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RESEARCH ARTICLE

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The changing dynamics of neutralizing antibody response within 10 months of SARS-CoV-2 infections

Aliye Bastug[1] | Hurrem Bodur[1] | Omer Aydos[2] | Nazlican Filazi[3] | Ergun Oksuz[4] | Aykut Ozkul[3,5]

[1]Department of Infectious Disease and Clinical Microbiology, Ankara City Hospital, University of Health Sciences, Gulhane Faculty of Medicine, Ankara, Turkey

[2]Department of Infectious Disease and Clinical Microbiology, Ankara City Hospital, Ankara, Turkey

[3]Department of Virology, Faculty of Veterinary Medicine, Ankara University, Ankara, Turkey

[4]Department of Family Medicine, Baskent University, Ankara, Turkey

[5]Biotechnology Institute, Ankara University, Ankara, Turkey

Correspondence
Aliye Bastug, Department of Infectious Disease and Clinical Microbiology, Ankara City Hospital, University of Health Sciences, Gulhane Faculty of Medicine, 1604, St, No 9, 06800 Cankaya/Ankara, Turkey.
Email: aliye.bastug@sbu.edu.tr and dr.aliye@yahoo.com

Abstract

There are limited data on how long neutralizing antibody (NAb) response elicited via primary SARS-CoV-2 infection will last. Eighty-four serum samples were obtained from a prospective cohort of 42 laboratory-confirmed COVID-19 inpatients at the time of discharge from the hospital and in the late convalescent phase. A virus neutralization assay was performed to determine the presence and titers of NAbs with authentic SARS-CoV-2. Long-term dynamics of NAbs and factors that may have an impact on humoral immunity were investigated. Mild and moderate/severe patients were compared. The mean sampling time was 11.12 ± 5.02 days (4–28) for the discharge test and 268.12 ± 11.65 days (247–296) for the follow-up test. NAb response was present in 83.3% of the patients about 10 months after infection. The detectable long-term NAb rate was significantly higher in mild patients when compared to moderate/severe patients (95.7% vs. 68.4%, $p = 0.025$). In the follow-up, NAb-positive and -negative patients were compared to determine the predictors of the presence of long-term humoral immunity. The only significant factor was disease severity. Patients with mild infections have more chance to have NAbs for a longer time. Age, gender, and comorbidity did not affect long-term NAb response. NAb titers decreased significantly over time, with an average rank of 24.0 versus 19.1 ($p = 0.002$). Multivariate generalized estimating equation analysis revealed that no parameter has an impact on the change of NAb titers over time. The majority of the late convalescent patients still had detectable low levels of neutralizing antibodies. The protective effect of these titers of NAbs from re-infections needs further studies.

KEYWORDS

COVID-19, humoral immunity, long-term immunity, neutralizing antibodies, SARS-CoV-2

1 | INTRODUCTION

Global SARS-CoV-2 pandemic is still ongoing, despite great global efforts. In addition to primary infections, there are rare reports of re-infections, as well. It is not known if reinfected patients had neutralizing antibody responses elicited by primary infection at the time of reinfection.[1] There is a knowledge gap in the literature regarding how long pre-existing immunity lasts after primary infection and whether it is protective for re-infection. In addition, it is not known whether boosting vaccination after primary infection is necessary, and if it is, the optimum time to perform this vaccination is also not known. Although there are more studies on acute phase antibody response after COVID-19,[2,3] knowledge about long-term immunity is still limited.[4,5] Recently, Dispinseri et al.[4] performed a neutralization

assay with pseudovirus and reported that most of the recovered COVID-19 patients had detectable NAb titers up to 8 months after primary infection, despite the progressive decrease in titers in the first 2 months.

To combat this pandemic, it is important to fill the paucity of information about the long-term dynamics of humoral and cellular immunity acquired by SARS CoV-2 infection.

In the present study, it was aimed to clarify the long-term persistence of the neutralizing antibody response after primary infection and to define the changing dynamics of the NAb titers over time. Viral neutralization assay (VNA), which is a gold standard, was performed in biosafety level-3 plus laboratory via authentic SARS-CoV-2 virus.[6]

2 | MATERIALS AND METHODS

2.1 | Study design and ethical statement

Serum samples were obtained from confirmed (PCR and/or ELISA IgM/IgG positive) COVID-19 patients for this prospective longitudinal cohort study. The study was approved by the Ethical Committee of a tertiary hospital (E1-21-1494). Informed consent was obtained from all patients.

A total of 129 consecutive laboratory-confirmed COVID-19 patients were admitted to our clinic between March and May 2020. They were asked whether they accept enrolling in this study and will come to the hospital for recurrent outpatient visits for longitudinal sampling. Forty-two patients who accepted were enrolled in the study. The samples were obtained twice: once during the discharge from the hospital and once in about the 10th month of infection. VNA was performed with discharge sera and follow-up sera with authentic SARS CoV-2. Patients were classified into two groups as mild and moderate/severe according to the National Institutes of Health (NIH) classification.[7] SARS-CoV-2 RT-PCR results of the oro/nasopharyngeal swab samples that were collected on admission day of hospitalization and clinical and demographic findings were recorded on case follow-up forms.

