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Immunology

LONGITUDINAL EVALUATION OF THE ANTI-NUCLEOCAPSID IGG AFTER THE CONFIRMED COVID-19 IN NORTHERN CROATIAN POPULATION

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ABSTRACT

BACKGROUND: This study longitudinally evaluated the IgG response against the N-protein after the onset of COVID-19 infection. We determined the kinetics and magnitude of the antibody response against SARS-CoV-2 in confirmed COVID-19 patients who were the first infected with SARS-CoV-2 in Krapina-Zagorje county in northern Croatia.

MATERIALS AND METHODS: We studied 177 blood specimens from 51 patients who tested positive by PCR for COVID-19 and provided longitudinal blood samples over a duration of several months, allowing to evaluate the IgG response against the N-protein. SARS-CoV-2 IgG assay was interpreted as positive (ratio1.4 S/C) or negative (ratio<1.4 S/C).

RESULTS: The majority of subjects (48/51) reported symptomatic disease. Among the 49 patients who underwent serological antibody testing at first time point (median: 47 days), 47/49 were positive for IgG 6.02 (0.24-10.54 S/C), while at sixth time point (median: 275 days) 4/16 patients were positive for IgG, 9/16 were in grey zone, and 3/16 were negative. Using Wilcoxon statistical analysis we found statistically significant decrease of SARS-CoV-2 N-protein IgG indices between the first and the sixth time point (median signal to cut-off ratio, S/C, 8.18 IQR 6.91, 9.51 to 0.94 IQR 0.56, 1.18, P=0.001).

CONCLUSIONS: We clarified the kinetics and magnitude of the antibody response against SARS-CoV-2 in confirmed COVID-19 patients. Our results provide critical evidence that N-protein IgG response persists in the majority of patients for at least six to eight months after COVID-19 infection.

KEYWORDS : COVID-19, IgG, SARS-CoV-2, serological assay

1. INTRODUCTION

Coronavirus disease 2019 (COVID-19), a novel severe respiratory infection caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was first identified in Wuhan, China in December 2019 and from thereon rapidly spread worldwide 1. Due to its swift global progression, the World Health Organization (WHO) proclaimed COVID-19 a pandemic in March 2020 2. The first case of COVID-19 in Croatia was confirmed on the 25th of February 2020 and during the next two weeks, only intermittent new cases were detected. However, daily new cases had been increasing steadily 3. By March 30th, 2021, a total of 267 522 confirmed cases, 252 321 recovered, and 5 911 deaths had been reported by the Croatian Institute of Public Health 4. The majority of COVID-19 patients have a three- to seven-day incubation period when there is no symptom onset, but there is a possibility of contagion 5. The usual spread of COVID-19 particles, in that case, happens via respiratory droplets 6. After the incubation period, the most common symptoms are fever, cough, and shortness of breath, but as COVID-19 continued to spread, new symptoms have emerged, such as chills, myalgia, headache, sore throat, and loss of taste and smell 7.

The SARS-CoV-2 genome encodes 30 proteins, four of which encode structural proteins Spike (S), Membrane (M), Envelope (E), and Nucleocapsid (N). The S protein is composed of two subunits, N-terminal S1 and C-terminal S2. The S1 subunit contains a receptor-binding domain (RBD) which is involved in binding to the host receptor angiotensin-converting enzyme 2 (ACE2). The spiral-shaped N-protein is involved in transcription, replication, and virus packaging. Both S and N-proteins show high antigenicity, so serological assays are most commonly based on the detection of specific antibodies against those two SARS-CoV-2 antigens 8.

A standard method for screening and diagnosis of acute SARS-CoV-2 infection is reverse-transcriptase polymerase chain reaction (RT-PCR) from respiratory samples acquired through the nasal or oropharyngeal swab. The sensitivity of this method depends on the sampling time, quality, and origin of the sample, as well as the viral load 9. Serological testing has great importance for the diagnosis of patients with characteristic COVID-19 symptoms, but with negative or undoable RT-PCR and it can be easily done from a blood sample 10. SARS-CoV-2 infection has an unusual immune response where specific IgM and IgG appear in serum practically simultaneously after two to three weeks from the disease onset. For that reason, it is not a common practice to measure IgM without IgG 11. Previous clinical findings show that anti-SARS-CoV-2 antibodies start developing six to ten days after the infection with the highest IgM titers occurring after 12 days and persisting for 35 days. On the other hand, IgG reaches the highest titers 17 days after infection and persists up to 49 days from the beginning of infection 12,13,14.

