ISSN 2304-3415, Russian Open Medical Journal
2022. Volume 11. Issue 2 (June). Article CID e0206
DOI: 10.15275/rusomj.2022.0206

Original article

Features of immune status in COVID-19 convalescents

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Received 3 January 2021, Revised 30 March 2022, Accepted 18 April 2022

Abstract: *Study objective* — assessment of the humoral and cell-mediated immunity features in COVID-19 convalescents three months after their discharge from the hospital.
Material and Methods — The study involved 78 COVID-19 convalescents who, depending on the profile of specific IgM and IgG antibodies to SARS-CoV-2, were divided into three groups. The control group consisted of 50 volunteers. Detection of IgM and IgG in blood serum was performed by ELISA. Determination of CRP concentration was conducted using the immunoturbidimetric assay. To determine the levels of IL-6, a sandwich version of the solid-phase ELISA was employed. Immunophenotyping of lymphocytes was performed via flow cytometry.
Results — Of 78 COVID-19 convalescents three months after their discharge from the hospital, 30.8% of them had a profile of specific antibodies IgM(+)IgG(+), 37.2% had IgM(-)IgG(+), and 32.0% were characterized by IgM(-)IgG (-). COVID-19 convalescents with an IgM(-)IgG(-) profile had the highest levels of NK cells, T helper cells, B lymphocytes ($p<0.001$) and were characterized by hyperproduction of proinflammatory IL-6 ($p<0.001$). COVID-19 convalescents with an IgM(+)IgG(+) specific antibody profile were characterized by the highest levels of cytotoxic T lymphocytes ($p<0.001$). In a COVID-19 convalescent with an IgM(-)IgG(+) specific antibody profile, we observed an increase in the number of lymphocytes expressing late activation/apoptosis molecules ($p<0.001$).
Conclusion — The collected data is of potential importance in clinical practice for developing a prognosis for epidemiological situation development, as well as for planning preventive measures to COVID-19.

Keywords: COVID-19, specific antibodies, immune status.

Cite as Shkaberda AS, Kordovskaya EA, Bakuta DYu, Plotnichenko LI, Gorbunov AA, Sorokina LE. Features of immune status in COVID-19 convalescents. *Russian Open Medical Journal* 2022; 11: e0206.

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Introduction

SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) is a new coronavirus strain identified at the end of 2019. It causes a dangerous infectious disease – coronavirus disease 2019 (COVID-19). Starting with a single case of the disease at a seafood market in Wuhan (China), the infection has rapidly spread throughout the world, covering virtually all countries. Following the global spread of SARS-CoV-2, World Health Organization (WHO) declared COVID-19 a public health emergency of international concern [1].

Worldwide studies contributed to understanding the clinic, diagnosis and treatment of patients with COVID-19 [2, 3]. However, the pathogenesis of SARS-CoV-2 infection was not fully clarified yet. Published data suggest that both humoral immunity and cell-mediated immunity are involved in the pathogenesis of COVID-19 [4]. For instance, according to Sethuraman N. et al. [5], specific antibodies of various classes appear 1-2 weeks after the onset of clinical symptoms in the majority of SARS-CoV-2 confirmed cases. In a study by Long Q.X. et al [6], seroconversion was found in 40-50% of patients with COVID-19 by days 5-7, and in all patients after days 17-19 from the onset of symptoms. However, by day 27, seroconversion was accompanied by twofold – fourfold increase of the antibody titer.

Simultaneously with the antibody response, cell-mediated immunity to SARS-CoV-2 is actively developed as well. Within 2-4 weeks after infection, a pool of virus-specific T lymphocytes is formed [7,8]. It is assumed that CD4 and CD8 T lymphocytes are capable of providing reliable immunity to SARS-CoV-2 reinfection for people without detectable antibodies.

However, it is worth noting that available published sources mainly discuss the features of seroconversion in patients with COVID-19 in its acute phase. A very few publications consider the specifics of the immune response to SARS-CoV-2 during the convalescence period [9,10], which is of utmost importance in terms of scientific knowledge and practical application. Moreover, the results of practice-oriented studies are fragmentary and thereby insufficient for unambiguous conclusions, which is probably due to the differences in sampling procedures and methodological approaches to designing the research.

The objective of our study was to assess the features of humoral and cell-mediated immunity in COVID-19 convalescents three months after their discharge from the hospital.

