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RESEARCH ARTICLE

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Long-term monitoring of the development and extinction of IgA and IgG responses to SARS-CoV-2 infection

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Abstract

Despite the great interest of the scientific community in the behavior of the human body after contact with the new coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), long-term (more than 6 months) monitoring of the immunological status of patients with coronavirus disease 2019 (COVID-19) having varying severity degrees and of the people with a low SARS-CoV-2 viral load is practically absent. The aim of this study is a 9-month monitoring of SARS-CoV-2 infection immune response development and extinction using quantitative assessment of IgA and IgG levels in the blood of healthy donors living in the context of the coronavirus pandemic and of the patients who have undergone COVID-19. The project involved 180 volunteers, of whom 51 persons (28.33%) fell ill with COVID-19 during the observation period. All people who underwent COVID-19 developed a stable humoral immune response but their individual immune status had a number of features. Approximately 39.22% (20 of 51 people) of project participants diagnosed with COVID-19 showed an unusual change in plasma anti-SARS-CoV-2 IgA levels. Relatively high levels of IgA (ratio ~ 3) after recovery persisted for a long time (more than 6 months). In one-third (17 of 51 people) of patients with COVID-19, the IgA level exceeded the IgG level. IgA antibodies appeared earlier and showed a stronger and more robust response to the SARS-CoV-2 virus than IgG. Increased levels of anti-SARS-CoV-2 IgA (ratio from 0.8 to 2.36) throughout the observation period were recorded in 28 of 180 project participants (15.56%) of whom only one person fell ill with COVID-19.

KEYWORDS
anti-SARS-CoV-2 immunoglobulins, COVID-19, humoral immunity, long-term monitoring

1 | INTRODUCTION

The global pandemic of the new coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in 2020 has challenged the entire global medical community. SARS-CoV-2 has shown extreme prevalence due to its extremely high contagiousness and the long incubation period before symptoms of coronavirus disease 2019 (COVID-19) appear. It is difficult to control asymptomatic carriers of the virus, newly infected individuals during the incubation period, and clinically recovered patients who are still infected with the virus. An additional problem in the fight against infection is the wide range of COVID-19 clinical manifestations from asymptomatic carriage to severe acute respiratory distress syndrome leading to severe complications and deaths.[1] Despite significant advances in the scientific description of the coronaviruses biology,[2,3] the development of antiviral vaccines and the creation of effective diagnostic test systems based on polymerase chain reaction (PCR),[4]

enzyme-linked immunosorbent assay (ELISA),[5] and chemiluminescence immunoassay,[6] a number of aspects of the COVID-19 development remain unclear. The study of the nature, severity, and duration of the human immune response after contact with the SARS-CoV-2 coronavirus and the assessment of the antibodies prevalence in the population will make it possible to predict the epidemiological situation development in distinct countries more accurately and to plan measures to prevent the infection. Today, it is obvious that the immune response to SARS-CoV-2 infection includes all elements of the humoral immunity system and the cellular immunity system.[3,7–9] Neutralizing antibodies play a protective role in limiting infection and preventing re-infection in the future.[9] Specific antibody monitoring is useful both for confirming SARS-CoV-2 infection in PCR-positive COVID-19 patients, which is important for infected but asymptomatic subjects and for patients with COVID-19 who are screened several weeks after onset. Serological tests are necessary to check the sensitivity or resistance to re-infection as well as for epidemiological studies and the implementation of control and surveillance activities because anti-SARS-CoV-2 IgM, IgA, and IgG levels are important indicators for assessing herd population immunity against SARS-CoV-2.[3,10] In this regard, long-term monitoring of the specific antiviral immunoglobulins levels is of undoubted interest both from a scientific and a practical point of view. Unfortunately, most of the immunological studies are associated with severe cases of viral disease caused by the new coronavirus. So far, there are very few publications devoted to long-term monitoring of the immunological status of patients who have undergone COVID-19 and people with a low viral load of SARS-CoV-2.[11,12] Today, there is no clear answer to the question of whether an immune response to SARS-CoV-2 is formed in people who have been forced to exist for a long time in the environment of COVID-19 infection, and how long the protective pool of antibodies lasts after the disease ends.

The present study is aimed at filling the gap in knowledge about the effects of the new coronavirus on the human body in terms of humoral immunity. The objectives of our project included a long-term analysis of the development dynamics of the immune response to SARS-CoV-2 infection through a quantitative assessment of IgA and IgG levels in the blood of healthy donors living in the context of the coronavirus pandemic and patients who had undergone COVID-19.

