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Post-COVID syndrome is associated with increased extracellular purine bases and neutrophil extracellular traps in the blood plasma

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ABSTRACT

Post-COVID syndrome is characterized by fatigue, reduced exercise tolerance, muscle and joint pain, and psycho-emotional disorders. In the development of a generalized body response in a viral infection, abnormal defense responses are of great importance. We studied neutrophils, neutrophil extracellular traps (NETs), DNA degradation products (purine nitrogenous bases, PNBs), and traditional biochemical parameters.

Aim. To determine biochemical parameters and the number of NETs and PNBs in the peripheral blood of patients with post-COVID syndrome.

Materials and methods. The study included outpatients ($n = 21$) aged 18–59 years (36 [27 ÷ 50]). The control group consisted of 20 individuals aged 18–59 years (38.5 [29 ÷ 51.5]) without a past medical history of the coronavirus infection. All patients underwent a physical examination, their medical history was assessed, and the level of NETs and PNBs in the venous blood was determined.

Results. 11 patients had a mild form of the disease in their past medical history, 7 – moderate, and 3 – severe. The most common symptoms in the patients were fatigue, headache, epigastric pain, dizziness, and joint pain. Hair loss and dyspnea were less common. The concentration of NETs and PNBs was higher in the patients with post-COVID syndrome than in the control group ($p < 0.05$). We detected NETs in the patients with post-COVID syndrome only in the form of filamentous structures. The concentration of extracellular purine bases in the blood of the patients with post-COVID syndrome was the highest in patients with moderate and severe acute periods. In patients with a mild acute period, the concentration of PNBs was 7.38 [0.0 ÷ 60.7] mg / ml, and in patients with moderate and severe acute periods – 19.15 [0.0 ÷ 33.5] and 34.19 [3.35 ÷ 70.0] mg / ml, respectively.

Conclusion. Extracellular purine bases in concentrations capable of causing secondary alteration of cells are found in the peripheral blood of patients with post-COVID syndrome. Post-COVID syndrome is accompanied by the formation of filamentous NETs in the blood of patients.

Keywords: post-COVID syndrome, extracellular purine bases, neutrophil extracellular traps, COVID-19, pathogenesis

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Постковидный синдром ассоциирован с повышением внеклеточных пуриновых оснований и нейтрофильных экстраклеточных ловушек в плазме крови

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РЕЗЮМЕ

Постковидный синдром характеризуется высокой утомляемостью, снижением толерантности к физической нагрузке, болями в мышцах и суставах, наличием психоэмоциональных проблем. В развитии генерализованной реакции организма при вирусном инфицировании большое значение имеют аномальные реакции защитных систем. Мы исследовали нейтрофилы и формируемые ими экстраклеточные ловушки (НЭЛ) совместно с продуктами деградации волокон ДНК (пуриновые азотистые основания, ПАО), а также традиционные клинико-лабораторные показатели.

Цель. Определение ряда лабораторных показателей, а также количества НЭЛ и уровня ПАО в периферической крови больных с постковидным синдромом.

Материалы и методы. В исследование включены амбулаторные пациенты ($n = 21$) в возрасте 18–59 лет (36 [27÷50]). Группу сравнения составили 20 лиц в возрасте 18–59 лет (38,5 [29÷51,5]) без перенесенной коронавирусной инфекции. Всем пациентам проводились сбор жалоб, оценка анамнеза, физикальный осмотр, определение НЭЛ и ПАО в венозной крови.