Material and Methods

Research design

A cross-sectional comparative study involved 78 COVID-19 convalescents: 56 women (71.4%) and 22 men (31.3%), with a mean age of 51.8±12.6 years. All examined subjects, depending on their profiles of specific IgM and IgG antibodies to SARS-CoV-2, were divided into three groups, similar in terms of their gender and age composition, severity of COVID-19, and comorbid somatic pathology: IgM (+)IgG(+) group (n=24), IgM(-)IgG(+) group (n=29), and IgM(-)IgG(-) group (n=25).

The control group consisted of 50 volunteers: 36 women (72.0%) and 14 men (28.0%), with a mean age of 52.1±11.9 years.

The study was performed in compliance with Good Clinical Practice standards and the requirements of the World Medical Association Declaration of Helsinki. The ongoing studies were approved by the Ethics Committee at S.I. Georgievsky Medical Academy of V.I. Vernadsky Crimean Federal University. Written informed consent was obtained from all participants prior to their inclusion in the study.

Eligibility criteria

The *inclusion criteria* for the study were ages within the range of 18 to 70 years, established diagnosis of COVID-19 in anamnesis, discharge from the hospital three months prior to the study, two consecutive negative PCR tests for COVID-19, and signed written informed consent to participate in the study.

The *exclusion criteria* for the study were discharge from the hospital over three months or less than three months prior to the study, a positive PCR test result for COVID-19, a history of infectious diseases and/or surgical interventions over preceding three months, immunodeficiency disorders, oncological diseases, pregnancy and lactation, and patient refusal to participate in the study.

The *criteria for inclusion in the control group* were as follows: volunteers similar to COVID-19 convalescents in terms of their age and gender; no history of COVID-19, oncological and immune deficiency diseases, allergic and inflammatory conditions at the time of the study; and written informed consent to participate in the study.

Patient examination methods

Detection of specific IgM and IgG antibodies to SARS-CoV-2 in blood serum was conducted via enzyme-linked immunosorbent assay (ELISA) using the following reagent kits: 'DS-ELISA-Anti-SARS-CoV-2-M' and 'DS-ELISA-Anti-SARS-CoV-2-IG' (*Diagnostic Systems* Scientific Production Association, Nizhny Novgorod, Russia).

Immunophenotyping of lymphocytes was performed on the Particle Analysing System PAS-III instrument by flow cytometry using dual-labeled monoclonal antibodies: CD3+CD19–, CD3+CD4+, CD3+CD8+, CD3-CD16+CD56+, CD3–CD19+, CD3+CD95+, CD3+CDHLA-DR+ (Becton Dickinson, USA); CD3-FITC/CD95-PE, CD3-FITC/HLA-DR-PE (Beckman Coulter, France).

Determination of the C-reactive protein (CRP) concentration in blood serum was carried out via the immunoturbidimetric assay on a biochemical analyzer AU 480 (Beckman Coulter, USA) using the original reagent. To identify the levels of IL-6 in blood serum, a sandwich version of the solid-phase ELISA (biotin-streptavidin signal amplification system and test systems of Vector-Best CJSC, Novosibirsk) was employed.

Statistical data processing

Statistical processing of collected data was performed using the STATISTICA 8.0 software package (StatSoft Inc., USA). The choice of statistical analysis method was determined by the data set type and data distribution pattern. To clarify the applicability of parametric tools, we evaluated the distributions of examined variables in relation to the normal distribution law via Shapiro–Wilk test. All quantitative indicators were normally distributed. Mean and its standard deviation (M ± σ) were used as descriptive statistics for quantitative parameters. Differences were considered statistically significant at $p<0.05$.

Results

Clinical characteristics of comparative study convalescents

Of 78 COVID-19 convalescents three months after their discharge from the hospital, 30.8% of them had a profile of specific antibodies IgM(+)IgG(+), 37.2% had IgM(-)IgG(+), and 32.0% were characterized by IgM(-)IgG (-). Characteristics of 78 convalescents who underwent COVID-19 infection are summarized in *Table* 1.

Women predominated among examined patients: n=56 (71.4% of all subjects), aged 54.1±9.2 years. The majority of subjects, n=42 (53.8%), underwent a moderate course of COVID-19. The structure of comorbid pathology was dominated by chronic respiratory and cardiovascular diseases: n=10 (12.8%) and n=9 (11.5%) convalescents, respectively.