2 | MATERIALS AND METHODS

2.1 | Study participants

The study involved 180 people: 84 men and 96 women. The age of the project participants: women from 22 to 53 years old (average age 33.51 ± 6.38), men from 23 to 48 years old (average age 34.70 ± 5.73). All study participants were office employees of a business company based in St. Petersburg (Russia).

The research was carried out in the Saint-Petersburg State University Hospital (St. Petersburg, Russia). The data from the SARS-CoV-2 coronavirus test were taken by reverse-transcription polymerase chain reaction (RT-PCR) and the levels of anti-SARS-CoV-2-specific IgA and IgG were taken using ELISA. The testing frequency RT-PCR and ELISA was 10–14 days. The monitoring was carried out for about 10 months: from May 27, 2020, to March 19, 2021. During the observation period, the total number of tests (PCR + ELISA) per person reached 32–36.

At the initial stage, COVID-19 was diagnosed with a positive PCR test. Subsequently, the disease severity was assessed based on the project participants' testimony. In all patients with COVID-19 (51 people) the disease was asymptomatic or relatively mild. No serious or critical conditions were recorded. No one was hospitalized. In most cases of mild COVID-19, symptoms of a mild respiratory illness were observed: malaise, accompanied by a slight increase in temperature for several days, headache, and runny cough. In 14 project participants, a short-term loss of smell was noted.

2.2 | Ethical statement

The authors declare that the use of human biological material (blood and flushes from the mucous membranes of the nose and throat) was approved by the Biomedical Ethics Board of the Saint-Petersburg State University Hospital, protocol # No. 07/20 from 16.07.2020 (Russia). All measurements were performed based on ethical guidelines of the 1975 Declaration of Helsinki. All study participants completed and signed informed consent to participate in this study project and to publish the results. All clinical data were depersonalized.

2.3 | Genetics testing

The presence of SARS-CoV-2 virus RNA was tested with RT-PCR using the swabs from nose and throat mucous membranes. A fully automatic Cobas 6800 platform and reagents manufactured by Roche (Switzerland) were used. All reactions were performed in accordance with the manufacturer's instructions.

2.4 | ELISA

Immunoassay for anti-SARS-CoV-2 IgA and IgG semiquantitative determination in serum was carried out using Euroimmun (Lubeck) reagent kits. The S1 domain of the SARS-CoV-2 thorn glycoprotein was used as an antigen. Venous blood was collected on an empty stomach using vacuum tubes Lind-Vac with a coagulation activator and gel. HydroFlex automatic microplate washer, Infinite F50 reader, and Magellan V.7.2. software (all by Tecan) were used.

Euroimmun recommends interpreting results as follows: ratio less than 0.8—negative for both IgA and IgG. Calculate the ratio

according to the following formula: Extinction of the control or patient sample/Extinction of calibrator.

2.5 | Statistical analysis

Statistical processing of research results was carried out using standard statistical functions of spreadsheets Microsoft Excel2007 (Statistical Package Microsoft Office 97 for Windows).

3 | RESULTS

3.1 | Project participants age characteristics and features of their immune status

Among 180 participants (84 men and 96 women) 129 people (58 men and 71 women) did not get COVID-19 during the observation period. Respectively, 51 people (28.33%) (26 men and 25 women) were diagnosed with COVID-19 using PCR analysis during the observation period. Consequently, there is no significant difference in the number of men and women with COVID-19. The peak incidence occurred in November 2020–January 2021 but there were several cases in June–July 2020.

The age of participants: women from 22 to 53 years old (average age 33.51 ± 6.38), men from 23 to 48 years old (average age 34.70 ± 5.73).

Age of non-COVID-19 women: 22–53 years (mean age 33.59 ± 6.82); non-COVID-19 men: 24–48 years (mean age 34.75 ± 5.66).

The age of women who had COVID-19 during the observation period: from 27 to 45 years (average age 33.43 ± 5.54), the age of men who had COVID-19 during the observation period: from 23 to 44 years (average age 34.58 ± 5.93).

The mean age of non-COVID-19 patients in both groups (men and women) was 34.11 ± 6.23 years and the average age of study participants who had COVID-19 during the observation period is 34.01 ± 5.73 years. Thus, no relationship was found between the age of the study participants and the susceptibility to SARS-CoV-2.

