Research Article

Journal of Immunology (ISSN 2995-861X)

POST-COVID SYNDROME - A MODEL OF PATHOGENESIS

Alexander N. Kazimirskii, Jean M. Salmasi, Gennady V. Poryadin, Marina I. Panina, Vera N. Larina, Ekaterina A. Stodelova

Pirogov Russian National Research Medical University, Ostrovityanova St.1, Moscow 117997, Russian Federation

Corresponding author

Alexander Kazimirskii ,
Pirogov Russian National Research Medical University, Ostrovityanova St.1, Moscow 117997, Russian Federation.
Email : alnica10@mail.ru

Received Date : April 16, 2024
Accepted Date : April 17, 2024
Published Date : May 17, 2024

ABSTRACT

Post- COVID is characterized by a decrease in the patients' quality of life, a tendency to hemocoagulation. Symptoms of post- COVID are diverse and difficult to classify.

Purpose : Study of neutrophil extracellular traps (NETs) in post-COVID patients to clarify the mechanisms of damage.

Patients and methods : Two groups of patients with post-COVID were examined. In the first group (21 patients), COVID-19 treatment was carried out in a hospital, in the second group (20 patients) - on an outpatient clinic. The comparison group consisted of 10 patients with acute appendicitis, the control group consisted of 20 healthy people.

Neutrophils were isolated using gradient centrifugation. NETs was visualized using fluorescence microscopy with SYBR Green dye (Evrogen; Russia), which specifically interacts with double-stranded DNA. Purine nitrogenous bases (PNB) were determined by the color reaction method based on their interaction with silver nitric acid.

Results : In patients with post- COVID treated on an outpatient clinic, the number of NETs (6.55±0.94%) was 7.6 times higher than in patients treated in a hospital (0.86±0.51%), and the tendency to increase the number of symptoms per patient (3.81±0.72) compared to outpatient patients (2.40±1.10), which indicates an effective suppression of the pathological process in the hospital. In all patients with post- COVID, NETs was detected only in the form of single strands of DNA. The concentration of extracellular PNB in the blood plasma of patients with post- COVID for more than 3 months was 7-35 mg / ml. In the comparison group, this index increased briefly (3-5 days) to 0.2-1.8 mg / ml. There are no extracellular PNB in the blood of healthy donors. We believe that patients with post- COVID develop spontaneous enzymatic degradation of DNA strands, which leads to an increase in extracellular PNB. Extracellular PNB are toxic because they can cause cell damage and inhibit the activity of T-lymphocytes, causing secondary immunodeficiency.

Conclusion : Virus-induced endothelial damage triggers contact interactions between neutrophils and fibroblasts, which causes neutrophils to form filamentous NETs. Spontaneous degradation of DNA fibers leads to an increase in extracellular PNB, which are factors of secondary alteration and damage endothelial cells and internal organs.

Keywords : post-COVID, neutrophil extracellular traps, NETs, NETs in filamentous form, post-COVID pathogenesis**.**

INTRODUCTION

Post-COVID or "long-COVID " is closely associated not only with a decrease in the quality of life of patients, but also with a tendency to hemocoagulation and thrombosis, which is supported by stable platelet activation with the risk of fibrin clot formation [1]. The state of hypercoagulation in patients with post-COVID syndrome leads to the appearance of a group of patients with cardiovascular complications. The combined rate of thrombosis (arterial and venous) in patients with COVID-19 on the 30th day after discharge from the hospital is 2.5 % [2]. And withine 12 months after COVID-19, patients were at increased risk of developing cerebral and cardiac complications, such as stroke, severe arrhythmia, myocarditis and pericarditis, and complications of coronary heart disease [3].

There are multiple numerous long-term symptoms,in some patients, who have recovered from COVID-19 which are very diverse, but there is no systematization of them. There was no correlation between the number of symptoms and the severity of the acute period of COVID-19.