Comparative assessment of CRP and IL-6 levels vs. the profile of specific IgM/IgG antibodies to SARS-CoV-2

The values of CRP content in the blood serum of all COVID-19 convalescents three months after their discharge from the hospital were within reference values. The highest level of IL-6 in blood serum was recorded for the IgM(-)IgG(-) group of convalescents, while the lowest level was in convalescents of the IgM(-)IgG(+) group ($p<0.001$) (*Table* 2).

Assessment of cell-mediated and humoral immunity indicators vs. the profile of specific IgM/IgG antibodies in COVID-19 convalescents

The CD3+ index in the blood serum of all COVID-19 convalescents three months after their discharge from the hospital was within reference values. Comparative intergroup analysis of CD3+CD8+ percentage in blood plasma demonstrated that convalescents of IgM(+)IgG(+) group had its highest content, whereas convalescents in the IgM(-)IgG(-) group had the lowest share ($p<0.001$). Intergroup comparison of CD3+CD4+, CD3–CD16+CD56+ and CD19+ content in blood plasma confirmed that convalescents in the IgM(-)IgG(-) group had the highest values of those, while convalescents in the IgM(+)IgG(+) group had the lowest values ($p<0.001$). The IgM(-)IgG(+) group of convalescents was characterized by an increase in the number of lymphocytes expressing late activation/apoptosis molecules (HLA-DR, CD95), whereas the lowest value of the studied parameter was recorded in convalescents of the IgM(+)IgG(+) group ($p=0.019$, $p<0.001$) (*Table* 3).

Table 1. Characteristics of COVID-19 convalescents included in the comparative study

Indicator	*IgM(+)IgG(+) (n=24)*	*IgM(-)IgG(+) (n=29)*	*IgM(-)IgG(-) (n=25)*
Age, years (M ± σ)	48.4± 12.7	50.1±11.4	52.6±11.9
Women (n, %)	17 (70.8)	21 (72.4)	18 (72.0)
Men (n, %)	7 (29.2)	8 (27.6)	7 (28.0)
Light course (n, %)	10 (41.7)	10 (33.3)	9 (36.0)
Moderate course (n, %)	11 (45.8)	16 (55.2)	15 (60.0)
Severe course (n, %)	3 (12.5)	4 (13.8)	1 (4.0)
CCVD (n, %)	2 (8.3)	3 (10.3)	4 (16.0)
CRD (n, %)	3 (12.5)	4 (13.8)	3 (12.0)
CCRVD (n, %)	1 (4.2)	1 (3.4)	1 (4.0)
Diabetes mellitus (n, %)	2 (8.3)	1 (3.4)	3 (12.0)
Hypo- and hyperthyroidism (n, %)	3 (12.5)	1 (3.4)	1 (4.0)

Table 2. Comparative assessment of CRP and IL-6 levels vs. the profile of specific IgM/IgG antibodies to SARS-CoV in COVID-19 convalescents, M±σ

Indicator	*Healthy donors (n=50)*	*Continuous sample (n=78)*	*IgM(+)IgG(+) (n=24)*	*IgM(-)IgG(+) (n=29)*	*IgM(-)IgG(-) (n=25)*
CRP, mg/L	3.6±0.7	3.5±0.9 p_{1-2}=0.51	3.6±2.7 p_{1-3}=1.00 p_{2-3}=0.78	3.4±1.1 p_{1-4}=0.33 p_{2-4}=0.63 p_{3-4}=0.72	3.5±0.5 p_{1-5}=0.53 p_{2-5}=1.00 p_{3-5}=0.86 p_{4-5}=0.67
IL-6, pg/mL	4.2±7.8	8.1±4.4 p_{1-2}<0.001	7.3±2.4 p_{1-3}=0.062 p_{2-3}=0.40	6.3±1.6 p_{1-4}=0.16 p_{2-4}=0.034 p_{3-4}=0.076	10.6±5.5 p_{1-5}<0.001 p_{2-5}=0.022 p_{3-5}=0.0096 p_{4-5}<0.001

Discussion

So far, sufficient number of studies clarified the features of seroconversion in patients with COVID-19 in the acute phase [4-6,11], while an issue regarding the specifics of the immune response during the convalescence period remains largely unexplored. Our research investigated various profiles of specific IgM and IgG antibodies to SARS-CoV and their association with the parameters of the immune status in COVID-19 convalescents three months after their discharge from the hospital.