Результаты. Легкое течение заболевания в анамнезе имелось у 11, среднетяжелое – у 7, тяжелое – у 3 пациентов. Наиболее частыми симптомами в нашей группе обследованных пациентов были слабость, головная боль, боль в эпигастрии, головокружение, боль в суставах. Более редкими симптомами являлись выпадение волос и одышка. Концентрация НЭЛ и ПАО была выше в основной группе, чем в группе сравнения ($p < 0,05$). Мы выявляли НЭЛ у больных с постковидным синдромом только в нитевидной форме. Концентрация внеклеточных пуриновых азотистых оснований в плазме крови больных с постковидным синдромом была наиболее высокой у больных со среднетяжелым и тяжелым течением острого периода. У больных, перенесших острый период заболевания в легкой форме, концентрация ПАО составляет 7,38 [0,0÷60,7] мг/мл, а у больных со среднетяжелой и тяжелой формой острого периода – 19,15 [0,0÷33,5] и 34,19 [3,35÷70,0] мг/мл соответственно.

Заключение. В периферической крови больных с посткоронавирусным синдромом обнаруживаются внеклеточные ПАО в концентрации, способной вызвать вторичную альтерацию клеток. Постковидный синдром сопровождался формированием в периферической крови больных НЭЛ в нитевидной форме.

Ключевые слова: постковидный синдром, внеклеточные пуриновые азотистые основания, нейтрофильные экстраклеточные ловушки, ковид-19, патогенез

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

Post-COVID syndrome is a complex of symptoms that occurs in 20% of people who have had the coronavirus infection (COVID-19). Patients with post-COVID syndrome suffer from headaches, asthenia, arthralgia, myalgia, consequences of organ dysfunction (lungs, heart, gastrointestinal tract (GIT), skin, kidneys, liver etc.), and neuropsychiatric disorders. Pathological manifestations of post-COVID syndrome persist for three or more months after the coronavirus infection. The syndrome is diagnosed clinically based on a past infectious medical history. Some researchers differentiate the concepts of “post-COVID” and “long COVID”, believing that post-COVID is a complication of the cured COVID-19, while long COVID is chronic persistence of the virus in the body. The pathogenesis of post-COVID syndrome is unclear and poorly understood. The etiology of post-COVID syndrome is directly related to the past coronavirus infection. It is assumed that residual inflammation, prolonged persistence of SARS-CoV-2 in latent foci, the formation of autoantibodies, and even social isolation can be significant in the pathogenesis of post-COVID syndrome.

At the same time, there are some reasons to believe that the manifestations of the post-COVID syndrome are associated with an increased concentration of extracellular purine nitrogenous bases (PNBs) and formation of neutrophil extracellular traps (NETs), leading to the development of immune thrombotic disorders.

The aim of the study was to compare clinical and laboratory parameters and the number of NETs and extracellular PNBs in the peripheral blood of patients with post-COVID syndrome, depending on the severity of the coronavirus infection.

MATERIALS AND METHODS

An open, comparative study included 21 patients aged 18 to 59 years (36 [27 ÷ 50]). Inclusion criteria: men and women aged 18–59 years with the confirmed diagnosis of COVID-19 in their past medical history; severe, moderate or mild forms of the coronavirus infection; not earlier than 1 month but not later than 12 weeks (3 months) from the onset of the first symptoms of COVID-19. Exclusion criteria: age over 60 years old; extremely severe course of COVID-19; the presence of chronic diseases incompatible with life or life expectancy of less than a year; asymptomatic carriers of the coronavirus infection (or those who contacted with COVID-19 patients but did not get ill).

The control group consisted of 20 individuals aged 18–59 years (38.5 [29 ÷ 51.5]) without a past medical history of the coronavirus infection. These patients went to the physician of the outpatient clinic for a regular check-up.

All patients underwent a physical examination, their medical history was taken, and anamnestic data were assessed. A past medical history of COVID-19 was confirmed by a positive test to detect SARS-CoV-2 RNA using nucleic acid amplification techniques or SARS-CoV-2 antigen using immunochromatography regardless of clinical manifestations or after patients with clinically confirmed COVID-19 were tested positive for immunoglobulin (Ig) A, IgM, and/or IgG antibodies. Standard criteria were used to assess the severity of disease manifestations.

