterile bandage was applied at the injection site and the affected limb. The patient’s condition was monitored after 3 days, then weekly during 1-2 month and then, if possible, monthly.

Re-introduction of cells after cryopreservation was performed at intervals of 1-2 months. In this case the injection points were 5-10 depending on the number of cells survived after malting.

The statistical analysis was carried out using the standard Microsoft EXCEL XP toolkit.

**Results**

**Patient conditions**

Developed as a result of this research approach to the treatment of DFS is represented in Figure 1.

The majority of patients (33 people) applied for medical assistance with the use of cell technologies with serious ulcer manifestations. According to the University of Texas classification they corresponded to categories 3-6. In 19 patients gangrenized tissues amputations have been performed already. Six patients applied directly for this procedure in the presence of categories 2-3 DFS.

As a result of treatment with autologous mononuclear fraction of bone marrow cells transplantation in most cases there were decreases in the DFS severity which can be noted as the preservation of category 3 but only due to the presence of a burdensome anamnesis. A significant improvement in the quality of life expressed in the healing of ulcers, the reduction of the level of ischemia and neuropathy was recorded absolutely in all the patients observed. According to the Karnofsky Performance Status patients demonstrate an increase in the life quality from the initial 40-50 points up to 90 [23]. If before the treatment patients require special care and medical care then after the course of the procedures they demonstrated sustained daily activity with a moderate degree of manifestation of the disease. Nevertheless caution should be exercised in evaluating such results considering that after a primary procedure and observation during a month a part of the patients (12 people) disappeared from the field of vision. Another part of the patients (19 people) disappeared from the field of vision after at least a year of observations, during this time there was an obvious improvement. The remaining 21 people are periodically observed and undergo secondary procedures with cell transplantation after cryopreservation. These patients have no signs of developing secondary ulcers risk. One of the most striking examples of ulcer healing is shown in Figure 2.

In the control group on the contrary there was a classical distribution of patients on the response to conservative treatment. Of the 46 patients 31 had a stabilized state while the risk of ulcers, angiopathy and ischemia was remained. During the observation for 1-2 years at 15 people the deterioration of the condition was recorded expressed in the development of ulcers involving deep layers of foot tissues. 11 of them required extensive surgical treatment later.

None of the patients from the experimental group demonstrated any side effects during the observation including autoimmune processes, oncological diseases, cardiac and vascular disorders. Hence the conclusion about the safety of the applied method follows freely.

**Selection of optimal conditions for bone marrow sampling**

For bone marrow sampling the ilium bone crest of the pelvis single-point puncture technique was chosen. This approach has a number of advantages such as the possibility of obtaining sufficiently effective bone marrow volumes, the use of local anesthesia and as a result a small traumatisms for the patient, a significant reduction in the cost of the procedure since only the procedural room is used instead of the operating room and the services of the anesthesia team are not required.

One of the main bone marrow sampling parameters was the prevention of coagulation. At the initial stages of the study coagulation clots were often formed even with the anticoagulant drugs use. As a result the technique was worked out when the bone marrow was collected in 50 ml tubes one-third filled with NaCl solution with heparin 50 IU/ml. This dosage was chosen as most closely matched to the tasks of preventing immediate coagulation. An important condition was fast enough aspiration so that the coagulation process did not start in the biopsy needle.

**Administred cells number and cryopreservation**

The number of procedures and the number of cells actual for the each group of patients are presented in Table 1.

As can be seen from the table the average number of cells introduced during the main procedure is about $3 \times 10^8$, varying from 1,980 to 3.5 x $10^8$ cells. Since the very procedure for isolating the cells mononuclear fraction did not change practically with time and the withdrawn bone marrow volume was practically identical the differences were due to the patients individual characteristics. The effect of the number of cells

<table>
<thead>
<tr>
<th>Group of Patients</th>
<th>Actual Number of Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.28 x $10^8$</td>
</tr>
<tr>
<td>Experimental</td>
<td>3.28 x $10^8$</td>
</tr>
</tbody>
</table>

**Figure 1:** Schematic presentation of DFS treatment developed.

**Figure 2:** Patient T, male 63 years old. (a) After an amputation with non-healing ulcer before cell therapy procedure. (b) Two month later the 3.28 x $10^8$ autologous bone marrow mononuclear cells injection around ulcer area.
administered on the time required for ulcer healing was not detected also. The most significant parameter is the number of procedures. Long-term stable progress in the patients’ condition was observed with repeated repetition of the main procedure alternating by replenishing a small number of cryopreserved cells. The limited number of cells for cryopreservation is caused firstly, by the desire to insert as much fresh cell material as possible during the main procedure and secondly, by the limited capacity of the standard cryovial with the optimal concentration of suspension of frozen cells for survival. The selected dose for freezing and subsequent replanting was about 2 × 10^7 cells. The most optimal is the alternation of 1 main procedure and 2 once with cells from liquid nitrogen with a periodicity of 2 months. With this approach the following basic procedure was performed 8–9 months after the start of treatment which completely avoided the formation of ulcers in these patients.