Figure 1 shows the most typical examples of anti-SARS-CoV-2 IgA level and IgG level dynamics in the blood plasma of some study participants who were diagnosed with RT-PCR of COVID-19 at different times. In all cases, the disease was asymptomatic or relatively mild. There were no severe or critical conditions in patients in the analyzed group. All people who had COVID-19 developed a stable humoral immune response but each individual immune status had a number of features.

Along with the typical [3] humoral immune response development and extinction during a viral illness and after recovery (Figure 1A) 20 of 51 project participants diagnosed with COVID-19 (39.22%) showed unusual behavior of plasma immunoglobulin A level slightly different from the "classic" humoral immune response to viral infections (Figure 1B,C). A relatively high level of IgA after suffering COVID-19 persisted for a long time (more than 6 months). At the same time, the IgG level dynamics corresponded to the concept of typical humoral immunity in viral infection.[3]

Figure 2 shows the dynamics of IgA and IgG mean values levels for all participants in the project who underwent COVID-19. A 4-month time period was analyzed: 2 weeks before the confirmed (RT-PCR) infection and the following 3.5 months.

The mean level of anti-SARS-CoV-2 IgA (Figure 2A) showed rather high values already after 2 weeks from the onset of the disease, and it reached maximum values approximately 2–3 weeks later. The average level of anti-SARS-CoV-2 IgG (Figure 2B) showed a significant increase later, after 1 month after the diagnosis of COVID-19, and peaked in another month. At the same time, IgA levels significantly exceeded IgG levels (1.5–3 times). Then a gradual decrease in antibody levels was recorded throughout the observation period. After recovery plasma, IgA remained at a fairly high level for a long time (ratio ~ 3) and one-third of participants diagnosed with COVID-19 (17 of 51 people, 33.33%) had IgA levels higher than IgG levels. Thus, we can state that IgA antibodies appeared earlier and demonstrated a stronger and more stable response to the SARS-CoV-2 virus than IgG.

3.2 | A group of study participants with consistently elevated anti-SARS-CoV-2 IgA level

Elevated plasma IgA levels (ratio ≥ 0.8) throughout the observation period were recorded in 28 of all 180 volunteers (15.56%). In that group, 27 people did not fall ill with COVID-19 and only one study participant was diagnosed with COVID-19 after 4 months from the beginning of observation (Figure 1D). The maximum IgA level recorded value for the entire observation period in this group of study participants who did not have COVID-19 had a ratio = 2.36. Figure 3A shows the dynamic change in the mean values of serum class A immunoglobulins over 7 months of observation in the group of patients with elevated IgA levels, who did not have COVID-19. Slight increase in the average IgA level (ratio 0.8–1.2) over the observation period was fixed.

At the same time, the IgG levels in this group did not exceed the borderline values characterizing the status of healthy patients (ratio < 0.8) and did not show any tendency to increase or decrease. The averaged data for this indicator are shown in Figure 3B.

4 | DISCUSSION

We found no relationship between age, sex, and SARS-CoV-2 susceptibility. All project participants diagnosed with COVID-19 had asymptomatic or mild disease. It is of importance that the study involved relatively young people leading an active life. These results correspond to modern ideas about the disease severity in the bulk of people infected with the new coronavirus: approximately 80% of infections are mild or asymptomatic, 15% are severe, and 5% are

