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Research article

Exploring immune response and lab markers across COVID-19 severity levels

Y. Desheva[1,*], T. Shvedova[2], A. Lerner[3], P. Kudar[1], O. Kopteva[1], D. Petrachkova[1], I. Koroleva[1], G. Leontieva[1], S. Ponkratov[2] and A. Suvorov[1]

[1] Federal State Budgetary Scientific Institution 'Institute of Experimental Medicine', 197022, Russian Federation, Saint-Petersburg, 12, Acad. Pavlov Street, Russian Federation
[2] State budgetary institution of health care of the Leningrad region "Vsevolozhsk clinical interdistrict hospital", 188643, Russian Federation, Leningrad region, Vsevolozhsk district, Vsevolozhsk, 20, Koltushskoe highway
[3] Northwestern State Medical University named after I.I. Mechnikov, 191015, Russian Federation, Saint-Petersburg, 41, Kirochnaya street

* **Correspondence:** Email: desheva@mail.ru; Tel: +79217863126.

Abstract: This retrospective cohort study investigated antigen-specific antibody responses to SARS-CoV-2 and laboratory markers in coronavirus disease 2019 (COVID-19) patients of varying severities. Serum or plasma samples collected early in the pandemic were analyzed for the presence of IgG antibodies and IgG subclasses which target the recombinant spike (S) and nucleocapsid (N) proteins of SARS-CoV-2. Correlation analyses were conducted to assess the possible relationship between the IgG subclasses to SARS-CoV-2 proteins, inflammatory markers, and the severity of the disease. It was shown that the severity of the disease positively correlated with the C-reactive protein (CRP), but most of all with the neutrophil/lymphocyte ratio (NLR) levels. IgG titers against the S- and N-proteins decreased in the most severe COVID-19 cases, which potentially attributed to a delayed IgG formation compared to milder infections. The presence of anti-spike IgG displayed a medium negative correlation trend (not statistically significant, which might be due to the small sample size in our study) with laboratory markers (such as CRP, Fibrinogen, Degree) which are suggestive of infection severity. anti-S IgG correlated positively but weakly with the serum histamine levels. The presence of anti-N IgG at the time of hospitalization correlated with the subsequent outcome. At the same time, anti-N IgG1 correlated with the detection of the viral RNA in the blood. Thus, seroconversion may occur later in patients with a more severe pneumonia. The summary data suggests correlations between the

expression of inflammatory markers and the antibody responses, which can also serve as early clinical markers.

Keywords: COVID-19; severity; serum IgG subclasses; blood tests

1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic has become a significant challenge of the 21st century. Clinical symptoms and laboratory signs that are involved in the development of hyperinflammatory syndrome with COVID-19 are still subject to comprehensive studies [1–3].

Evidence regarding the relation between the options of innate and adaptive immunity in acute COVID-19 are still far from clear. During different periods of infection, antibodies to viral antigens are produced, which are primarily the immunoglobulin M (IgM) and subsequently the immunoglobulin G (IgG) [4]. Of the entire pool of immunoglobulins in normal blood serum, 80% of all the immunoglobulins are IgG, which are the main protective immunoglobulins, only a small part of which are pathogen-specific [5]. Some types of antibodies are used for serological diagnoses of past infections. The best characterized antibodies for COVID-19 are the spike (S) protein, the nucleocapsid (N) protein, and the receptor binding domain (RBD) of the SARS-Cov-2 virus. Antibodies to the RBD and the N-terminal domain of the S-protein exhibit neutralizing properties by interfering with the S-protein interaction with the human angiotensin-converting enzyme 2 receptor (ACE2) [6].

Antibody detection provides important clinical information about the infection process during COVID-19. The different characteristics of the IgG isotypes and subclasses in severe and non-severe groups of COVID-19 will support the development of future therapeutic measures such as the use of convalescent plasma [7]. Moreover, the increase in antibody titers to SARS-Cov-2 proteins in the paired sera of COVID-19 patients can provide an additional diagnostic tool for past infections in the absence of a positive PCR test [8].

A number of publications reported that virus-specific IgG in acute COVID-19 was identified in hospitalized patients within 2 weeks after the onset of symptoms [8–11]. However, as with other systemic viral infections, the most noticeable increase in the level of immunoglobulins during COVID-19, especially IgG, was observed during the recovery stage [10]. The predominant antibody response to the virus SARS-Cov-2, as with other viral infections, are represented by the IgG1 and IgG3 subclasses [11]. The increase of IgG1 and IgG3 in respiratory viral infections are associated with immune functions such as virus neutralization, opsonization, and complement activation [12]. The study of virus-specific IgG subclasses in combination with age, medical history, and the severity of primary infection can serve to predetermine the likelihood of developing the currently widely studied post-acute COVID-19 syndrome [13]. In addition, studying the IgG antibody subclasses can provide insight into the strength and durability of the immune memory following either natural infection or vaccination, which is important to predict the level of protection. For example, the production of RBD-specific IgG1 corresponds to the activation of Th1 lymphocytes [14].

There is some evidence that virus-specific IgG was increased in severe COVID-19 infections compared to mild COVID-19 infections [15–17]. At the same time, the lack of anti-SARS-Cov-2 IgM and IgG antibodies at the time of hospitalization was negatively correlated with in-hospital mortality [18]. Antibody-dependent enhancement (ADE) theory has been proposed to play a role in the pathogenesis

of viral infections, including COVID-19. Antibodies that bind to Fc receptors can activate antibody-dependent complement deposition, antibody-dependent cell-mediated phagocytosis, and antibody-dependent cell-mediated cytotoxicity. It was previously hypothesized that patients with pre-existing high levels of pathogen-specific antibodies appeared to have a more severe clinical course [19]. There is an assumption about the relationship between systemic antibodies and macrophage or mast cell activation syndromes in coronavirus infections [20]. Antibodies that bind to Fc receptors can activate antibody-dependent complement deposition, antibody-dependent cell-mediated phagocytosis, and antibody-dependent cell-mediated cytotoxicity. These reactions of the innate immune system limit viral replication, and contribute to increased inflammatory reactions [21].