This study aimed to longitudinally evaluate the IgG response against the N-protein after the onset of COVID-19 infection. We determined the kinetics and magnitude of the antibody response against SARS-CoV-2 in confirmed COVID-19 patients who were the first infected with SARS-CoV-2 in Krapina-Zagorje county in northern Croatia and followed up for several months to determine how sustainable the antibody response against SARS-CoV-2 is.

2. MATERIALS AND METHODS

2.1 Subjects

A cohort study involved 177 blood specimens from 51 patients who presented to General Hospital Zabok and Croatian Veterans Hospital

(Krapina-Zagorje county) between March and April 2020 with symptoms consistent with a respiratory tract infection and tested positive by PCR for COVID-19. In period from 25th of February to 30th of April 2020, there were total of 139 cases of confirmed COVID-19 infection in Krapina-Zagorje county 15. Sampling was random during the day, with the patient's informed signed consent for scientific research. It was performed at the Department of Medical Biochemistry Laboratory in General Hospital Zabok and Croatian Veterans Hospital. Blood samples were taken into clot activator tubes (Greiner bio-one, Austria) according to CLSI venous blood sample guidelines 16. Serum samples were centrifuged for 10 minutes at 3000g and they were analyzed the same day. Most subjects provided longitudinal blood samples over a duration of several months (two to six timepoints), allowing for longitudinal assessment of immune memory in a subset of the cohort. At first timepoint, median: 47 days after disease onset, 49/51 patients came to the sampling, at second timepoint, median: 79 days after disease onset 51/51, at third timepoint, median: 116 days after disease onset 30/51, at fourth timepoint, median: 161 days after disease onset 17/51, at fifth timepoint, median: 200 days after disease onset 12/51, and at sixth timepoint, median: 275 days after disease onset, 16/51 patients came to the sampling. General characteristics and clinical findings in 51 patients with confirmed COVID-19 are shown in Table 1. Subjects (32/51 female, 19/51 male) represented a range of asymptomatic, mild, moderate, and severe COVID-19 cases, and were recruited from multiple sites throughout the Krapina-Zagorje county. To corroborate our findings, antibody indices of 49 prototypical patients with repetitive sampling were analysed, as well for two unusual patients who did not develop a measurable IgG-N response while their PCR results were positive.

Table 1. General characteristics and clinical findings in 51 patients with confirmed COVID-19

Patient characteristic	No=51
Age median (range)	44 (5-77)
Male/female	19/32
PCR positive	50
Asymptomatic	3
Mild disease	43
Hospitalized severe	5
Hospitalized (ICU)	0
Non-hospitalized	46
Respiratory symptoms	
Cough	48
Fatigue	23
Shortness of breath	24
Sore throat	12
Nasal congestion	7
Systemic symptoms	
Fever (temperature >38.0˚C)	17
Chills	16
Myalgia and arthralgia	11
Headache	9
Diarrhea	4
Loss of smell	6

2.2 METHODS

The Abbott SARS-CoV-2 IgG assay was performed on an Abbott Architect i1000SR (Abbott Diagnostics, USA) according to the manufacturer's instructions. The method was verified according to the EPA (the United States Environmental Protection Agency) guidelines. This assay is an automated, two-step immunoassay for the qualitative detection of IgG antibodies to N-protein of SARS-CoV-2 in human serum and plasma using chemiluminescent microparticle immunoassay (CMIA) technology. The presence or absence of IgG antibodies to SARS-CoV-2 in the sample is determined by comparing the chemiluminescent relative light unit (RLU) in the reaction to the calibrator RLU, which is calculated by the system as an Index (signal to cut-off ratio, S/C) and can be interpreted as positive (ratio≥1.4 S/C) or negative (ratio<1.4 S/C).

2.3 Statistical analysis

Statistical analysis was performed using Medcalc version 10.1.2. (Medcalc software bvba, Ostend, Belgium). The normality of data distribution was examined by D'Agostino-Pearson statistical test. The kinetics of antibody levels determined by different sampling timepoints from the onset of symptoms were calculated using Wilcoxon statistical analysis. A significant difference was determined as $P<0.05$.