The experimental work carried out to identify the most significant links in the pathogenesis of post-COVID is of interest. For example, circulating microclusters that are persistent and resistant to fibrinolysin and trypsin have been

Journal of Immunology (ISSN 2995-861X)

found in the blood of post-COVID patients; the origin of these circulating microclusters is unknown [4]. An increase in the concentration of antimicrobial active proteins (RBP2 and BST2) and MATN2 and COL6A3 proteins involved in extracellular matrix remodeling [5], was also found in these patients. Long-COVID is accompanied by an increase in ACE levels, and this, according to the authors, may induce the development of an aberrant long-term immune response [6]. In fact, the abundance and diversity of symptoms, a large number of detectable disorders in the functioning of the central nervous system and internal organs create significant difficulties for doctors and researchers in developing the concept of the pathogenesis of post-COVID syndrome [7, 8].

Despite some noteworthy experimental findings and clinical observations presented in the world scientific literature, the overall picture of the pathogenesis of post-COVID does not add up.

Since the pathogenesis of post-COVID is associated with virus-induced damage to the vascular endothelium, which leads to the development of an inflammatory process, we assume that in patients with post -COVID there is a change in the functional activity of the leading inflammatory cells – neutrophils. One of the manifestations of the involvement of these cells in the pathogenesis of inflammatory process is the formation of neutrophil extracellular traps (NETs).

PURPOSE

Study of neutrophil extracellular traps (NETs) in post-COVID patients to clarify the mechanisms of damage.

PATIENTS AND METHODS

Patients

The study included two groups of patients with post-COVID. In the first group (21 patients), acute COVID-19 infection was treated in a hospital setting. In this group, 11 people had a mild acute period of COVID-19, 7 patients had a moderate acute period, and 3 patients had a severe course of COVID-19. The second group (20 patients) received treatment for acute COVID-19 infection on an outpatient clinic. All patients in this group had a mild acute period of the disease. Patients diagnosed with acute appendicitis after surgery were selected as comparison groups (10 patients).

The control group consisted of 20 healthy people without previous COVID-19 infection.

Methods

Determination of the content of neutrophil extracellular traps

Method for obtaining cell fractions

Venous blood samples from patients and healthy people were collected using a vacutainer with EDTA to prevent clotting. Neutrophil isolation from the blood was performed by gradient centrifugation according to the generally accepted method. The blood was diluted 2 times with a sodium-phosphate buffer solution, pH 7.4, and layered on a double density gradient of Ficoll-Verografin solutions 1.077/1.190 g/cm3. After centrifugation (1600 rpm, 30 min), a ring of granulocytes with a purity of 98-100% appears at the boundary between the gradients. A ring of neutrophilic granulocytes was taken from the interphase ring, transferred to centrifugation tubes, and washed twice from ficoll impurities with a buffer solution using centrifugation for cell deposition (1200 rpsm, 15 min). Sterile isolated neutrophils were transferred to RPMI-1640 medium and used in culture experiments. The viability of isolated neutrophils was at least 95%, which was determined in a test with 0.1% trypan blue solution.

Fluorescent staining of neutrophil extracellular traps

Fluorescence microscopy was used to detect and count NETs. The method was described in detail earlier [9]. The results were expressed as a percentage as the ratio of the number of NETs to the total number of neutrophils. The fluorescent dye SYBR Green (Evrogen) specifically interacting with double-stranded DNA was used to detect NETs. Counting and photoregistration of cells and extracellular structures were performed at magnification x 1000.

Determination of purine nitrogenous bases

The method is based on the reaction of purine nitrogenous bases with silver nitrate to form a colored compound. Blood plasma of patients and patients from the comparison group was subjected to high-speed centrifugation of 20000g for 30 minutes and stored at -26o° C. Purine nitrogenous bases were extracted from blood plasma using chloroform. For this purpose, 2 ml of chloroform was added to 0.5 ml of blood plasma and processed on a vibrating platform for 1 hour at room temperature. Chloroform (1 ml) with purine nitrogenous bases dissolved in it was taken and the samples were dried in a vacuum evaporator. The dry precipitate containing purine nitrogenous bases was dissolved in 3 ml of a 10% NaOH solution and 500 µl of a 5% AgNO3 solution prepared in a 10% aqueous ammonia solution was added. As a result of the reaction, a light brown color develops. The samples were photometric at a wave length of 610 nm. Adenine (Sigma) was used to construct the calibration curve. The calibration graph was linear in the range of 0-10 mg / ml.