Thus, in order to effectively combat COVID-19, it is necessary to study all the factors of immunity to better understand their participation in pathogenesis. The aim of this work is to understand the relationship between factors of adaptive immunity, such as virus-specific IgG subclasses, and the severity of the disease, which is accompanied by an increase in inflammatory markers.

2. Materials and methods

2.1. Ethics statement

A retrospective study was conducted using serum/plasma samples that remained from clinical examinations of COVID-19 patients hospitalized at the beginning of the first wave of the pandemic caused by SARS-Cov-2. The protocol of the study was approved by the local Ethics Committee of the Institute of Experimental medicine (protocol 3/20 from 06/05/2020). After obtaining Ethics Committee approval, the sera were released to the investigators, none of whom had access to the patients' personal data. When analyzing the clinical data, the primary patient data was anonymized and the employees did not have access to the patients' personal data. Because this was a retrospective study, informed consent was not required, although all patients signed an informed consent upon admission to the hospital.

2.2. Study participants and specimens

A total of 45 blood samples were collected from COVID-19 patients during the first three days of their hospital stay. The study examined the serum and plasma samples that remained from ongoing laboratory studies of patients with COVID-19 of varying severities. The serum was used to assess the antibody levels and determine the C-reactive protein (CRP), and the plasma alongside sodium citrate was used to assess the fibrinogen levels. The serum samples were provided for serological testing in aliquots, which were stored at −20 ℃ until testing, so that they were thawed only once.

The study cohort was divided into 3 groups, depending on the severity of the course of the disease at the time of their respective hospitalization admissions to the hospital. In February-April 2020, the patients were hospitalized at the Vsevolozhsk Clinical Interdistrict Hospital of the Leningrad Region of the Russian Federation. The COVID-19 severity was estimated according to the "Interim guidelines for the prevention, diagnosis and treatment of coronavirus disease 2019 (COVID-19), Version 8" (https://static-0.minzdrav.gov.ru/system/attachments/attaches/000/064/610/original/%D0%92%D0%9C%D0%A0_COVID-19_V18.pdf, accessed on the 3rd of September, 2020). A mild course of disease was characterized by a body temperature <38 ℃, a cough, weakness, a sore throat, with a lack of criteria for moderate and severe courses. The moderate course was characterized by a body temperature >38 ℃,

a respiration rate >22/min, dyspnea on exertion, changes on X-ray typical of a viral lesion (the extent of damage is minimal or moderate), an oxygen saturation <95%, and a serum C-reactive protein (CRP) >10 mg/L. A severe course of infection was characterized by a respiratory rate >30/min, an oxygen saturation <93%, unstable hemodynamics (systolic blood pressure less than 90 mm Hg or diastolic blood pressure less than 60 mm Hg, urine output less than 20 mL/hour), changes in the lungs on radiographs typical of a viral lesion (the volume of damage >70%), and an arterial blood lactate >2 mmol/L. The severe course of the infection was characterized by the addition of acute respiratory distress syndrome (ARD) to the previous symptoms. Nasopharyngeal and pharyngeal swabs were tested by real-time polymerase chain reaction at hospitalization.

2.3. PCR test

PCR analyses of the nasal swabs were determined using RealBest RNA SARS-CoV-2 kits (Vector best, Novosibirsk, Russia) accordingly to the provider's manual. Although the quantitative measurement of viral RNA in blood is not routinely applied in the diagnosis of COVID-19, we measured the blood viremia levels using the above kit for PCR SARS-CoV-2 detection. Detection of the SARS-CoV-2 genetic material in the blood serum were performed using the 'Intifica-SARS-CoV-2' kit (Alcor Bio, Saint-Petersburg, Russia).

2.4. Laboratory data

The concentrations of CRP and fibrinogen in the blood serum were studied by the turbidimetric method using the BioSystems reagents (BioSystems, Barcelona, Spain). The serum histamine levels were detected using the Histamine ELISA kit (Cloud-Clone Corp, Wuhan, Hubei) according to the manufacturer's instructions. All blood tests were performed with unheated blood samples. The neutrophil/lymphocyte ratio (NLR) was calculated based on clinical blood test data using the following formula: Absolute neutrophil count/absolute lymphocyte count. The reference values for laboratory markers were as follows: 0.0–5.0 mg/L for CRP; 1.13–3.79 conventional units for NLR; 2.0–4.0 mg/L for fibrinogen; and 0.0–9.3 nm/L for histamine.

2.5. Detection of antibodies to SARS-CoV-2 S- and N- proteins using enzyme-linked immunosorbent assay (ELISA)

The blood samples were tested using an in-house developed enzyme-linked immunosorbent assay (ELISA)for the detection of either IgG or the IgG subclasses specific for proteins of SARS-CoV-2 [8]. For this purpose, 96-well Nunc MaxiSorp plates (Thermo Fisher Scientific, Waltham, United States) were sensitized with either recombinant S-protein at a concentration of 2 μg/mL that contained 496–646 amino acids (GenBank: OL447006.1) obtained in *E. Coli* as described previously [22] or 2 μg/mL of commercial SARS-CoV-2 N-protein (AtaGenix, Wuhan, China). After washing 3 times, serial dilutions of sera in 1:4 increments were added to the well of the sensitized plates. All tests were performed in duplicates. After 1.5 hours of incubation at 37 °C, a series of washes was used to excise unbound antibodies. The horseradish peroxidase (HRP)-linked goat anti-human IgG antibody (Sigma, St. Louis, USA) was used to detect serum IgG as described earlier [8]. To identify the IgG subclasses, a specially developed technique was established using rabbit anti-human IgG1, IgG2,

IgG3, IgG4 (‘Polygnost’, Saint Petersburg, Russia) as conjugates. ELISA end-point titers were expressed as the highest dilution that gave an optical density at 450 nm (OD450), which was greater than the mean OD450 plus 3 standard deviations (SD) of the control wells. The control well means were determined for each 4× to 6× dilution of negative blood sera obtained before the emergence of SARS-CoV-2.