Discussion

The successful use of cell biology technologies for the treatment of lower limb ischemia and ulcers caused by has been described in the literature since 2002 [11]. Noteworthy in later works it is noted that this methods have not yet introduced mass use in clinical practice [17]. It can be assumed that the possible causes of this phenomenon are in the combination of a number of social, medical and biological factors. It is noted that the following issues remain unclear: 1) the optimal dose and method of introducing cell material, 2) specific molecular mechanisms that cause a reduction in the level of ischemia and lead to the healing of ulcers, 3) the possibility of combining cell and gene therapy to improve efficiency, 4) possible complications first of all the risk of carcinogenesis.

In the presented study the number of cells administered to a patient is relatively small. Compared with analogous studies [9] and [16] when the number of injected bone marrow mononuclear cells was about 1–2 × 10^8 the number 0.3 × 10^8 seems significantly smaller. Nevertheless the clinical effect was quite comparable. Apparently the advantages of the chosen approach are precisely the multiple applying and much less traumatisms for the patient. The use of a single-point aspiration biopsy under local anesthesia seems much more convenient than the method of multipoint aspiration under general anesthesia. A plausible mechanism for such effect of maintaining the therapy effectiveness with less cell number is maintaining a pool of pluripotent cell concentration in tissues with progressive ischemia. These cells are probably CD34+, CD133+ and KDR+. It is noted [11] that upon a large volume (more than 500 ml) of bone marrow sampling the concentration of CD34+ cells can be significantly reduced due to dilution with peripheral blood flow. This does not occur with a relatively small (100–150 ml) taking from a single point.

The different pluripotent cells role in the angiogenesis of susceptible limb ischemia is also a matter of debate. Vascular growth is possible in the presence of endothelial progenitor cells secreting the growth factors VEGF, FGF-2, GM-CSF and several others. CD34+ cells are well suited for this role which has been repeatedly demonstrated in various studies [24-26]. On the other hand the study [9] showed that with the restoration of a limb from an ulcer was associated only with the introduction of a subpopulation of KDR+ AMBMFC while the CD34+ and CD133+ subpopulations did not have a similar effect. From our point of view the separation of AMBMFC into subpopulations before administration can hardly be effective. In addition to endothelial progenitor and hemopoietic cells that stimulate angiogenesis the mesenchymal stem cells expressing on their surface CD54/CD102, CD166, CD49, CD73 and CD90 are contained in the bone marrow [27,28]. Despite the fact that the potential for differentiation in these cells is significantly lower than in embryonic stem, induced pluripotent and a number of other types of stem cells they are able to migrate and demonstrate the effect of homing in damaged tissues promoting regeneration processes, secreting trophic factors and paracrine mediators of intercellular contacts [29-32]. Due to the above properties it is the mesenchymal stem cells that can compensate for the diabetic neuropathy that accompanies the DFS.

Thus it is the autotransplantation of the whole fraction of bone marrow mononuclear cells that can provide delivery to the affected limb of all components for the healing of diabetic ulcers.

After the introduction of cell biology technologies into medical practice both researchers and clinicians are asking about the safety of these methods and possible complications. The majority of reports indicate good tolerability of cell autotransplantations with complete absence of side effects. Only one study describes such side effects of autologous mononuclear peripheral blood therapy specifically cardiovascular complications leading to myocardial infarction [33]. At ones it is noted that the patients belonged to the group with the age of 76.7 ± 9.7 years. The blood cells underwent G-CSF induction in vitro.

In connection with the special concern about the possibility of developing such a side effect as carcinogenesis after the application of autotransplantation of stem cells all cases of oncological diseases in patients undergoing cell therapy procedures are closely monitored. It is noted that the use of AMBMFC is absolutely safe and the incidence of cancer in patients does not exceed the average statistical values even in older age group. Using of laboratory animals models allows expanding the scope of the experiment. An original study was carried out to test the possibility of