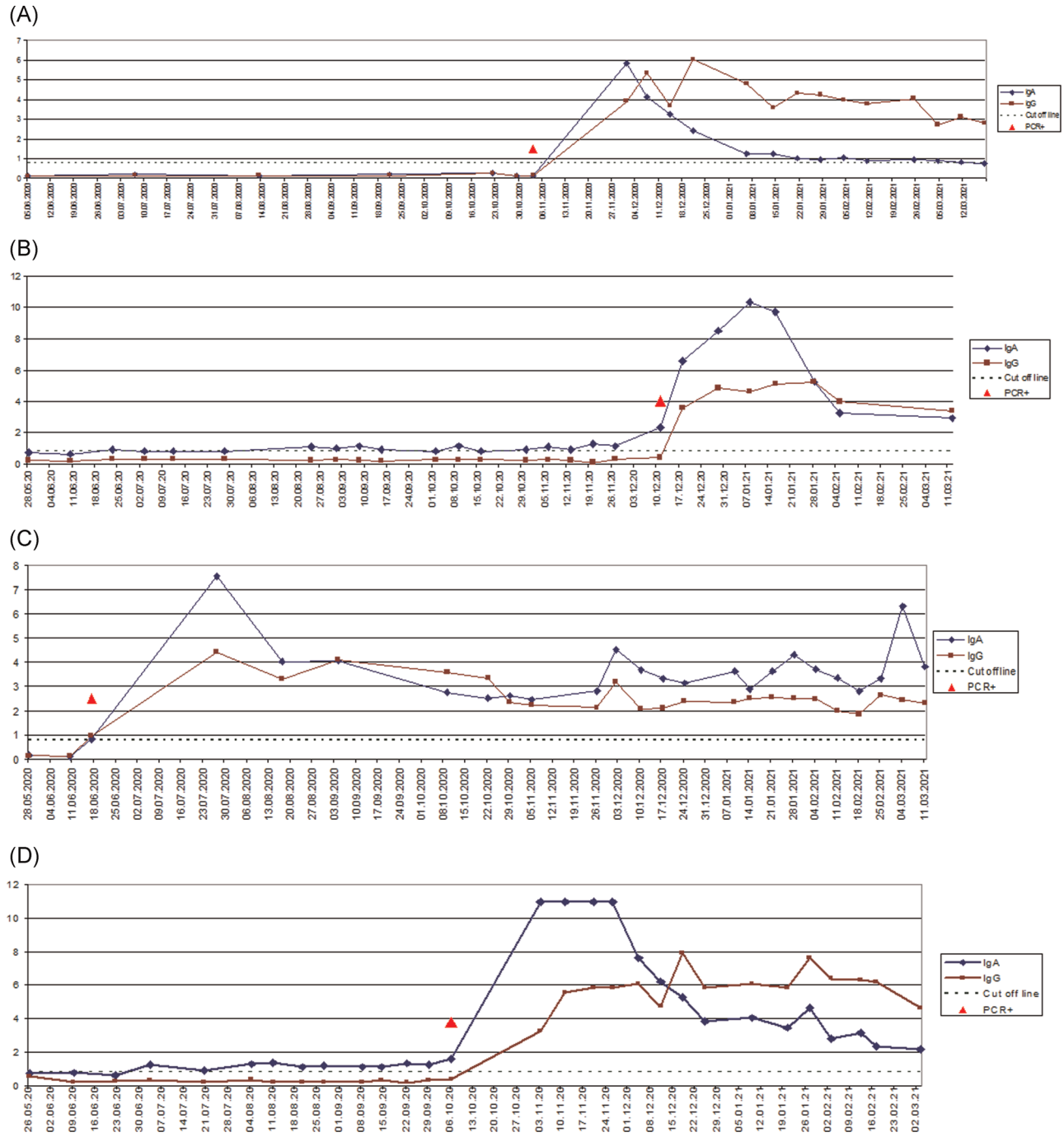

FIGURE 1 Typical examples of changes over time in anti-SARS-CoV-2 IgA and IgG levels in the blood plasma of study participants diagnosed with COVID-19. (A) Relatively "classic" variant of the humoral immune response to a viral disease. IgA level dropped to normal values. IgG level was gradually decreasing. (B) An example of IgA and IgG levels dynamics typical for the majority of study participants. After the illness, the concentrations of immunoglobulins A and G in plasma decreased but remained at fairly high levels (ratio ~3). (C) An example of the long-term persistence of relatively high antibody levels after illness. Small peaks in early December and March may be associated with re-exposure to the virus. Due to persistent immunity, the disease did not occur but the immune status changed fractionally. (D) An example of the disease with the background of elevated IgA. Changes in IgA and IgG levels in the only study representative of the group of people with initially elevated IgA levels, who were diagnosed with COVID-19 in October 2020. The abscissa shows testing dates; the ordinate is immunoglobulins ratio. Blue icons are IgA, red icons are IgG. Dotted line is the cut-off 0.8 ratio. Red triangles are the points of the PCR positive test. COVID-19, coronavirus disease 2019; PCR, polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2