2.6. Statistical analysis

Statistical analyses of the data were performed using the Statistica 12.0 software package (StatSoft, Inc. Tulsa, Oklahoma, USA) and the graphics data were generated using Prism 8 (GraphPad software, San Diego, USA). The data was normalized using the mean normalization method (Z-normalization). The medians (Me) and lower and upper quartiles (Q1; Q3) were calculated and used to represent the antibody response and blood tests levels. To compare two independent samples with a normal distribution, the Student’s t-test was used for unpaired samples with a preliminary analysis of the equality of variances using the D’Agostino & Pearson omnibus normality test. If the data were not normally distributed, then comparisons of the two independent groups were made with the nonparametric Mann-Whitney test. For the nominal data, either Fisher exact 2-tailed tests or McNemar’s chi-square tests were used for the same purpose. The presence of a statistical relationship between the variables was assessed using a correlation analysis in Python 3 by applying the Pandas library ‘Corr’ function by Spearmen’s method. The level of statistical significance of the correlation was determined as follows: $P < 0.05 > 0.01$ − low statistical significance, $P \leq 0.01 > 0.001$ − moderate statistical significance, and $P \leq 0.001$ − high statistical significance. The p-value < 0.05 was considered to be statistically significant.

3. Results

3.1. Patient’s characteristics

In this retrospective cohort research, we assessed serum/plasma samples obtained from 45 patients with mild, moderate, and severe forms of COVID-19 who were hospitalized in February-April 2020 at the Vsevolozhsk Clinical Interdistrict Hospital of the Leningrad Region.

Table 1 presents the patient characteristics among the group of 45 COVID-19 patients at the time of hospitalization. The exclusion criteria included oncological diseases and immunodeficiency states. The cohort included 22 men and 23 women, aged 27 to 92 years old, and the median age was 62 years old. There were no significant differences between the main indicators between men and women (Table S1). It was shown that 65% of the examined patients over 60 years of age had concomitant diseases compared to younger people ($P = 0.016$), and the average level of CRP was 1.5 times higher than in the younger subjects ($P = 0.03$), (Table S2). The other markers considered in the study did not differ between these age groups (Table S2). The most common comorbidities in the hospitalized patients with COVID-19 were arterial hypertension and ischemic heart disease (55.5%), diabetes (13.3%), and chronic pulmonary disorders such as bronchial asthma and chronic obstructive bronchitis (4.4%). It was shown that there were no differences in age or time of admission to the hospital from the beginning of the onset of symptoms among the studied groups. A positive PCR test for SARS-Cov-2 was observed most frequently in the mild COVID-19 patients and to a lesser extent in the severe cases.

Bacterial complications were identified in 26.6% of the examined patients. Bacteriological analyses revealed opportunistic pathogenic microorganisms, such as *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and *Candida albicans*. For mild COVID-19, all patients were recovered; for medium severe COVID-19, four patients died (25.0%); and in the severe course of the disease, the lethal outcome was observed in 11 cases (73.3%).

Table 1. Characteristics of patients with COVID-19.

Parametrs	Patient categories		
	Group 1-mild (n = 14)	Group 2-moderate (n = 16)	Group 3-Severe (n = 15)
Age, Me (Q25; Q75)	59 (51; 69)	61 (57; 66)	64 (53; 72)
Males	4 (28.6%)	11 (68.7%)	7 (46.8%)
Females	10 (71.4%)	5 (31.3%)	8 (53.3%)
Days from onset of illness, Me (Q25; Q75)	7.00 (4.00; 8.00)	7 (5.00; 7.00)	8.00 (5.00; 10.00)
Positive PCR test for SARS-Cov-2 on the day of hospitalization	7 (50%)	6 (37.5%)	4 (26.7%)
Viremia (positive serum PCR-test)	2 (14.3%)	5 (31.2%)	5 (33.3%)
Comorbididies:			
Cardiovascular	6 (42.9%)	10 (62.5%)	8 (53.3%)
diabetes	3 (21.4%)	1(6.2%)	2 (13.3%)
chronic pulmonary disorders	0	1 (6.2%)	1 (6.7%)
Bacterial coinfections	2 (14.2%)	7 (43.8%)	2 (13.3%)
Lethal outcome	0	4 (25%)[1]	11 (73.3%)[2]

The moderate COVID-19 patients were less likely to have lethal outcomes than the severe COVID-19 patients ($P = 0.014$, Fisher's exact test).

The severe COVID-19 patients were more likely to have lethal outcomes than the mild COVID-19 patients ($P < 0.001$, Fisher's exact test).