Statistical processing

The obtained results were processed in the Statistica 12.0 program (StatSoft, Inc.). The data are presented as the mean value (M)±standard error of the mean (m). Quantitative features were compared using the Mann-Whitney rank U test

Journal of Immunology (ISSN 2995-861X)

and Kruskal-Wallis analysis of variance. The differences were considered statistically significant at $p<0.05$.

RESULT

The results of a comparative study of clinical symptoms that form post-COVID and a laboratory indicator that reflects the state of innate immunity - the average number of neutrophil extracellular traps in the blood, in the two studied groups of patients with post-COVID are shown in Table 1. Patients of the second group demonstrate a tendency to increase the average number of symptoms per patient (3.81±0.72) compared with the same indicator in patients of the first group who were treated in a hospital (2.40±1.10).

Patients of these two groups differed statistically significantly in the number of NETs. In patients of the first group, the number of NETs did not exceed 1% (0.86±0.51%), and in patients of the second group, the number of NETs in the blood was recorded 7.6 times higher (6.55±0.94%). A likely explanation for this discrepancy in results is the different treatment given to patients in the acute period of COVID-19 infection.

More effective suppression of the pathological process was observed in patients who received treatment in a hospital setting than in outpatient clinic.

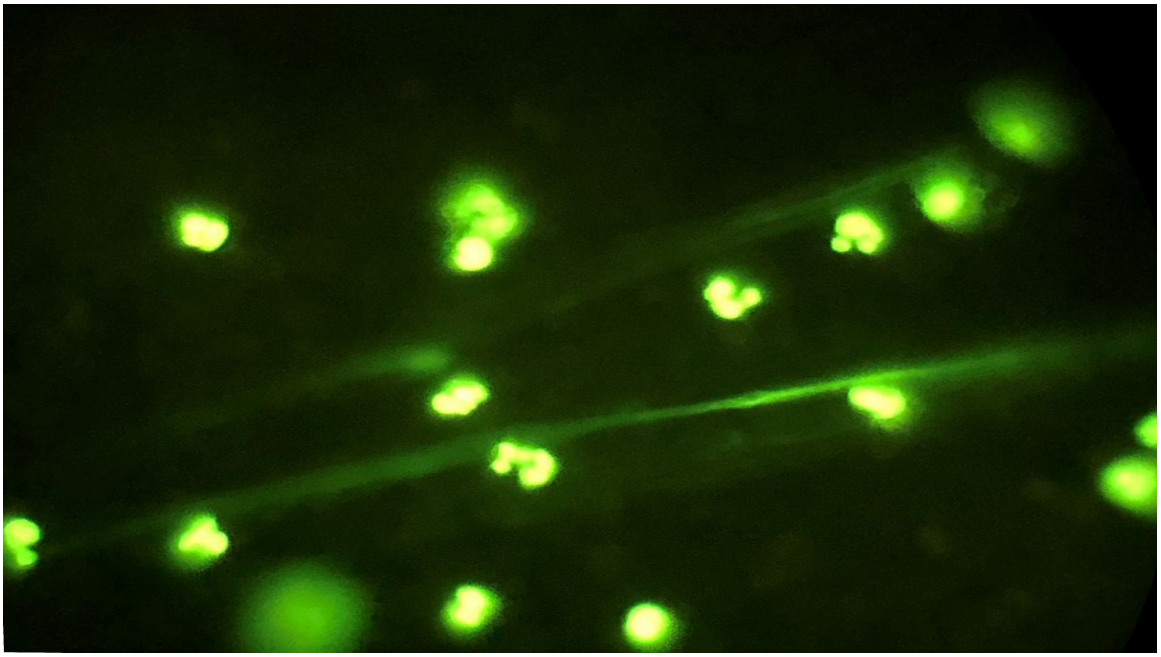
In our studies, we found that the characteristic feature of post-COVID for these groups of patients is NETs in the morphological form of single strands (Fig. 1).