According to the traditional ideas of contemporary immunology, the detection of IgM antibodies to infectious agents in patients is typically considered evidence of an acute phase or a recent illness, whereas the detection of IgG implies the formation of a long-term immunity to a specific antigen. Our study demonstrated that specific IgG antibodies to SARS-CoV-2 were detected in more than half of convalescents three months after their discharge from the hospital: in 68.0% of cases. Our results are consistent with the data of a previous study by Popova A.Yu. et al. [12], who established that IgG to SARS-CoV-2 were revealed in 48.7-87.7% of the subjects with an apparent form of COVID-19. It should be pointed out that the serological response to coronaviruses is temporary. Antibodies to other human coronaviruses habitually disappear within a few months of infection. Previously published data suggested that SARS-CoV-2 antibody profile was similar to that of SARS-CoV [13]. In our study, three months after the patient discharge from the hospital, IgM antibodies were found in 30.8% of individuals recovering from COVID-19. The absence of specific IgM and IgG antibodies was observed in 32.0% of COVID-19 convalescents. Similar to our results, previous studies reported the absence of antibodies in patients infected with SARS-CoV-2 as well. E.g., Tan W.E. et al. [14] reported the absence of specific antibodies in 10-20% of patients in their convalescence phase. Similar data were collected in the study by Shen Y. et al. [10], according to which, six months after discharge from the hospital, specific antibodies to SARS-CoV-2 were absent in 36.4% of convalescents. Medical scientific community debated an assumption that an absence of IgG in the long term may indicate a mild course of SARS-CoV-2, which was effectively eliminated by the components of nonspecific immune system. However, innate immunity does not ensure the formation of long-term immunological memory [15].

Given our data on variability of humoral immune responses, it becomes necessary to study the immune status features in COVID-19 convalescents vs. their IgM/IgG blood serum profiles to SARS-CoV-2.

In infectious pathologies, including COVID-19, activation of the populations of alveolar macrophages, along with dendritic, immune, and endothelial cells, is accompanied by increased production of molecular inflammatory markers [17]. IL-6 plays the key role in the pathogenesis of SARS-CoV-2: via positive feedback, it activates T lymphocytes and other immunocompetent cells that regulate local and systemic inflammation [18]. Besides, regarding COVID-19, an increase in the content of the surrogate biomarker IL-6 (an acute-phase C-reactive protein that reflects the severity of the disease rather than hyperproduction of proinflammatory mediators alone) is of particular diagnostic importance [19-21]. Our study demonstrated that in all COVID-19 convalescents three months after their discharge from the hospital, CRP values were within the reference values, which was consistent with the results of previous studies [10]. In convalescents with an IgM(-)IgG(-) profile, IL-6 values exceeded the reference values and were 1.7 times higher, compared with the results in the IgM(+)IgG(+) and IgM(-)IgG(+) groups, which does not fully fit into conventionally accepted paradigm of the concentration reduction in proinflammatory mediators during the recovery period after an infectious process and emphasizes the necessity of additional study of the issue.

Analyzing the immune status indicators in IgM(+) convalescents, some researchers associate the long-term level of specific IgM with damage to the function of T helper cells (CD3+CD4+ lymphocytes) that are essential for switching IgM production to IgG, as well as with a decrease in the total number of T lymphocytes and suppressor T cells, which could later provoke an uncontrolled hyperresponse to the virus [22]. In our study, an assessment of the cell-mediated immunity revealed that the presence of IgM antibodies in the blood serum of convalescents after COVID-19 was associated with a substantial reduction in the relative content of CD3+CD4+ lymphocytes and a statistically significant increase in the proportion of CD3+CD8+, compared with healthy donors and IgM(-)IgG(-)/IgM(-)IgG(+) groups against the background of maintaining the reference values of the total number of T lymphocytes (CD3+). We believe that the decrease in the number of CD3+CD4+ lymphocyte subpopulations in patients who have undergone the COVID-19 infection implies insufficient T cell proliferative response to coronavirus antigens, while an increase in the cytotoxic potential of the immune system may reflect an improved level of IgM+ bacterial load in patients.