3.2. The results of blood tests

Figure 1 shows a comparative analysis of the individual blood samples of the COVID-19 patients with various forms of COVID-19 severities. Blood sampling for testing in the first days of hospitalization was performed in 60% of cases before the appointment of any specific therapy, so the effect of drugs was minimal. The level of CRP was higher than normal in 96% of those examined COVID-19 patients, with an average of 136.7 mg/L. The CRP levels were apparently different in the mild and medium severe forms of the COVID-19 patients (Figure 1A). Thus, in patients with mild COVID, the average CRP level was 77.8 mg/L; with a medium-severe COVID-19 case, it was 117.95 mg/L; and with a severe disease course of the disease, it reached 214.2 mg/L.

For laboratory markers such as NLR, fibrinogen, and histamine, statistically significant differences between the COVID-19 patients with mild and moderate or severe infections were revealed (Figure 1A). In contrast to other parameters, the histamine levels in the severe COVID-19 patients were statistically and significantly lower than in the patients with mild to moderate disease courses (Figure 1B).

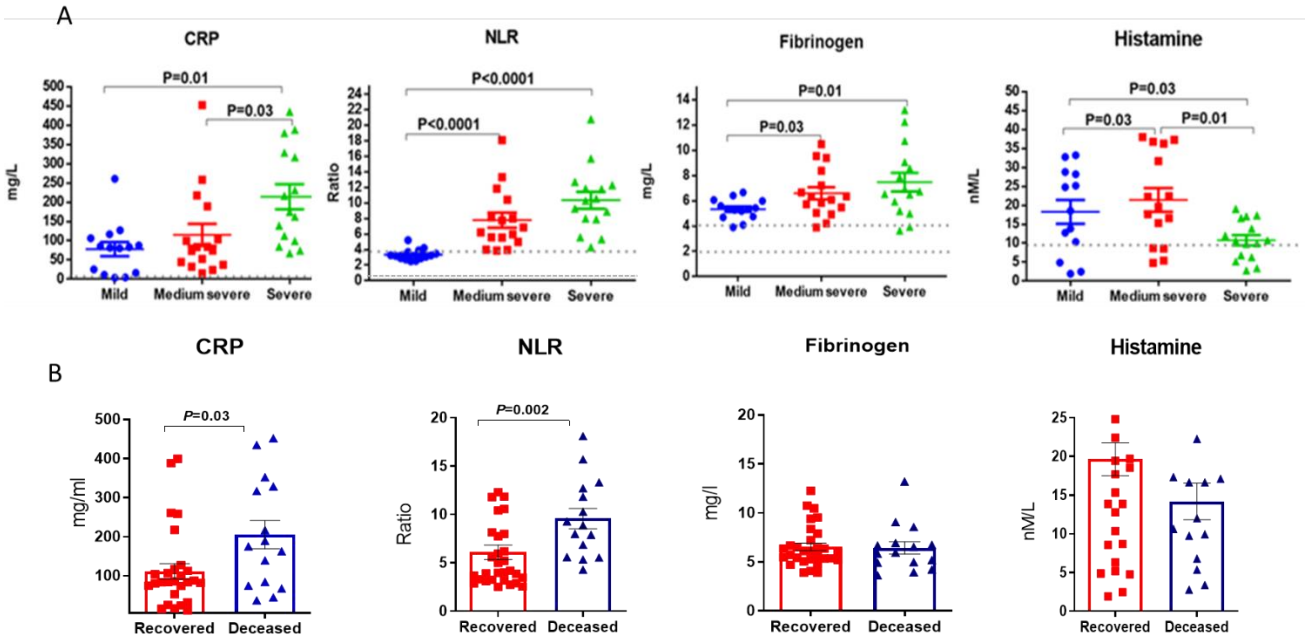

Figure 1. The blood tests in COVID-19 patients. A. Comparisons of blood tests in COVID-19 patients with mild (n = 14), moderate (n = 16) and severe (n = 15) conditions upon admission to the hospital. Mean values and standard errors of the means (SEM) are presented. The p-values were determined using the Mann-Whitney U test. The dotted line shows the upper reference values or upper and lower limits of the reference interval. B. Comparisons of blood tests in COVID-19 patients with different disease outcome (recovered, n = 30, deceased, n = 15).

It should be noted that out of the 30 recovered patients from the observed cohort, the mean CRP levels were 101.5, which were significantly lower than those of the 15 patients who died during hospitalization, in whom this indicator was on average 207.0 (P = 0.03) (Figure 1B). The mean levels of NLR were also significantly higher among the patients with poor outcomes (Figure 1B). Among the 11 patients with an NLR level that did not exceed the reference values (1.13–3.79), all the patients recovered, while 15 people died (44.1%), (P = 0.0054; Fisher's exact test) among the 34 patients with increased NLR levels. It turned out that the mean fibrinogen levels among the survivors were slightly lower than those of the non-survivors, although the differences were not statistically significant (Figure 1B). The histamine levels were higher among the surviving patients, although the differences were not statistically significant.

3.3. Serum IgG subtypes in COVID-19 patients

Figure 2 shows the results of the IgG to S- and N-protein of SARS-CoV-2 among the COVID-19 patients of varying severities. In patients with medium severe COVID-19, the levels of IgG to SARS-CoV-2 were higher than in the severe COVID-19 patients, although the differences were only statistically significant for the IgG to S-protein.

Anti-spike IgG1, IgG2, IgG3, and IgG4 levels in the patients with moderate COVID-19 were higher compared to those in the patients with mild or severe COVID-19, although statistically significant differences were noted only for IgG1 and IgG3 (Figure 3A,C).

Anti-N IgG1 and IgG2 antibodies in the patients with severe COVID-19 were slightly lower compared to the moderate disease (Figure 3A,B). Anti-N IgG4 levels were lower in the medium severe COVID-19 patients compared to those with the mild disease (Figure 3D). Moreover, the IgG and IgG1 to SARS-CoV-2 N-protein were significantly lower in the subsequent poor outcomes compared to those in the recovered patients.