A mild form of the infection was characterized by a body temperature lower than 38°C, cough, weakness, and sore throat. A moderate form of infection was characterized by a body temperature of 38°C or higher, respiratory rate higher than 22 breaths / min, dyspnea on exertion, pulmonary changes on chest computed tomography (CT) or X-ray typical of a viral infection (the volume of the lesion is minimal or moderate; stage 1–2 according to CT findings), oxygen saturation (SpO₂) of less than 95%, and an increase in the level of C-reactive protein above 10 mg / l. A severe form of infection was characterized by respiratory rate higher than 30 breaths / min, SpO₂ ≤ 93%, PaO₂ / FiO₂ ≤ 300 mmHg, and hemodynamic instability (systolic blood pressure lower than 90 mmHg or diastolic blood pressure lower than 60 mmHg, diuresis less than 20 ml / hour). The volume of lung damage is significant or subtotal (stage 3–4 according to CT findings). Data on the disease severity were obtained from patients' medical histories, epicrisises, and health records.

To determine the blood biochemical parameters of COVID-19 patients and patients of the control group, an automatic clinical chemistry analyzer Olympus 5800 (JP, Olympus Corporation, USA) was used. Standard methods using manufacturer's reagents were employed to carry out tests in the laboratory of the City Government-Funded Healthcare Institution “Diagnostic Clinical Center No. 1 of the Moscow City Health Department”. The study was approved by the protocol No. 203 at a meeting of the Ethics Committee at Pirogov Russian National Research Medical University on December 21, 2021. Each patient signed an informed consent to participate in the study. The study was conducted at the City Government-Funded Healthcare Institution “Diagnostic Clinical Center No.

1 of the Moscow City Health Department". The Department of Polyclinic Therapy and the Department of Pathophysiology and Clinical Pathophysiology of Pirogov Russian National Research Medical University were used as the clinical site.

In addition to standard clinical laboratory studies, two new laboratory methods were used in the study: determination of the NET concentration and the PNB level in the blood plasma.

Determination of the number of neutrophil extracellular traps

Cell fractionation. Cell fractions of neutrophils were used in the study. Venous blood (10 ml) of patients was placed in a siliconized tube containing EDTA to prevent clotting. To isolate neutrophils from EDTA-treated venous blood, the blood was diluted two-fold with sodium phosphate buffer solution (pH 7.4) and layered on a double Ficoll – Verografin density gradient. The density of the gradient upper layer was 1.077, the density of the bottom layer was 1.190. Centrifugation (1,600 rpm, 30 min) resulted in a ring of granulocytes with purity of 98–100% at the boundary between the gradients. Erythrocytes were deposited on the bottom of the tube. The ring of neutrophil granulocytes was collected, placed into centrifuge tubes, and washed twice with a buffer solution to remove Ficoll. Centrifugation was used to pellet the cells (1,200 rpm, 15 min). Sterilely isolated neutrophils were transferred to the RPMI-1640 medium and then used in cell culture experiments. The viability of the isolated neutrophils was at least 95%, which was determined in the test with 0.1% Trypan Blue solution.

Immunofluorescent staining of neutrophil extracellular traps. Fluorescence microscopy was used to detect and quantify NETs. The technique is proprietary and is described in detail in the RF Patent Application No. 2021104936. The results were expressed in percentage as the ratio of the number of NETs to the total number of neutrophils. The fluorescent dye Syber Green (Evrogen, Russian Federation) which specifically binds to double-stranded DNA was used to detect NETs.

Detection of purine nitrogenous bases

The method is based on the interaction between PNBs and silver nitrate to form a colored compound. The blood plasma was subjected to high-speed centrifugation at 20,000 rpm for 30 min and stored at –26°C. Chloroform was used to extract PNBs from the blood plasma. To do this, 2 ml of chloroform was added to

0.5 ml of the blood plasma, and this mixture was processed on a vibration platform at room temperature for 1 hour. Chloroform (1 ml) with PNBs dissolved in it was collected, and the samples were dried in a vacuum evaporator. The dry precipitate containing PNBs was dissolved in 3 ml of 10% NaOH solution, and 500 µl of 5% AgNO₃ solution prepared in 10% aqueous ammonia was added. The reaction resulted in a light brown staining. The samples were photometered at a wavelength of 610 nm. Adenine (Sigma, USA) was used to construct a calibration curve. The calibration curve was linear in the range of 0–10 mg / ml.