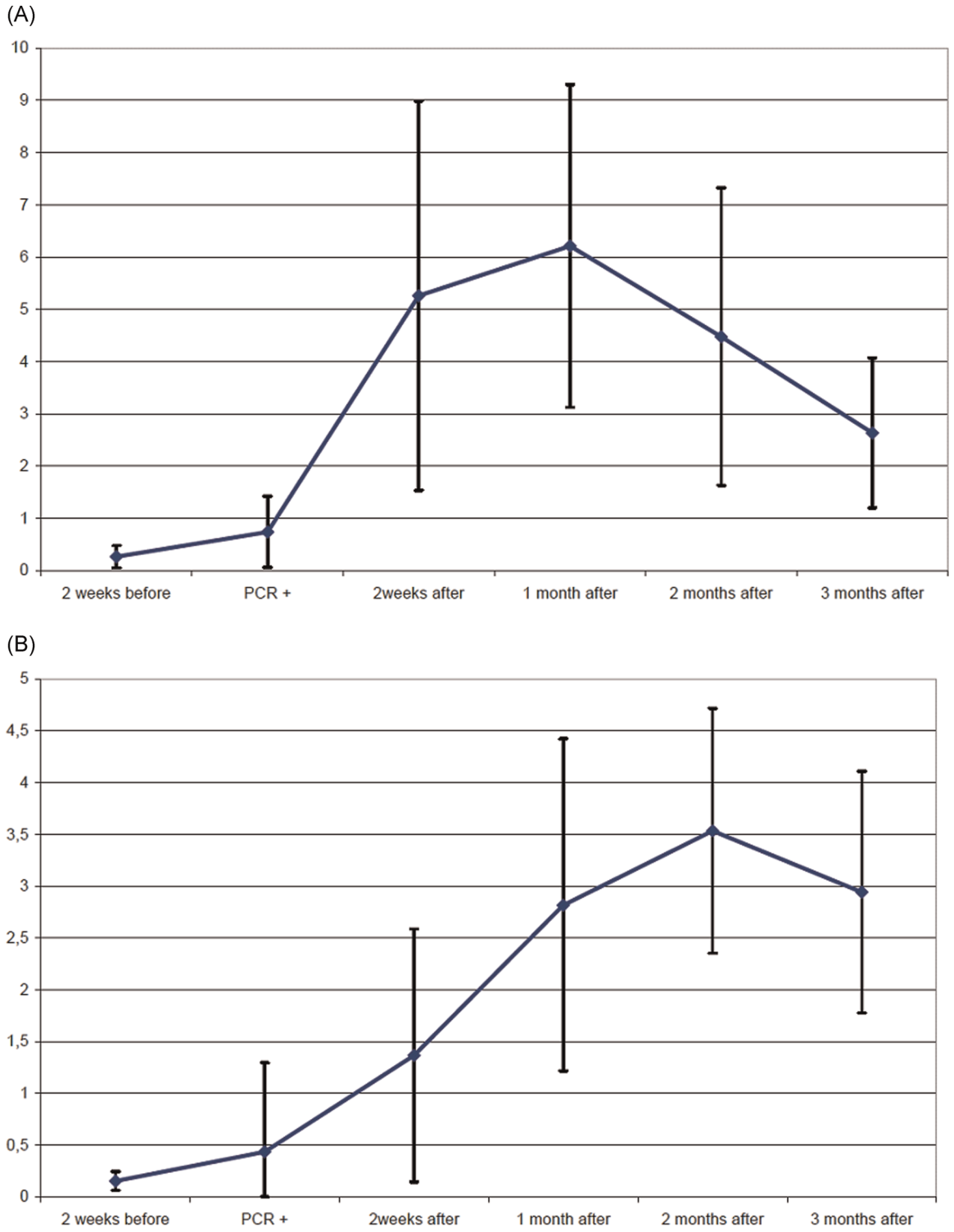

FIGURE 2 Dynamics of the mean levels of anti-SARS-CoV-2 IgA and IgG for study participants diagnosed with COVID-19. (A) Mean level of anty-SARS-CoV-2 IgA. (B) Mean level of anty-SARS-CoV-2 IgG The abscissa is testing frequency; the ordinate is immunoglobulins ratio. Points are the average value of IgA and IgG ratio after a certain period of time after the COVID-19 diagnosis. Whiskers are mean standard deviation. COVID-19, coronavirus disease 2019; PCR, polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2