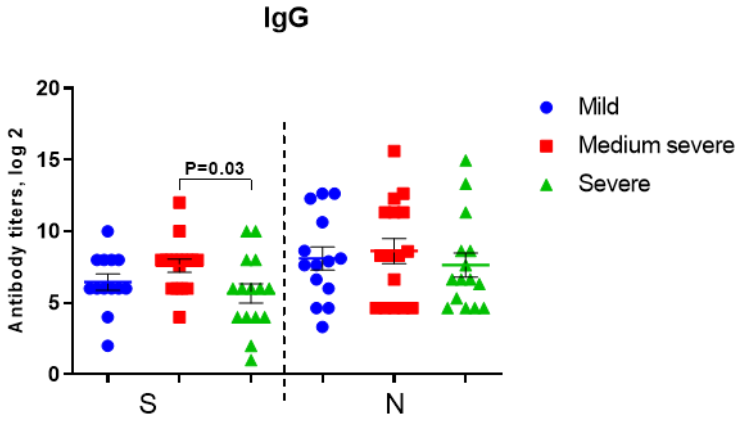

Figure 2. Detection of IgG in the blood sera of the examined patients. IgG to S- and N-proteins of SARS-CoV-2 in COVID-19 patients with mild (n = 14), moderate (n = 16) and severe (n = 15) infections upon admission to the hospital. Mean values and SEM are presented. We used Student's test to determine differences in anti-spike IgG and Mann-Whitney U-test was used for anti-N IgG analysis.

3.4. Correlation analysis

Figure 4 shows the results of a correlation analysis of the clinical features, the markers of systemic inflammation, and the IgG titers to SARS-CoV-2 proteins. The correlation P-values are presented in Table S3.

A medium-strength relationship between the levels of anti-spike IgG1 and IgG3 in the same blood sera ($r_s = 0.41$, $P = 0.005$) was demonstrated (Figure 4A). Additionally, anti-S IgG1 weakly correlated with IgG and IgG 4 ($r_s = 0.33$, $P = 0.027$; $r_s = 0.33$, $P = 0.029$), while anti-S IgG3 moderately and positively correlated with anti-S IgG4 ($r_s = 0.4$, $P = 0.007$). A noticeable correlation was observed in relation to anti-N IgG1 and IgG3 ($r_s = 0.52$, $P = 0.042$); at the same time, anti-N IgG1 positively correlated with anti-N IgG ($r_s = 0.53$, $P < 0.0001$) and with anti-N IgG4 ($r_s = 0.48$, $P = 0.004$); anti-N IgG2 was noticeable positively correlated with IgG4 ($r_s = 0.54$, $P = 0.011$) (Figure 4A).

As for the correlation between the anti-S and anti-N IgG subtypes, a weak-strength positive relationship between the levels of anti-spike IgG4 and anti-N IgG ($r_s = 0.34$, $P = 0.021$) was shown (Figure 4A). Anti-S IgG2 antibody titers were intermediately and positively correlated with anti-N IgG2 ($r_s = 0.41$, $P = 0.005$) and with anti-N IgG4 ($r_s = 0.38$, $P = 0.011$) (Figure 4A).

A low to medium negative relationship was shown between anti-spike IgG1 with CRP and fibrinogen ($r_s = -0.32$, $P = 0.03$; $r_s = -0.41$; $P = 0.005$, respectively) (Figure 4B). At the same time, there was a weak positive relationship between –the anti-spike IgG levels and the histamine levels ($r_s = 0.27$; $P = 0.019$).

A weak to moderate relationship was found between the favorable disease outcome and anti-N IgG-and IgG1 ($r_s = 0.33$, $P = 0.028$; $r_s = 0.45$, $P = 0.002$, respectively) (Figure 4B).

Quite expectedly, the severity of the disease was positively correlated with inflammatory markers, such as CPR ($r_s = 0.46$, $P = 0.001$), but more with the NLR levels ($r_s = 0.76$, $P < 0.0001$), and to a lesser extent with the fibrinogen levels ($r_s = 0.37$, $P = 0.012$) (Figure 4C). Detection of viremia was intermediately and positively correlated with the CRP and fibrinogen levels ($r_s = 0.4$, $P = 0.007$; $r_s = 0.39$, $P = 0.009$, respectively). In addition, detection of viremia was positively but weakly correlated with the levels of anti-N IgG1 ($r_s = 0.31$, $P = 0.041$). The favorable outcome of the disease was negatively correlated with the COVID-19 severity ($r_s = -0.63$, $P < 0.0001$), as well as with the content of CRP and NLR ($r_s = -0.36$, $P = 0.015$; $r_s = -0.46$, $P = 0.002$, respectively), but not depended on the fibrinogen content (Figure 4C).