Statistical processing of the data was performed using STATISTICA 12.0 (StatSoft). Descriptive statistics were presented as continuous quantitative data: as the mean and the standard error of the mean ($M \pm m$) in normal distribution and as the median and the interquartile range ($Me [25\div 75]$) in non-normal distribution. Comparison of quantitative variables was performed using the Mann – Whitney U test and Kruskal – Wallis one-way analysis of variance. The difference was considered statistically significant at $p < 0.05$.

RESULTS

Weakness, headache, epigastric pain, dizziness, and joint pain were the most common symptoms in patients with post-COVID syndrome. Hair loss and dyspnea were less common. A similar study named weakness, myalgia, headaches, and vegetative symptoms as the most common symptoms in patients with post-COVID syndrome [1]. The authors suggest that these symptoms are more typical of post-COVID syndrome, that they do not require specific treatment and may be associated with microangiopathy and endothelial dysfunction.

The group of patients with post-COVID syndrome demonstrated the activity of alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGT), and alkaline phosphatase (ALP), increased by 1.7, 2.1, and 3.7 times, respectively. These changes indicate the development of hepatotoxicity, but do not allow for a complete understanding of the post-COVID syndrome pathogenesis.

The most significant changes in patients with post-COVID syndrome were detected when studying NETs and PNBs levels in the blood plasma.

The study of NETs in patients with post-COVID syndrome shows that they are formed as thin single filaments of considerable length. The dimensions of DNA strands exceed several tens of cell diameters.

The peculiarity of NETs in patients with post-COVID syndrome is that DNA fibers do not form a network structure and are not capable of capturing with subsequent retraction of apoptotic cells (Fig. 1–3).

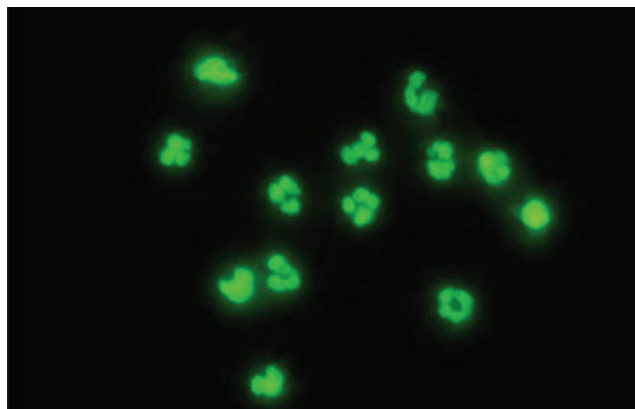


Fig. 1. Intact neutrophils of healthy donors

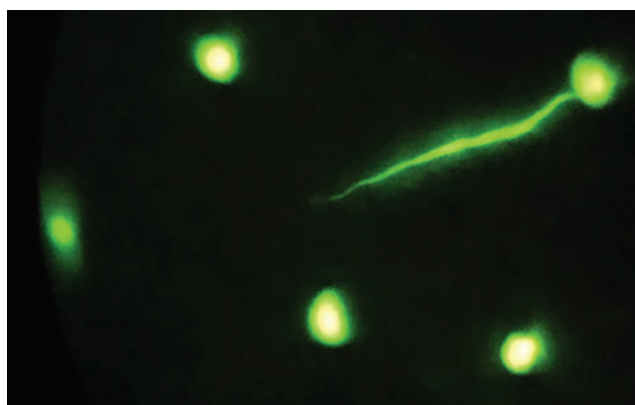


Fig. 2. Initial stages of NET formation in post-COVID syndrome. Ejection of a single strand of nuclear DNA from the cell nucleus; neutrophil incubation is 30 min