3. RESULTS

A total of 177 samples were obtained from 51 COVID-19 patients with a median age of 44 (5-77) and collected for more than 250 days after disease onset. The majority of subjects had a mild case of COVID-19, not requiring hospitalization. 46/51 of subjects were never hospitalized for COVID-19, 5/51 of subjects were hospitalized, but did not require intensive care unit (ICU). The majority of subjects (48/51) reported symptomatic disease with fever, cough, and shortness of breath as the most common symptoms. Patients were categorized as shown in Table 2. Among the 49 patients who underwent serological antibody testing 28-60 days after disease onset (median: 47 days, Index 1), 47/49 were positive for IgG 6.02 (0.24-10.54 S/C) antibodies. Two subjects were negative for IgG. Among the 51 patients who were tested for serological antibody 56-92 days after infection (median: 79 days, Index 2), 47/51 were positive for IgG 5.25 (0.25-11.95 S/C) antibodies while 4/51 were negative. Among the 30 patients who underwent serological antibody testing 96-137 days after disease onset (median: 116, Index 3), 24/30 were positive for IgG 3.81 (0.27-9.75 S/C) antibodies. 6/30 patients became negative for antibodies. Among the 17 patients who were tested for serological antibody 150-191 days after infection (median: 161 days, Index 4), 11/17 were still positive for IgG 1.79 (0.12-7.67 S/C) antibodies, and 6/17 were negative for antibodies. Among the 12 patients who were tested for serological antibody 182-249 days after infection (median: 200 days, Index 5), 6/12 were still positive for IgG 1.39 (0.10-4.76 S/C), while 2/12 patients were in the grey zone and 4/12 patients were negative for IgG antibodies. Among the 16 patients who underwent serological antibody testing for more than 250 days after infection (median: 275 days, Index 6), 4/16 patients were still positive for IgG 1.29 (0.09-4.07 S/C) antibodies, 9/16 patients were in the gray zone and 3/16 patients were negative for antibodies.

Table 2. Summary statistics of IgG (S/C) indices in 177 samples from 51 patients measured longitudinally up to more than 250 days, categorized by sampling intervals in days after disease onset.

Sampling interval in days (median)	2019-nCoV IgG: Sample number Median (min-max) Positive/Total
Index 1 28-60 (47)	49 6.02 (0.24-10.54) 47/49
Index 2 56-92 (79)	51 5.25 (0.25-11.95) 47/51
Index 3 96-137 (116)	30 3.81 (0.27-9.75) 24/30
Index 4 150-191 (161)	17 1.79 (0.12-7.67) 11/17
Index 5 182-249 (200)	12 1.39 (0.10-4.76) 6/12
Index 6 >250 (275)	16 1.29 (0.09-4.07) 4/16

Relative change in index antibody level determined by different sampling time points from the disease onset are shown in Table 3. The SARS-CoV-2 N-protein indices decreased between the first (Index 1) and second (Index 2) follow-up (median signal to cut-off ratio, S/C, 6.05 IQR [4.16, 8.91] to 5.07 IQR [2.95, 7.92], $P<0.001$). Further SARS-CoV-2 N-protein indices follow the decrease between the second (Index 2) and third (Index 3) follow-up (median signal to cut-off ratio, S/C, 6.78 IQR [3.42, 8.32], to 3.81 IQR [2.26, 6.18], $P<0.001$). The SARS-CoV-2 N-protein indices were significantly decreased between the third (Index 3) and fourth (Index 4) follow-up (median signal to cut-off ratio, S/C, 3.47 IQR [1.39, 6.18] to 1.75 IQR [0.64, 3.24], $P=0.001$). Furthermore, SARS-CoV-2 N-protein indices continue to decrease between the fourth (Index 4) and fifth (Index 5) follow-up (median signal to cut-off ratio, S/C, 2.35 IQR [0.72, 4.18] to 1.32 IQR [0.46, 1.82], $P=0.001$). The SARS-CoV-2 N-protein indices were significantly decreased between the fifth (Index 5) and sixth (Index 6) follow-up (median signal to cut-off ratio, S/C, 1.67 IQR [0.84, 2.12] to 0.94 IQR [0.56, 1.10], $P<0,001$). There is statistically significant decrease of SARS-CoV-2 N-protein indices between the

first (Index 1) and the last (Index 6) follow-up (median signal to cut-off ratio, S/C, 8.18 IQR [6.91, 9.51] to 0.94 IQR [0.56, 1.18], P=0.001).

Table 3. Relative change in index antibody level determined by different sampling time points from the disease onset.

Sampling time point from the disease onset	Median signal to cut-off ratio, S/C (IQR)	P-value
Index 1/Index 2	6.05 (4.16, 8.91)	P<0.001
	5.07 (2.95, 7.92)	
Index 2/Index 3	6.78 (3.42, 8.32)	P<0.001
	3.81 (2.26, 6.18)	
Index 3/Index 4	3.47 (1.39, 6.18)	P=0.001
	1.75 (0.64, 3.24)	
Index 4/Index 5	2.35 (0.72, 4.18)	P=0.001
	1.32 (0.46, 1.82)	
Index 5/Index 6	1.67 (0.84, 2.12)	P<0.001
	0.94 (0.56, 1.10)	
Index 1/Index 6	8.18 (6.91, 9.51)	P=0.001
	0.94 (0.56, 1.18)	

IQR, interquartile range

4. DISCUSSION

SARS-CoV-2 IgG-N-protein was positive in 47/49 of the patients at the first time point (Index 1, median: 47 days from disease onset) and remained at a relatively high percentage until the fourth sampling (Index 4, median: 161 days from disease onset). The SARS-CoV-2 N-protein indices reached their peaks at the second timepoint (Index 2) with the highest index 11.95 from one patient and then recorded a decrease of the indices till the end of the observation period at more than 250 days from symptom onset (Index 6).