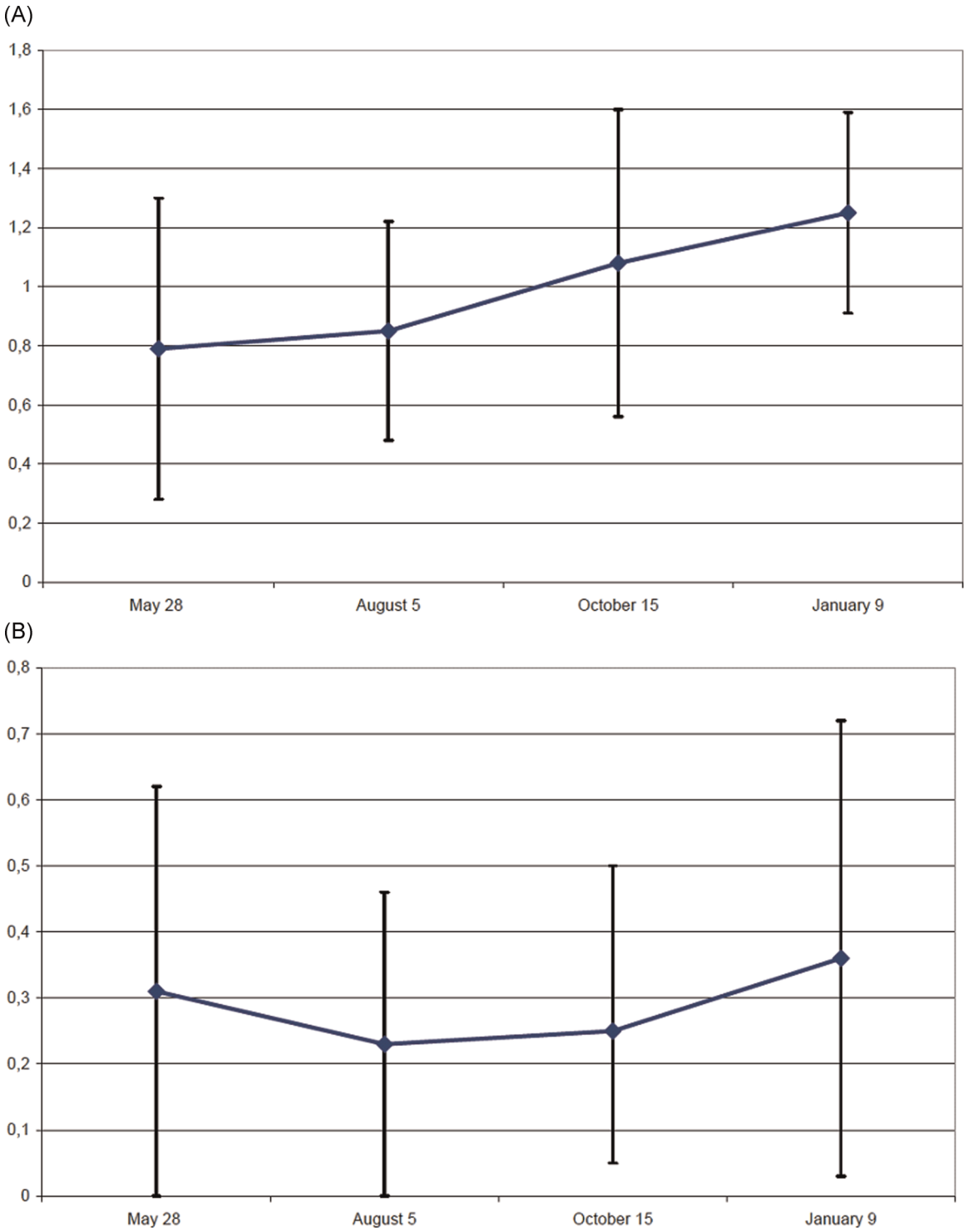

FIGURE 3 Dynamics of the mean levels of class A and G serum immunoglobulins in the group of the patients with elevated IgA levels (ratio ≥ 0.8). (A) Mean IgA level in the group with stable elevated IgA level not sick with COVID-19. (B) Mean IgG level in the group with stable elevated IgA level not sick with COVID-19. The abscissa is the date of testing; the ordinate is the immunoglobulin ratio. Points are the average value of IgA and IgG ratio after a certain period of time after the COVID-19 diagnosis. Whiskers are mean standard deviation. COVID-19, coronavirus disease 2019

critical conditions requiring lungs ventilation.[13] Most often patients experience 5–7 days of malaise character of respiratory viral infections followed by the development of the neutralizing antiviral T- and B-cell immunity and specific antibodies formation.[3]