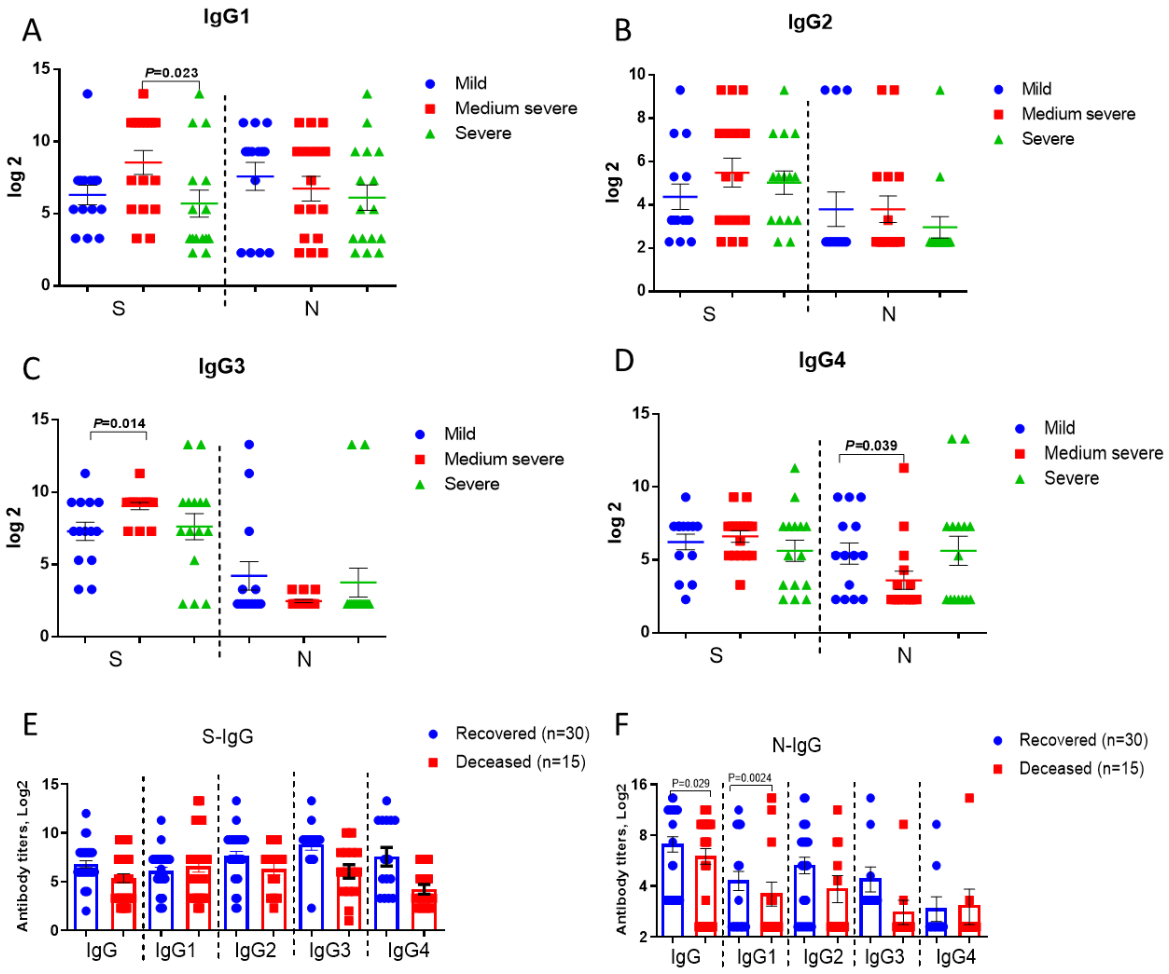

Figure 3. Results of studying IgG subclasses for COVID-19 of varying severity. A–D. IgG to S- and N-proteins of SARS-CoV-2 in COVID-19 patients with mild (n = 14), moderate (n = 16) and severe (n = 15) patients upon admission to the hospital. Mean values and SEM are presented. Mann-Whitney U-test was used for statistical analysis in all cases except for anti-spike IgG3 and IgG4 when we used Student's test. E. IgG subclasses to S-protein in COVID-19 patients with different disease outcome. F. IgG subclasses to N-protein in COVID-19 patients with different disease outcome.