### Table 1: Administered cells number and cryopreservation

<table>
<thead>
<tr>
<th>Patient Group</th>
<th>Patient Number</th>
<th>Main Procedure Number</th>
<th>The average number of mononuclear cells per procedure</th>
<th>Number of administrations after cryopreservation</th>
<th>The average number of mononuclear cells after cryopreservation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Who received cells autotransplantation once</td>
<td>25</td>
<td>1</td>
<td>2.88 × 10^8 ± 1.13 × 10^8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Who underwent the procedure from 2 to 5 times</td>
<td>4</td>
<td>2</td>
<td>3.16 × 10^8 ± 0.74 × 10^8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3</td>
<td>3.02 × 10^8 ± 1.25 × 10^8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5</td>
<td>2.44 × 10^8 ± 0.59 × 10^8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Who underwent the procedure with subsequent use the cells that underwent cryopreservation</td>
<td>5</td>
<td>1</td>
<td>3.5 × 10^8 ± 0.66 × 10^8</td>
<td>1</td>
<td>3.3 × 10^7</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1</td>
<td>2.96 × 10^8 ± 0.18 × 10^8</td>
<td>2</td>
<td>1.95 × 10^7 ± 0.45 × 10^7</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
<td>1.98 × 10^8</td>
<td>3</td>
<td>2 × 10^6 ± 0.52 × 10^8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2</td>
<td>2.88 × 10^8 ± 0.51 × 10^8</td>
<td>3</td>
<td>1.9 × 10^7 ± 0.4 × 10^8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2</td>
<td>2.9 × 10^8 ± 0.7 × 10^8</td>
<td>5</td>
<td>1.5 × 10^7 ± 0.35 × 10^7</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3</td>
<td>2.42 × 10^8 ± 0.81 × 10^8</td>
<td>6</td>
<td>0.95 × 10^7 ± 0.72 × 10^7</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>4</td>
<td>3.00 × 10^5 ± 0.85 × 10^5</td>
<td>4</td>
<td>1.18 × 10^6 ± 0.33 × 10^6</td>
</tr>
</tbody>
</table>

stimulating carcinogenesis [34]. As a result the tumors developed in the mice after the bone marrow cells autotransplantation actually. However when treated in vitro the cells were subjected to a mutagenic effect. It also should be noted that diabetes and myocardial infarction were artificially stimulated in mice during the experiment. According to the results of the study the authors recommend controlling the possibility of chromosomal abnormalities when working with cells implying an increased possibility of mutations. However in reality the view on cell therapy as a cause of carcinogenesis seems rather controversial.

A much larger number of studies reports about the protective role of cellular therapy against cancer on the contrary [35]. Suchlike data speak in favor of autologous bone marrow transplantation for the treatment of DFS in particular. The use of allogeneic preparations despite the pronounced perspectives conditioned by the very nature of the cellular material taken from healthy people and the possibility of an accurate selection by the HLA system does not have carcinogenic safety [36].

Conclusion

As a result of this study a rather original approach to the treatment of DFS has been developed. It is based on combining previously described in the literature methods of cell therapy using autologous mononuclear bone marrow fraction cells (AMBMFC) using cryopreservation and biobanking methods. The advantages of the chosen approach include first of all the demonstrated high efficiency expressed in an increase in the percentage of favorable outcomes compared with the control group treated with conservative methods, reducing the healing time of ulcers and minimizing the relapses possibility. Another important factor is the possibility of preventive application of AMBMFC therapy after cryopreservation to prevent ulceration recurrences. A significantly greater efficacy of multiple cell administration to a patient has been demonstrated in several studies [9,10]. The use of cryo technologies allows reducing the effect of traumatism which accompanies the procedure of bone marrow sampling. Another important advantage is the greater availability for the patient due to lower overall cost of the procedure.

This trial in Russia has the status of an approved methodology. Despite relative completeness at this initial stage it undoubtedly requires continuation of work which can result in: 1) conducting a multicenter randomized double-blind trial, 2) evaluating results at the physiological level, for example, angiographic studies, 3) characterizing and searching for active molecular level mechanisms including a description of cell surface markers. Particular attention should be paid to the possibility of combining the potentials of cell therapy and cryotechnology presented in the present work with gene therapy probably by the genes of some growth factors.

An additional safety level in the presented study was provided by the use of autologous serum when working with cells and during cryopreservation procedures. With considering the above facts the described technique allows for more effective and safe treatment of DFS.

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Author Contributions

•  A.V.I. contributed to the study design; work with cells and cryotechniques, conduct of the study, data analysis, and writing of the manuscript.
•  A.V.K. contributed to the study design, medical supervision of the study, all work with the patients.
•  Y.N.F. contributed to patient recruitment, conduct of the study, global supervision.
•  All authors critically reviewed the manuscript and approved the final version for publication. A.V.I. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
•  Prior Presentation. Parts of this study were presented in inner meetings of University Hospital of Saint-Petersburg State University.

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