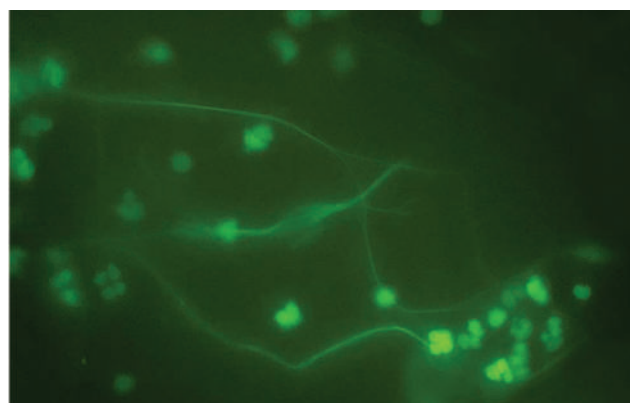


Fig. 3. NETs in post-COVID syndrome; neutrophil incubation is 4 hours

In our previous studies, we found that the morphological structure of NETs depends on the type of inflammation. The formation of NETs in the form of single strands of nuclear DNA (Fig. 2, 3) indicates sterile inflammation in patients.

The number of NETs in patients with post-COVID syndrome is insignificant, but they are constantly reproduced over a long period of time (3 months and longer). The results of determining the number of NETs in patients with post-COVID syndrome, depending on the severity of the disease, are presented in the table.

Our studies showed that the number of NETs in the post-COVID period was on average 2.6 times greater in patients who had a moderate form of the coronavirus infection, compared with patients with a mild form of the disease. At the same time, NETs were not detected at all in patients with a severe form of the disease in the past (Table).

Table

Neutrophil extracellular traps and extracellular purine nitrogenous bases in patients with post-COVID syndrome who have experienced an acute period of the disease in mild, moderate, and severe forms, *Me* [25÷75], *M* ± *m*

Parameter	Control group, <i>n</i> = 20	Patients with post-COVID syndrome, <i>n</i> = 21		
		Mild form, <i>n</i> = 11	Moderate form, <i>n</i> = 7	Severe form, <i>n</i> = 3
NETs, %	0.00	0.00 [0.00÷1.31]* 0.61 ± 0.23	0.00 [0.00÷0.56]* 1.61 ± 1.52	0.00
Extracellular PNBs, mg / ml	0.00	7.38 [0.0÷60.7]* 23.27 ± 8.9	19.15 [0.0÷33.5]* 22.89 ± 8.36	34.19 [3.35÷70.0]* 35.84 ± 19.25

* $p < 0.05$ compared with the control group (according to the Kruskal – Wallis analysis of variance).

We suggest that the formation of filamentous NETs in patients with post-COVID syndrome is one of the reasons for a significant increase in the concentration of PNBs in the blood. Thin strands of DNA are destroyed by the effect of DNases localized on chromatin. An increase in the activity of nuclear DNases

occurs during chromatin despiralization, which is observed in the formation of NET networks. The interaction of DNases with decondensed chromatin leads to the accumulation of nucleotides in the intercellular space. Ectoenzymes CD39 and CD73 localized on the surface of blood cells and endotheliocytes catalyze

hydrolysis of nucleotides to nitrogenous bases and can cause an increase in the concentration of PNBs. Tests studying the blood serum of patients with COVID-19 showed increased levels of extracellular DNA and specific NET markers [2].

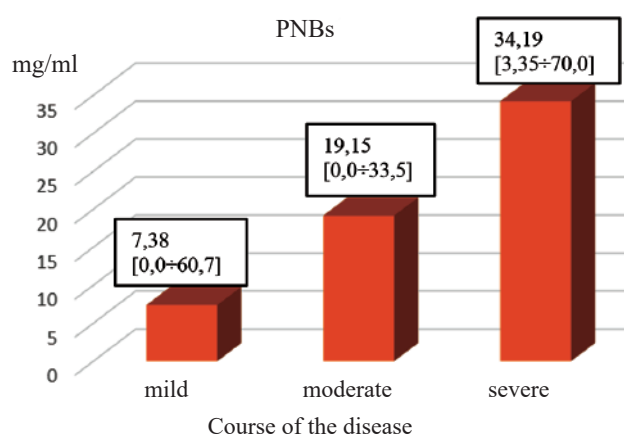


Fig. 4. Extracellular purine nitrogenous bases (PNBs) in patients with post-COVID syndrome who have had the disease in mild, moderate, and severe forms, mg / ml, *Me* [25÷75]