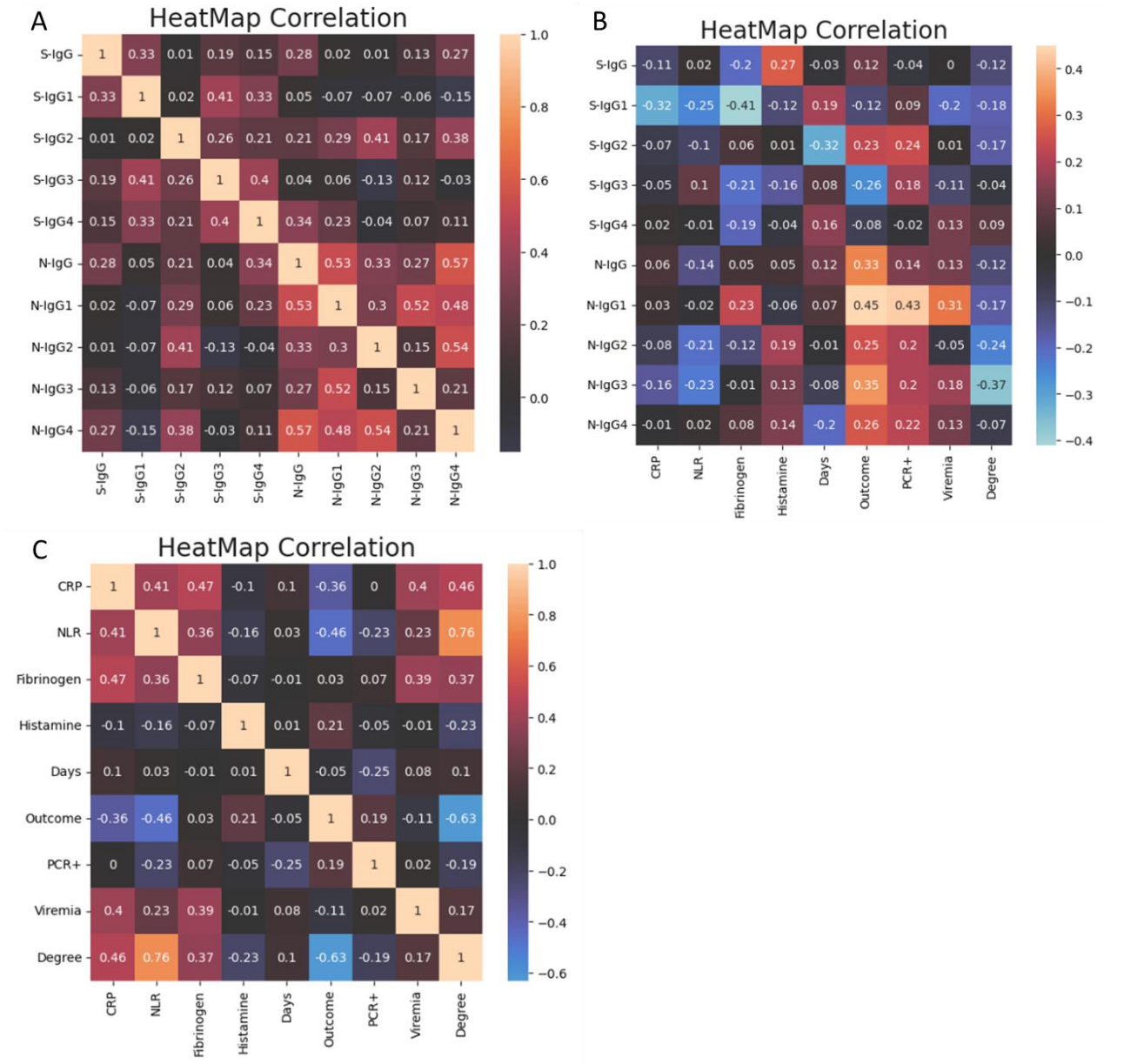

Figure 4. Correlation analysis of IgG subclasses, blood test and clinical data (n = 45 samples obtained upon admission to the hospital). Hierarchical clustering based on raw signal intensity for blood tests. Data were normalized using the mean normalization method (Z-normalization). The feature intensity picture was obtained using the built-in functions of the Pandas&Seaborn library in Python 3. Class variables have been converted to numeric as follows: Outcome-Died-0, Survived-1; severity-Light-1, Medium severe-2, Severe-3; PCR+ or viremia-yes-1, no-0. The strength of the relationship between variables was estimated according to the 'Chaddock scale': Correlation coefficient (r_s) from 0.1 to 0.3–weak; from 0.3 to 0.5–moderate; from 0.5 to 0.7–noticeable; from 0.7 to 0.9–high. A. Correlation analysis of IgG antibodies. B. Correlation analysis of IgG and clinical data. C. Correlation analysis of clinical data and blood tests.

A moderate to strong negative correlation was obtained between the anti-N IgG1 and IgG3 titers

and the threshold cycles (Ct) when detecting SARS-CoV-2 RNA in the studied blood samples using RT-PCR (Figure 5).

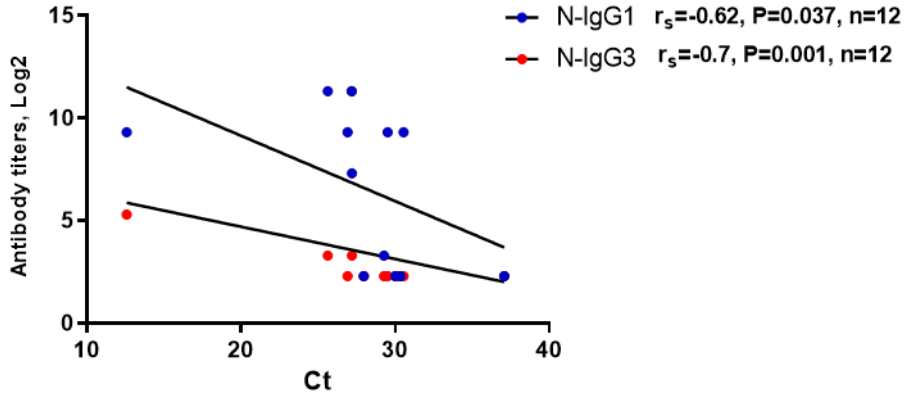

Figure 5. Scatterplot of RT-PCR Ct values of the S-protein gene as a function of anti-N protein IgG subclasses levels (n = 12). RT-PCR analysis of serum samples was performed 'Intifica-SARS-CoV-2' kit (Alcor Bio, Saint-Petersburg, Russia) according to the manufacturer's instructions.

Thus, some degree of relationship between the clinical indicators and the antibodies to SARS-CoV-2 has been identified. To summarize, our data suggest that the presence of anti-spike IgG shows an intermediate negative correlation trend (not statistically significant which might be due to the small sample size in our study) with the laboratory markers (such as CRP, fibrinogen, degree), which are suggestive of the infection severity. The presence of anti-N IgG at the time of hospitalization correlated with a subsequent recovery. At the same time, anti-N IgG1 correlated with the detection of the viral RNA in the blood.

4. Discussion

Sera from patients in this study were collected early in the pandemic, when the pathogenicity of the virus was much higher than in the later stages of the pandemic [23], and severe forms of COVID-19 accompanied by a high mortality were observed. This was associated with the development of a 'cytokine storm', namely the acute respiratory distress syndrome (ARDS) [24]. Similar to other studies, our study showed that IgG1 and IgG3 prevailed among other subclasses of virus-specific antibodies in acute COVID-19 [11,15,25]. However, the features and kinetics of the formation of types of immune responses during COVID-19 are still insufficiently studied [26]. The presentation of S and N proteins to the immune system during coronavirus infection may vary depending on their role and the nature of the immune response they produce. As shown in this study, seroconversion of both the anti-spike and the anti-N IgG may occur later in patients with a more severe pneumonia. The delayed seroconversion to intracellular pathogens such as SARS-CoV-2 may be a result of the intricate and time-consuming processes involved in the activation and maturation of the adaptive immune response [27]. Antigen-presenting cells, such as dendritic cells, need time to process and present viral antigens to T cells, which, in turn, stimulate B cells to produce antibodies [28]. All of these stages of antigen presentation

are important in the development of the immune response and contribute to the delayed seroconversion. Moreover, there is severe impairments of helper T cells in severe cases of COVID-19; as a result, there are fewer antigen-specific antibodies and a longer symptomatic period of the disease [29].