Table 1. Comparison of the number of symptoms and the number of neutrophil extracellular traps (NETs) in the blood

Some characteristics of post-COVID syndrome	**Patients with post-COVID syndrome**	
	Acute period of the disease of varying severity (patients treated in hospital) (n=21)	**Acute period of the disease in a mild form (patients treated in outpatient clinic) (n=20)**
Number of symptoms in one patient in the post-COVID period	2.40±1.10	3.81±0.72
Neutrophil extracellular traps, %	0.86±0.51	6.55±0.94*

Note: * - $p<0.05$ compared to the control group (according to the Kruskal-Wallis analysis of variance).

Figure 1



Journal of Immunology (ISSN 2995-861X)

Figure 1: Neutrophil extracellular traps in the form of single strands of DNA in postcovoid syndrome. Incubation time is 1 h. Coloring with CYBR Green. Magnification x1000.

The formation of neutrophil extracellular traps in the form of single strands of DNA, which we previously identified in post-COVID [10, 11], differs sharply from the visualization of extracellular structures of neutrophils obtained during the study of infectious inflammatory processes in the abdominal cavity. An uncomplicated inflammatory process in the abdominal region (appendicitis) is always accompanied by the formation of neutrophil extracellular traps only in the morphological form of neutrophil networks (Fig. 2).

It is obvious that such a significant discrepancy in the morphological structure of NETs has both its own cause and significant consequences. Without dwelling on all the details of the process, we highlight the main consequence that follows from the filamentous nature of NETs in post-COVID syndrome.

Figure 2 :