In our study, the humoral immune response in patients with RT-PCR confirmed SARS-CoV-2 infection was highly variable. However, in a considerable group of people class A immunoglobulins appeared and their levels rose at an earlier stage reaching rather high levels as early as 2 weeks after the onset of disease signs. It was a stronger and more stable response in comparison to IgG. Apparently, we estimated the level of IgA1 monomer as more than 90% of blood IgA was present in the form of IgA1.[14] The maxima in IgA concentrations were recorded approximately 1 month after the diagnosis of COVID-19. Subsequently, plasma IgA remained at a fairly high level for a long time (ratio ~ 3) and in 17 of 51 project participants who had been ill with COVID-19 (33.33%) the IgA level exceeded the IgG level. Thus, the IgA behavior (growth dynamics after infection with SARS-CoV-2 and maintaining relatively high levels for a long time) was different from that for SARS-CoV during the 2003 epidemic.[15] The dynamics of the anti-SARS-CoV-2 IgG level generally corresponded to the classical concepts of the humoral immune response development in viral infections.[3]

Among all the project participants, there was a fairly large group of people (15.56%) in whom elevated levels of plasma anti-SARS-CoV-2 IgA (ratio from 0.8 to 2.36) were recorded throughout the observation period. The simplest explanation for this phenomenon was the cross-reactivity of antibodies to proteins from non-SARS-CoV-2 coronaviruses. Indeed cross-reactivity is a potential problem in the interpretation of the serological test as positive results may be due to past or present infection with other human coronaviruses.[16,17] Several anti-SARS-CoV Mab (human monoclonal antibodies) show cross-neutralizing activity against the S-protein (spike transmembrane glycoprotein) SARS-CoV-2.[18,19] In particular cross-neutralizing monoclonal antibodies MAb362 are described that interact with SARS-CoV-2 RBD (receptor-binding domain of protein S) with high affinity and block interactions with the receptor ACE2 (human angiotensin-converting enzyme 2).[20] Such IgA present as a monomer in serum or secretory dimer neutralizes both the pseudotypic SARS-CoV virus particles and the true SARS-CoV-2 virus in cells expressing ACE2. Interestingly, the efficiency of neutralizing IgG which blocks the interaction with ACE2 is significantly lower. Analysis of the COVID-19 patients' plasma samples interaction with antibodies against the S-protein from SARS-CoV-2 and SARS-CoV also reveals cross-reactivity.[21] About 3% of a fairly large group of healthy volunteers without COVID-19 gave a positive response to the presence of SARS-CoV-2 specific IgA.[22] In 7% of blood samples from patients with COVID-19, borderline cross-reactivity of IgA and IgG antibodies with human coronaviruses NL63 and OC43 is found.[23]

However, in the present study, the percentage of people with elevated IgA levels was higher. In addition, there was a slight increase in the mean anti-SARS-CoV-2 IgA level (ratio 0.8–1.2) over time in this group (Figure 3A). Apparently, this phenomenon was not the result of the cross-reactivity alone. It is possible that SARS-CoV-2-specific IgA plays an independent role in providing protective immunity at low viral loads. However, elevated IgA levels were not absolute protection against COVID-19 disease: one of the project participants who demonstrated elevated plasma IgA values for a long period was diagnosed with COVID-19 after 4 months of observation (Figure 1D).

So, a characteristic feature of the manifestation of humoral immunity in response to a new infection namely the atypical behavior of anti-SARS-CoV-2 IgA was described. A long-term IgA level increase in a substantial group of healthy donors with an active lifestyle was established (leading an active life или with an active lifestyle). The persistence of IgA elevated levels for a long time, which did not decrease after the termination of the acute phase of the COVID-19 disease, was monitored in patients.

Further research is needed on the functions of virus-specific anti-SARS-CoV-2 antibodies and their protective efficacy over time. Given the underestimated role of IgA in COVID-19 and the importance of long-term monitoring of SARS-CoV-2 specific IgA levels[20,22] more attention should be paid to IgA for COVID-19 diagnosis and in assessing the immune status of SARS-CoV-2 infected patients.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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REFERENCES