Several previous studies that examined the progression of seroconversion in COVID-19 have found that neutralizing antibody responses, which occur within the first two weeks after the symptom onset, correlate with the recovery; alternatively, antibodies generated after this period do not contribute to viral clearance and recovery of the patients [30]. In this case, it has been suggested that the coronavirus at certain stages of infection becomes inaccessible to antibodies when located in infected tissues; additionally, in later stages of infection, antibody-mediated pathological reactions may develop. For instance, antibodies from patients with severe COVID-19 show pro-inflammatory Fc modification signatures, including high levels of afucosylated IgG1, which could potentially drive pathologic responses [31].

The S-protein is a primary target for neutralizing antibodies, which are essential to prevent viral entry, while the involvement of the N-protein in the immune response is more associated with as a marker of viral infection. The N-protein is involved in packaging the viral RNA, thus forming the nucleocapsid. It plays a crucial role in the assembly of the virus [32]. While antibodies against the N-protein can be detected during infection, they may not necessarily confer a strong neutralizing activity [33]. The immune response to the N-protein is often more associated with a cellular immunity that involves T-cells [34]. It has been previously shown that the high concentration of the anti-N immunoglobulin may predict poor COVID-19 outcomes [35]. In contrast, another study showed that the level of IgG to the N-protein in the blood of individuals who had recovered from COVID-19 increased faster than the level of antibodies to the spike protein [36]. Our study showed that the anti-N IgG1 correlated with a viral load in the blood. This may indicate that the formation of these antibodies coincides with viral replication. Nevertheless, the presence of anti-N IgG at the time of hospitalization correlated with the subsequent outcome. Thus, understanding the immune response to both proteins is important for vaccine development, diagnostic testing, and gaining insights into the dynamics of the immune response against coronaviruses. Along with this, it should be noted that the dynamics of the immune response can vary between individuals, and the factors that influencec seroconversion are complex. Additionally, the severity of the infection may not be the only determinant of the timing of seroconversion; individual variability, genetics, and other factors also play a role.

Interestingly, the serum histamine concentrations were significantly higher in moderate COVID-19 than in mild or severe COVID-19, with a trend toward higher concentrations in the recovered patients compared with the deceased patients. Histamine is the main product of macrophages (MCs), which belong to the innate immune system [37]. There is evidence that MCs may play a positive role in the body's defenses in the early stages of infection. Recent studies have shown that macrophages can also produce histamine and are involved in the histamine-mediated pathogenesis [38]. The increase in histamine in the moderate COVID-19 versus the mild COVID-19 may be due to an increased antibody-dependent Fc-mediated phagocytosis by monocytes and macrophages; although this stops the viral replication, it also leads to an increased inflammation. It was found that 6% of the peripheral blood monocytes from patients with COVID-19 were infected with SARS-CoV-2; abortively, an infection of these cells with the virus was mediated by antibodies [39]. In severe COVID-19, the hyper-inflammatory response may lead to myeloid cell depletion [40], which potentially results in lower circulating levels of histamine. The role of the histamine pathway in the pathogenesis of the COVID-19 cytokine storm is discussed in the literature [41]. There is a hypothesis that histamine may play a protective role during COVID-19 by either enhancing the immune response or inhibiting viral entry

into cells. However, these hypotheses are not generally accepted, and the available evidence is limited. The findings that increased the histamines levels might play a role in the COVID-19 pathogenesis may support the use of antihistamines or histamine receptor blockers in the management of patients with coronavirus infections [42]. Understanding of the pathology of COVID-19 is still evolving, and a number of studies continue to explore the interaction between the virus and the immune system [43].

In conclusion, it should be noted that COVID-19 is a complex disease, and multiple factors contribute to its progression and severity. Research is ongoing to understand the complexities of the immune response to SARS-CoV-2 and its implications for disease severity and vaccine development.

A preprint has previously been published [44].

5. Study limitations

This study has several limitations. The serological results of this study were obtained from a small number of subjects. Because this was a retrospective study, the researchers did not have access to the patients' personal information. We were able to associate each of the samples analyzed with varying degrees of severity of the disease according to specific signs determined by the recommendations of the Ministry of Health of the Russian Federation specified in in the Materials and Methods. From the previous anamnesis, it is known that none of the examined patients were vaccinated with the Sputnik V vaccine since the study was conducted using blood sera obtained in March-April 2020 when vaccines against COVID-19 were not used.

Supplementary Materials: Table S1: COVID -19 patient characteristics depending on the gender of the participants; Table S2: COVID -19 patient characteristics depending on the age of the participants. Table S3: The correlation P-values.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

Author contributions

Conceptualization, Y.D. and A.L.; methodology, G.L.; validation, G.L., and I.K.; formal analysis, D.P.; investigation, P.C., O.K.; data curation, T.Sh. and S.P.; writing—original draft preparation, Y.D.; writing—review and editing, Y.D. and G.L.; supervision, A.S.; project administration, Y.D. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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Institutional review board statement

The study was approved by the Local Ethics committee of the FSBSI "IEM" (protocol 3/20 from 06/05/2020).

Informed consent statement

All patients signed written informed consent upon admission to the hospital.

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