Previous studies have shown that antibody responses against SARS-CoV and MERS-CoV infections can persist for at least two years 17, 18. However, the kinetics and durability of SARS-CoV-2 antibody responses have rarely been reported. We clarified the kinetics and magnitude of the antibody response against SARS-CoV-2 in confirmed COVID-19 patients who were the first infected with SARS-CoV-2 in Krapina-Zagorje county and followed up for several months to determine how sustainable the antibody response against SARS-CoV-2 is. A positive rate of IgG antibodies was measured in time series following the disease onset.

The survey from Wu *et al*. was probably the first one with six months observational period on the dynamics of antibody responses 19. They found that SARS-CoV-2-specific IgM recognizing S and N-proteins was only transient and disappeared after around 12 weeks, which meant that it would not contribute to sustained immunity against SARS-CoV-2. However, IgG recognizing S and N-proteins was maintained at high positive rates and titers for six months which is very important in the case of IgG recognizing the RBD of the S protein, whose titer correlated with neutralizing activity and was associated with early virus control, highlighting the relevance of IgG for disease protection in humans. In their study, the positive rate of IgG-N-protein rose rapidly to 87% of the patients at the second week and stayed at very high levels thereafter, which coincides with the results of our study where we observed the highest N-protein indices at the second timepoint (Index 2) 56-92 days after disease onset and 11/17 of respondents were still positive at the fourth timepoint (Index 4) 150-191 days after the symptom onset.

In our research, SARS-CoV-2 IgG-N-protein was positive in 47/49 of the patients at the first time point (Index 1, median: 47 days from symptom onset) and remained at a relatively high percentage until the fourth sampling (Index 4, median: 161 days from symptom onset) when N-protein indices were significantly decreased. Gaebler C. *et al*. also observed decreased anti-N antibodies between timepoints 1.3 and 6.2 months after the symptom onset as measured by enzyme-linked immunosorbent assay (ELISA) 20. Additionally, Pyoeng Gyun *et al*. serosurvey 21 confirmed that rates of antibody positivity according to three commercial kits were still high eight months after the infection, even in asymptomatic or mildly symptomatic participants (69.0%-91.4%) which is similar to the results of our survey. We detected anti-N IgG in 4/16 of participants at more than 250 days after their infection, while their anti-N IgG (ELISA) were found in 25.9% of the participants.

Understanding the complexities of immune memory to SARS-CoV-2 is crucial to gain insight into the likelihood of durability of protective immunity against re-infection with SARS-CoV-2 and secondary COVID-19 disease. Dan *et al*. assessed immune memory of all three branches of adaptive immunity (CD4+ T cell, CD8+ T cell, and humoral immunity) in a predominantly cross-sectional study of 188 recovered COVID-19 cases, extending up to eight months post-infection 22. Their data showed that the immune memory in at least three immunological compartments was measurable in 95% of subjects five to eight months after the symptom onset, indicating that durable immunity against secondary COVID-19 disease is possible in most individuals.

Even though we present some conclusive findings of the N-protein IgG dynamics, there are certain limitations in our research. We had a small number of patients who participated in the study, timepoints of sampling were different, and the number of participants decreased according to the last timepoint of sampling. Moreover, we determined IgG antibody against the N-protein which is more conserved between SARS-CoV-2 and other pathogenic human coronaviruses compared with the S protein RBD, which makes it less than ideal target for highly accurate COVID-19 diagnosis 23.

5. CONCLUSION

Our results provide critical evidence that IgG N-protein response persists in the majority of patients for at least six to eight months after COVID-19 infection. The IgG testing is useful to support the diagnosis of COVID-19 in symptomatic individuals who test negative by molecular detection methods, such as RT-PCR. Our study findings can inform the design of future serological studies and the development of SARS-CoV-2-targeted vaccines.

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Conflicts of Interest

The authors have declared that there is no conflict of interest.

Ethical approval

Research involving human subjects complied with all relevant national regulations, institutional policies and is in accordance with the tenets of the Helsinki Declaration (as revised in 2013), and has been approved by the local Ethical Committee (General Hospital Zabok and Croatian Veterans Hospital, Zabok, Croatia)**.**

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