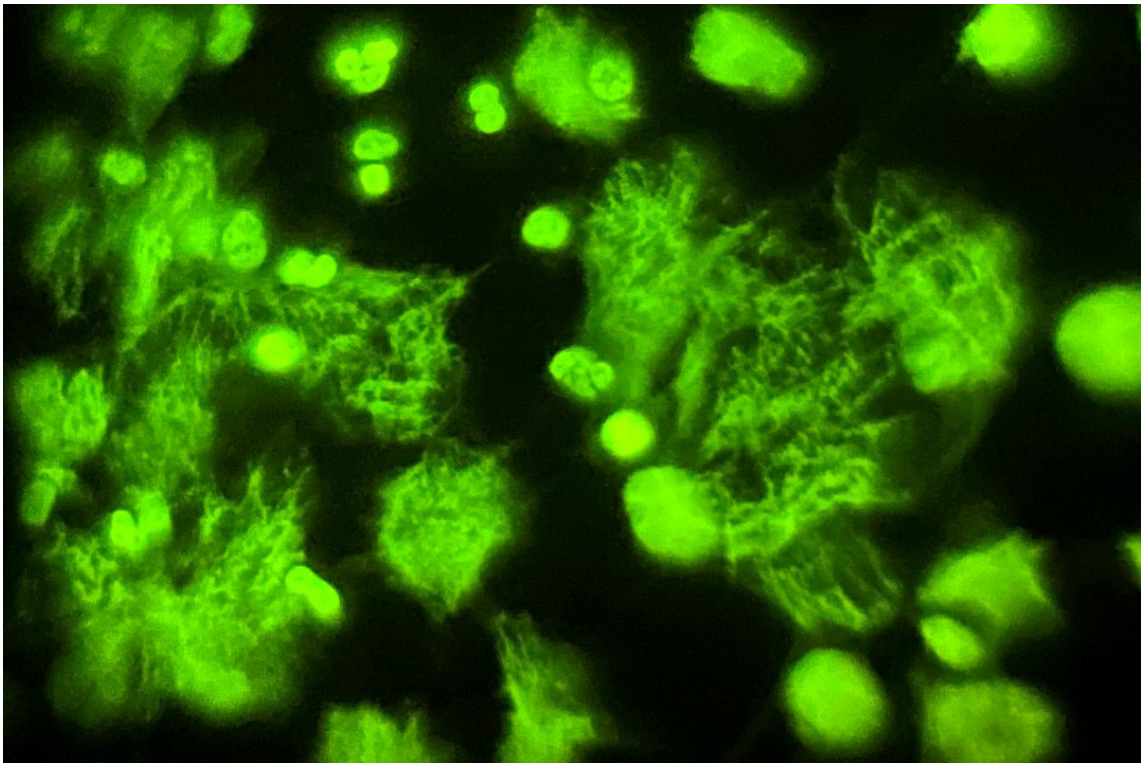


Figure 2 : Neutrophil extracellular traps in the network-like morphological form. Uncomplicated appendicitis after surgery. Incubation time is 1 h. Coloring with CYBR Green. Magnification x1000.

The formation of NETs in the morphological form of single strands of DNA in post-COVID is the reason for a significant increase in the concentration of extracellular purine nitrogenous bases in blood plasma. Spontaneous enzymatic degradation of DNA fibers leads to an increase in the concentration of free nucleotides in the extracellular space first, and then, after they lose phosphate groups and deoxyribose, the nucleotides turn into extracellular purine nitrogenous bases.

In our preliminary studies, we found that the formation of NETs in the morphological form of single strands of DNA occurs after contact interactions of neutrophils from healthy donors with fibroblasts under cell culture conditions.

Our measurements of the concentration of extracellular purine nitrogenous bases in the blood plasma of post-COVID patients showed that this parameter increases significantly. Thus, the range of registered concentrations of purine nitrogenous bases in the blood plasma of patients reaches 7.38-34.19 mg/ml. The peculiarity of post-COVID is a very long (3 months or more) period of registration of NETs in the form of single strands of DNA and, in accordance with this, a long period of increased concentration of extracellular purine nitrogenous bases in the blood plasma.

Acute bacterial inflammation can also lead to an increase in the concentration of extracellular purine nitrogenous bases in the blood plasma. Our research has shown that this does happen, but the increase is extremely small. In a group of patients with acute abdominal inflammation, we found an increase in the concentration of extracellular purine nitrogenous bases in blood

Journal of Immunology (ISSN 2995-861X)

plasma in the range from 0.2 to 1.8 mg/ml. It should be noted that this slight increase was recorded for a short time - only in the acute period of the inflammatory process (within 3-5 days).

The study of this parameter in healthy people shows the complete absence of extracellular purine nitrogenous bases in the blood plasma.

Spontaneous enzymatic degradation of DNA strands of neutrophil extracellular structures, which we have identified in patients with post-COVID syndrome, leads to an increase in the concentration of extracellular purine nitrogenous bases in the blood of sick people. Extracellular purine nitrogenous bases are toxic to the body, since they are capable (due to their high lipophilicity) of attaching to the cell surface, causing their damage, and are also able to inhibit the activity of T-lymphocytes and cause the formation of secondary immunodeficiency.

The results obtained indicate a long-term and persistent increase in the concentration of extracellular purine bases in patients with post-COVID (more than 3 months), and we suggest that they are an endogenous source of damaging effects in the pathogenesis of post-COVID, that is, factors of secondary alteration, which may also explain the multi-symptom nature of the post-COVID [12], especially in people with residual symptoms of a previous infection: drowsiness, joint pain, headache, weakness and hair loss, etc.

The formation of NETs in the morphological form of single strands of DNA in post-COVID is the reason for a significant increase in the concentration of extracellular purine nitrogenous bases in blood plasma. Spontaneous enzymatic degradation of DNA fibers leads to an increase in the concentration of free nucleotides in the extracellular space first, and then, after they lose phosphate groups and deoxyribose, the nucleotides turn into extracellular purine nitrogenous bases.