1. Matricardi PM, Dal Negro RW, Nisini R. The first, holistic immunological model of COVID-19: implications for prevention, diagnosis, and public health measures. *Pediatr Allergy Immunol*. 2020; 31(5):454-470.
2. Zhou P, Yang X-L, Wang X-G, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature*. 2020; 579(7798):270-273.
3. Azkur AK, Akdis M, Azkur D, et al. Immune response to SARS-CoV-2 and mechanisms of immunopathological changes in COVID-19. *Allergy*. 2020;75(7):1564-1581.
4. Mizumoto K, Kagaya K, Zarebski A, Chowell G. Estimating the asymptomatic proportion of coronavirus disease 2019 (COVID-19) cases on board the Diamond Princess cruise ship, Yokohama, Japan, 2020. *Euro Surveill*. 2020;25(10):2000180.
5. Amanat F, Stadlbauer D, Strohmeier S, et al. A serological assay to detect SARS-CoV-2 seroconversion in humans [published online ahead of print March 17, 2020]. *MedRxiv*. 2020.
6. Padoan A, Cosma C, Sciacovelli L, Faggian D, Plebani M. Analytical performances of a chemiluminescence immunoassay for SARS-CoV-

2 IgM/IgG and antibody kinetics. *Clin Chem Lab Med*. 2020;58(7):1081-1088.
7. Li G, Fan Y, Lai Y, et al. Coronavirus infections and immune responses. *J Med Virol*. 2020;92(4):424-432.
8. Baruah V, Bose S. Immunoinformatics-aided identification of T cell and B cell epitopes in the surface glycoprotein of 2019-nCoV. *J Med Virol*. 2020;92(5):495-500.
9. Gorse GJ, Donovan MM, Patel GB. Antibodies to coronaviruses are higher in older compared with younger adults and binding antibodies are more sensitive than neutralizing antibodies in identifying coronavirus-associated illnesses. *J Med Virol*. 2020;92(5):512-517.
10. Deng W, Bao L, Liu J, et al. Primary exposure to SARS-CoV−2 protects against reinfection in rhesus macaques. *Science*. 2020;369(6505):818-823.
11. Mai HK, Trieu NB, Long TH, et al. Long-term humoral immune response in persons with asymptomatic or mild SARS-CoV-2 infection, Vietnam. *Emerg Infect Dis*. 2021;27(2):663-666.
12. Wu J, Liang B, Chen C, et al. SARS-CoV-2 infection induces sustained humoral immune responses in convalescent patients following symptomatic COVID-19. *Nat Commun*. 2021;12(1):1813.
13. Rokni M, Ghasemi V, Tavakoli Z. Immune responses and pathogenesis of SARS-CoV-2 during an outbreak in Iran: comparison with SARS and MERS. *Rev Med Virol*. 2020;30(3):e2107.
14. Schroeder HW, Cavacini L. Structure and function of immunoglobulins. *J Allergy Clin Immunol*. 2010;125(202):41-52.
15. Woo P, Lau S, Wong B, et al. Detection of specific antibodies to severe acute respiratory syndrome (SARS) coronavirus nucleocapsid protein for serodiagnosis of SARS coronavirus pneumonia. *J Clin Microbiol*. 2004;42:2306-2309.
16. Patrick DM, Petric M, Skowronski DM, et al. An outbreak of human coronavirus OC43 infection and serological cross-reactivity with SARS coronavirus. *Can J Infect Dis Med Microbiol*. 2006;17:330-336.
17. Cheng MP, Papenburg J, Desjardins M, et al. Diagnostic testing for severe acute respiratory syndrome-related coronavirus 2: a narrative review. *Ann Intern Med*. 2020;172(11):726-734.
18. Pinto D, Park Y-J, Beltramello M, et al. Cross-neutralization of SARS-CoV-2 by a human monoclonal SARS-CoV antibody. *Nature*. 2020;583:290-295.
19. Yuan M, Wu NC, Zhu X, et al. A highly conserved cryptic epitope in the receptor-binding domains of SARS-CoV-2 and SARS-CoV. *Science*. 2020;368:630-633.
20. Ejemel M, Li Q, Hou S, et al. A cross-reactive human IgA monoclonal antibody blocks SARS-CoV-2 spike-ACE2 interaction. *Nat Commun*. 2020;11:4198.
21. Lv H, Wu NC, Tsang OT, et al. Cross-reactive antibody response between SARS-CoV-2 and SARS-CoV infections. *Cell Rep*. 2020;31(9):107725.
22. Huang Z, Chen H, Xue M, et al. Characteristics and roles of severe acute respiratory syndrome coronavirus 2-specific antibodies in patients with different severities of coronavirus 19. *Clin Exp Immunol*. 2020;202(2):210-219.
23. Beavis KG, Matushek SM, Abeleda APF, et al. Evaluation of the EUROIMMUN anti-SARS-CoV-2 ELISA assay for detection of IgA and IgG antibodies. *J Clin Virol*. 2020;129:104468.

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