In the group of IgG(–) convalescents, despite the absence of specific antibodies, the cell-mediated immune response was characterized by an increase in the relative content of CD3+CD4+

cells and a reduction in CD3+CD8+ lymphocytes against the background of normal values for the pool of CD3+ cells. This may be indirect evidence of ongoing viral persistence, which is implemented via reduction in the expression of the own genome below the visibility threshold of effector T cells, or else via infecting immunologically privileged tissues.

A significant role in the implementation of antiviral immunity belongs to innate lymphoid cells. For instance, in particular, natural killer (NK) cells, expressing inhibitory and activating receptors on their surface that regulate their cytotoxicity, are capable of inducing the lysis of infected cells. The latter are characterized by an increased expression of proteins of viral origin and stress-induced ligands, which are then recognized by receptors activating NK cells [23,24]. A number of foreign studies reported a decrease in the number of NK cells in the peripheral blood in the acute phase of COVID-19, which was associated with the severity of the disease [25,26].

Amanat et al. demonstrated that the secretion of IgG1 and IgG3 antibodies in the course of the SARS-CoV-2 infection could induce the activation of $CD56^{dim}CD16^{+}$ innate lymphoid NK cells through the recognition by Fc receptors of antibodies, associated either with surface antigens expressed on infected cells, or with extracellular virions as part of immune complexes. Perhaps, this interaction provokes both the production of cytokines by NK cells and the lysis of infected cells as a result of antibody-dependent cellular cytotoxicity (ADCC), as was observed in influenza infection [27].

Our study showed that the most significant decrease in the pool of CD3-CD16+CD56+ cells in the blood serum three months after discharge from the hospital was observed solely in convalescents with retained IgM antibodies, while a statistically significant increase in the relative content of NK cells was detected in the group of seronegative convalescents. Therefore, the presence of specific IgG and IgM antibodies in the blood serum of convalescents three months after their discharge from the hospital, recorded against the background of immune system imbalance, is an early warning factor regarding the risk of developing autoimmune reactions.

To date, we are aware that it is the B-cell response that is crucial for the elimination of cytopathic viruses and is an essential part of the secondary immune response preventing reinfection.

According to our data, IgG(-) convalescents had higher statistically significant values of the relative content of B lymphocytes, compared with those of convalescents with IgM(+)IgG(+) and IgM(-)IgG(+) profiles, which was undoubtedly a detrimental manifestation against the background of the absence of antibody formation.

Modulation of immunocompetent cell activation, caused by exposure to pathogenic viral agents, is characterized by a functional change in surface molecules that consistently reflect the processes of activation, proliferation, differentiation, and apoptosis occurring in the cell, which allows assessing their contribution to the pathological process formation. All examined COVID-19 convalescents three months after their discharge from the hospital exhibited the signs of chronic T cell activation, associated with an increase in the number of CD95/Fas and HLA-DR expression. It is noteworthy that the highest values of apoptotic markers were recorded in convalescents with an IgM(-)IgG(+) profile, whose immunocompetent cell parameters were closest to those of healthy donors.

Conducting a comprehensive assessment of the functional state of the immune status indicators in patients with a history of coronavirus infection, we established a variability in the activation level of peripheral blood lymphocytes, implying the presence of an individual response from the immune system. An imbalance in the cell-mediated immune response, recorded in seronegative patients who have had COVID-19, could be indirect evidence of ongoing infection with this virus. The presence of specific IgG and IgM antibodies in blood serum in the long term after clinical recovery, detected against the background of the immune system imbalance, was an alarming factor regarding the pronounced humoral response, formation of autoantibodies to the own tissues, and development of autoimmune disorders.

Hence, our data on the relationship between the profiles of specific IgM and IgG antibodies to SARS-CoV-2 and immunological parameters in COVID-19 convalescents could contribute to our better understanding of the long-term immune response specifics in these patients and be of potential importance in clinical practice for developing a dynamic prognosis of epidemiological situation, as well as for planning measures for specific and nonspecific prevention of COVID-19.