The data obtained show that the activity of enzymes catalyzing the hydrolysis of extracellular nucleotides to free nitrogenous bases is increased on the surface of patients' blood cells [3]. The results demonstrate that the concentration of extracellular PNBs in the blood of patients with post-COVID syndrome was elevated in proportion to the severity of the disease (Table, Fig. 4).

DISCUSSION

Molecular inducers of NET formation are currently unknown. However, it has been established that this process is accompanied and possibly initiated by the components of pathogenic microorganisms, activated platelets, complement system proteins, autoantibodies, and proinflammatory cytokines. NETs take part in thrombosis and occlusion of small vessels through activation of the contact pathway of blood coagulation, electrostatic interactions between histone proteins and platelet phospholipids, destruction of antithrombin III by neutrophil elastases, and induction of interleukin (IL) 1 β synthesis. Activation of IL1 β and platelets leads to an increase in the NET formation, and an increase in the concentration of its components (extracellular DNA, myeloperoxidase (MPO) – DNA complex, citrullinated histone H3) in the blood serum of COVID-19 patients correlates with the severity of

the disease and the development of thrombotic disorders [2,4,5].

In the present study, we detected NETs in patients with post-COVID syndrome only in the form of filamentous structures. No other morphological variants of NETs were found in the examined patients. Moreover, NETs were absent in patients with a severe course of the disease. At the same time, the PNB concentration in patients with post-COVID syndrome was increased in proportion to the severity of the disease. Patients who had had a severe form of the coronavirus infection did not have NETs in the post-COVID period, but we assume that filamentous networks of DNA fibers are produced by other blood cells. Therefore, an increase in the PNB concentration in patients with post-COVID syndrome in proportion to an increase in the disease severity seems to be quite logical and uncontroversial.

Extracellular PNBs are toxic to the body, but the mechanism of their damaging effect is unknown. Animal models with chronic adenine diet showed rapid progression of kidney disease with extensive tubulointerstitial fibrosis, tubular atrophy, crystallization, pronounced vascular calcification, and cardiovascular disorders [6–9].

Myocardial ischemia significantly increases the activity of CD39 and CD73 ectoenzymes localized on the surface of platelets, catalyzing extracellular hydrolysis of adeny nucleotides to adenine, in patients after myocardial infarction. As a result, the levels of extracellular adenine and troponin increase in such patients [10].

We observed an increase in the concentration of extracellular PNBs in patients with post-COVID syndrome (more than 3 months) and suggest that they are an endogenous damaging factor in the pathogenesis of the post-COVID period (secondary alterations), especially in patients with drowsiness, joint pain, headache, weakness, and hair loss. We believe that asthenic syndrome, which persisted in more than half of patients in the post-COVID period, is based on persistent and prolonged increased production of extracellular PNBs, which act as a damaging factor. The results of our study make it relevant to clarify the causes of a significant long-term increase in the concentration of extracellular PNBs in post-COVID syndrome.

CONCLUSION

Extracellular PNBs found in the peripheral blood of patients with post-COVID syndrome can cause secondary cell alterations.

Post-COVID syndrome is accompanied by the formation of filamentous NETs in the peripheral blood.

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Authors contribution

Poryadin G.V., Larina V.N. – conception and design. Ryzhikh A.A. – collection and processing of the material. Kazimirskii A.N. – illustrations, drafting of the article. Panina M.I. – statistical processing of the material. Salmasi J.M. – editing of the article.

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