In our preliminary studies, we found that the formation of NETs in the morphological form of single strands of DNA occurs after contact interactions of neutrophils from healthy donors with fibroblasts under cell culture conditions.

Our measurements of the concentration of extracellular purine nitrogenous bases in the blood plasma of post-COVID patients showed that this parameter increases significantly. Thus, the range of registered concentrations of purine nitrogenous bases in the blood plasma of patients reaches 7.38-34.19 mg/ml. The peculiarity of post-COVID is a very long (3 months or more) period of registration of NETs in the form of single strands of DNA and, in accordance with this, a long period of increased concentration of extracellular purine nitrogenous bases in the blood plasma.

Acute bacterial inflammation can also lead to an increase in the concentration of extracellular purine nitrogenous bases in the blood plasma. Our research has shown that this does happen, but the increase is extremely small. In a group of patients with acute abdominal inflammation, we found an increase in the concentration of extracellular purine nitrogenous bases in blood plasma in the range from 0.2 to 1.8 mg/ml. It should be noted that this slight increase was recorded for a short time - only in the acute period of the inflammatory process (within 3-5 days).

The study of this parameter in healthy people shows the complete absence of extracellular purine nitrogenous bases in the blood plasma.

Spontaneous enzymatic degradation of DNA strands of neutrophil extracellular structures, which we have identified in patients with post-COVID syndrome, leads to an increase in the concentration of extracellular purine nitrogenous bases in the blood of sick people. Extracellular purine nitrogenous bases are toxic to the body, since they are capable (due to their high lipophilicity) of attaching to the cell surface, causing their damage, and are also able to inhibit the activity of T-lymphocytes and cause the formation of secondary immunodeficiency.

The results obtained indicate a long-term and persistent increase in the concentration of extracellular purine bases in patients with post-COVID (more than 3 months), and we suggest that they are an endogenous source of damaging effects in the pathogenesis of post-COVID, that is, factors of secondary alteration, which may also explain the multi-symptom nature of the post-COVID [12], especially in people with residual symptoms of a previous infection: drowsiness, joint pain, headache, weakness and hair loss, etc.

CONCLUSION

The results of this study, combined with data from other studies, allow us to formulate the following concept of post-COVID pathogenesis.

Virus-induced damage to the vascular endothelium leads to activation of contact interactions between neutrophils and fibroblasts.

As a result of this process, neutrophils form extracellular traps in the morphological form of single strands of DNA.

Spontaneous enzymatic degradation of single strands of DNA causes an increase in the concentration of extracellular purine bases, which are factors of secondary alteration of cells of the central nervous system and internal organs.

Secondary alteration factors damage the vascular endothelium and induce the formation of new neutrophil extracellular traps in the form of single strands of DNA.

As a result, a vicious circle is formed, which is induced and maintained by damaged vascular endothelium.

Overcoming the consequences of the COVID in the form of post-COVID can be carried out, including the inclusion

of therapeutic measures for the regeneration of vascular endothelium.

Compliance with the principles of ethics

The study of blood samples was carried out at the Department of Pathophysiology and Clinical Pathophysiology of the Faculty of Medicine of the

Pirogov Russian National Research Medical University of the Ministry of Health of the Russian Federation. All procedures were performed in accordance with the ethical standards of the Helsinki Declaration of the WMA (as amended in 2004) and the written informed consent of patients. The study was approved by the Ethics Committee of the Russian National Research University (Protocol No. 203 dated 12/21/2021).

Authors' participation : the concept and design of the study – Poryadin G.V. Salmasi J.M. Larina V.N.; collection, processing of material and staging of experiments - Stodelova E.A., Kazimirskii A.N.; preparation of illustrative material, writing of text – Kazimirskii A.N.; statistical processing of material – Panina M.I.; editing – Panina M.I., Larina V.N.

Approval of the final version of the article, responsibility for the integrity of all parts of the article – all co-authors.