Table 3. **Assessment of cell-mediated and humoral immunity indicators vs. the profile of specific IgM/IgG antibodies in COVID-19 convalescents three months after discharge from the hospital, M±σ**

Indicator	*Healthy donors (n=50)*	*Continuous sample (n=78)*	*IgM(+)IgG(+) (n=24)*	*IgM(-)IgG(+) (n=29)*	*IgM(-)IgG(-) (n=25)*
CD3+, %	73.3±7.4	72.2±7.2 p_{1-2}=0.41	71.1±10.7 p_{1-3}=0.31 p_{2-3}=0.56	73.5±8.8 p_{1-4}=0.91 p_{2-4}=0.44 p_{3-4}=0.37	74.4±10.6 p_{1-5}=0.60 p_{2-5}=0.24 p_{3-5}=0.28 p_{4-5}=0.73
CD3+CD4+, %	46.1±4.1	50.1±4.9 p_{12}<0.001	38.4±4.7 p_{13}<0.001 p_{23}<0.001	40.6±5.2 p_{14}<0.001 p_{24}<0.001 p_{34}=0.12	71.4±4.8 p_{15}<0.001 p_{25}<0.001 p_{35}<0.001 p_{45}<0.001
CD3+CD8+, %	25.9±5.5	41.0±8.9 p_{12}<0.001	52.7±7.2 p_{13}<0.001 p_{23}<0.001	40.6±10.2 p_{14}<0.001 p_{24}=0.84 p_{34}<0.001	29.6±9.2 p_{15}<0.001 p_{25}<0.001 p_{35}<0.001 p_{45}<0.001
CD3–CD16+CD56+, %	10.4±4.8	22.4±6.9 p_{1-2}<0.001	17.7±7.4 p_{1-3}<0.001 p_{2-3}<0.001	19.9±6.9 p_{1-4}<0.001 p_{2-4}=0.10 p_{3-4}=0.27	29.7±6.3 p_{1-5}<0.001 p_{2-5}<0.001 p_{3-5}<0.001 p_{4-5}<0.001
CD19+, %	10.8±3.1	15.2±4.4 p_{1-2}<0.001	11.3±3.0 p_{1-3}=0.51 p_{2-3}<0.001	14.2±3.8 p_{1-4}<0.001 p_{2-4}=0.28 p_{3-4}=0.0038	20.2±6.3 p_{1-5}<0.001 p_{2-5}<0.001 p_{3-5}<0.001 p_{45}<0.001
CD95+, %	20.4±7.1	28.7±4.1 p_{1-2}<0.001	27.2±4.0 p_{1-3}<0.001 p_{2-3}=0.12	29.6±3.2 p_{1-4}<0.001 p_{2-4}=0.29 p_{3-4}=0.019	29.5±5.0 p_{1-5}<0.001 p_{2-5}=0.42 p_{3-5}=0.083 p_{4-5}=0.93
HLA-DR+, %	15.5±4.0	16.3±3.6 p_{1-2}=0.24	14.0±2.8 p_{1-3}=0.10 p_{2-3}=0.0050	20.0±4.2 p_{1-4}<0.001 p_{2-4}<0.001 p_{3-4}<0.001	14.8±3.8 p_{1-5}=0.47 p_{2-5}=0.076 p_{3-5}=0.41 p_{4-5}<0.001

Conclusions

1. Of 78 COVID-19 convalescents, three months after their discharge from the hospital, 30.8% had a profile of specific antibodies IgM(+)IgG(+), 37.2% had IgM(-)IgG(+), and 32.0% were characterized by IgM(-)IgG(-) profile.

2. COVID-19 convalescents with a specific antibody profile IgM(-)IgG(-) had the highest levels of NK cells, T helper cells and B lymphocytes, and were characterized by hyperproduction of proinflammatory IL-6.

3. COVID-19 convalescents with a profile of specific antibodies IgM(+)IgG(+) were characterized by the highest levels of cytotoxic T lymphocytes.

4. In COVID-19 convalescents with an IgM(-)IgG(+) specific antibody profile, we observed an increase in the number of lymphocytes expressing molecules of late activation/apoptosis.

Study limitations

The results of our research should be interpreted with some caution. First, we had a relatively small sample of patients. Another limitation of our study is caused by a wide age range of examined subjects on accordance with chosen eligibility criteria. Also, among the main limitations of our study, it should be noted that it included patients with a history of comorbid pathology, which did not allow obtaining complete information on the degree of immunological disorders in COVID-19 convalescents.

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Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with 1964 Declaration OF Helsinki and its later amendments, or comparable ethical standards.

Conflict of interest

We declare that we have no conflicts of interest.

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