Conflict of interest : The authors declare that there is no conflict of interest.

Financing : The study had no sponsorship.

REFERENCES

1. Martins-Gonçalves R., Hottz E.D., Bozza P.T. Acute to post-acute COVID-19 thromboinflammation persistence: Mechanisms and potential consequences. Curr Res Immunol. 2023; 4: 100058. doi: 10.1016/j.crimmu.2023.100058.

2. Patell R., Bogue T., Koshy A., et al. Postdischarge thrombosis and hemorrhage in patients with COVID-19. Blood 2020; 136 (11): 1342–1346. doi: https://doi.org/10.1182/blood. 2020007938

3. Xie, Y., Xu, E., Bowe, B., et al. Long-term cardiovascular outcomes of COVID-19. Nat Med 28, 583–590 (2022). https://doi. org/10.1038/s41591-022-01689-3.

4. Pretorius E, Vlok M, Venter C, Bezuidenhout JA, Laubscher GJ, Steenkamp J, Kell DB. Persistent clotting protein pathology in Long COVID/Post-Acute Sequelae of COVID-19 (PASC) is accompanied by increased levels of antiplasmin. Cardiovasc Diabetol. 2021 Aug 23;20(1):172. doi: 10.1186/s12933-021-01359-7. PMID: 34425843; PMCID: PMC8381139.

5. Kubiak JZ, Kloc M. Coronavirus Disease Pathophysiology: Biomarkers, Potential New Remedies, Comorbidities, Long COVID-19, Post Pandemic Epidemiological Surveillance. Int J Mol Sci. 2023;24(15):12236. Published 2023 Jul 31. doi:10.3390/ijms241512236 https://www.mdpi.com/1422-0067/24/15/12236

6. Mitchell PD, Buckley C, Subramaniam A, Crowther S, Donnelly SC. Elevated serum ACE levels in patients with post-acute COVID-19 syndrome. QJM. 2022;115(10):651-652. doi:10.1093/qjmed/hcac119

7. Zawilska JB, Kuczyńska K. Psychiatric and neurological complications of long COVID. J Psychiatr Res. 2022;156:349-360. doi:10.1016/j.jpsychires.2022.10.045

8. Parotto M, Gyöngyösi M, Howe K, et al. Post-acute sequelae of COVID-19: understanding and addressing the burden of multisystem manifestations. Lancet Respir Med. 2023;11(8):739-754. doi:10.1016/S2213-2600(23)00239-4

9. Kazimirskii AN, Salmasi JM, Poryadin G .V., Panina M.I . New opportunities for diagnosis and investigation of the pathogenesis of various types of inflammation. Patologicheskaya Fiziologiya i Eksperimental`nayaterapiya. (Pathological Physiology and Experimental Therapy, Russian Journal). 2022; 66(2): 34-42 . (in Russian) . DOI: 10 .25557/0031-2991 .2022 .02 .34-42

10. Salmasi JM, Poryadin GV, Panina MI, Larina VN, Ryzhikh AA, Stodelova EA, et al. Neutrophil and monocyte extracellular traps in the diagnosis of post-COVID syndrome. Bulletin of RSMU. 2022; (6): 80–4. DOI: 10.24075/brsmu.2022.057

11. Kazimirskii AN, Salmasi J.M., Poryadin G.V., Panina M.I., Larina V.N., Ryzhikh A.A. Post-COVID syndrome is associated with increased extracellular purine bases and neutrophil extracellular traps in the blood plasma. Bulletin of Siberian Medicine. 2022;21(2):41-47. https://doi.org/10.20538/1682-0363-2022-2-41-47

12. Larina VN, Stodelova E.A., Salmasi Zh.M. et al. The Role of the Innate Immune System in the Development of Postcovid Syndrome and its Complications. The Russian Archives of Internal Medicine. 2023; 13(4): 263-271. (in Russian) DOI: 10.20514/2226-6704-2023-13-4-263-271. EDN: DUUQGV