The patients were also grouped based on sampling time for discharge antibody test after symptom onset as follows: 4–9 days, 10–14 days, and 15–28 days.

2.2 | Virus neutralization assay

Serum samples were diluted twofold in quadruplicate, starting from 1:5 (which is threshold dilution for positivity), in a microtiter tissue culture plate and mixed with an equal volume of $100TCID_{50}$ (equals to 1:10,000 dilution) SARS-CoV-2 Ank1 isolate. The plate was incubated at 37°C for 1 h for neutralization. Then, the mixture of virus and serum was inoculated into 90% confluent Vero E6 cells grown in 96-well plates. Virus control cells were prepared with $100TCID_{50}$ dilution test virus. Therefore, 100% cytopathogenic effect occurrence in control wells was accepted for the best time for test assessment. Reciprocals of serum dilutions that neutralize a minimum of 50% of $100TCID_{50}$ virus infection were accepted as virus-neutralizing antibody (NAb) titer of each serum sample.

2.3 | Statistical analysis

Mean and standard deviation, minimum, maximum, median, and interquartile range were used for continuous variables. Number and percentage were used for categorical variables. The Kruskal–Wallis and Mann–Whitney *U*-tests were used for the data that did not have a normal distribution. The categorical data were compared using the $\chi 2$ test or Fisher's exact test. The Wilcoxon signed-rank test was used to determine baseline and follow-up antibody test titers. *p*-values were calculated using the Wilcoxon rank-sum test to compare titer values between groups of different categories of disease severity and sampling days at a given time point. $p \leq 0.05$ was considered significant. The association between the variation of neutralizing antibody titers and potential factors such as gender, age, clinical disease severity, and time from onset of symptoms was calculated with the generalized estimating equation (GEE) model, which takes into account the correlation between repeated measurements. In the analyses, hypothesis testing was performed bi-directionally with an α value of 0.05. All analyzes were performed using SPSS 24.0 (IBM Corp) software.

3 | RESULTS

A total of 42 laboratory-confirmed COVID-19 inpatients with a mean age of 40 ± 10.2 years were enrolled. Of the patients, 83.3% was PCR (+) on admission. The remaining patients had SARS CoV IgM and or IgG positivity at the time of hospital discharge in the pre-vaccine era. It was found that 57.1% were male and 21.4% had comorbidity. Twenty-three patients (54.7%) were in the mild group, 14 (33.3%) were moderate, and the remaining 5 (11.9%) were severe. Hypertension was the most frequent comorbidity with a ratio of 11.9% and it was statistically more frequent in the moderate/severe group ($p = 0.015$). Cough, fever, tachypnea, and myalgia were the most frequent symptoms. The mean length of time after symptom onset was 11.12 ± 5.02 days (4–28 days) for the sampling of the first NAb tests and 268.12 ± 11.65 days (247–296 days) for the follow-up NAb tests. The presence of NAb response was observed in 50% of the patients at the time of hospital discharge, then it reached a level of 83.3% on the follow-up sera. In none of the patients, re-infection occurred after discharge. The detectable NAb rate was lower on discharge as 52.4% of the patients were tested 4–9 days after symptom onset at the time of discharge from the hospital. In other words, the reason for this result may be that 10–14 days required for antibody development has not passed yet at the test time of discharge NAbs.

In the follow-up test samples, the detectable NAb rate was significantly higher in mild patients compared to moderate/severe patients (95.7% vs. 68.4, $p = 0.025$). The follow-up NAb positive and negative patients were compared to determine the predictors of the presence of long-term humoral immunity. The only significant factor was determined as the degree of disease severity. Age, gender, and comorbidity had no effect on long-term NAb response.

A total of seven patients had negative follow-up NAb titers. Discharge NAb titers were also negative in three of them. The other four patients had positive discharge NAb titers (1:1250, 1:125, 1:25, and 1:7.5 respectively) which became negative on the follow-up test (Figure 1).

Mild patients constituted 54.7% of the study population. When the mild and moderate/severe groups were compared, the median NAb titers on discharge was higher in the moderate/severe group (median, 7.5; range, 0–1250 vs. median, 0.0; range 0–125) whilst it did not reach statistical significance at the Wilcoxon rank analysis (Table 1). The median NAb titers according to post-onset symptom time are summarized in Table 2. The changes in the NAb titers over time and the age and gender characteristics of each patient are shown in Figure 1. The changes in median levels of NAb titers over time in the mild and moderate/severe groups are summarized in Figure 2A–D.

NAb titers decreased on the follow-up test compared to discharged NAb titers, with an average rank of 24.0 versus 19.1. The Wilcoxon signed-rank test shows that the observed difference between both measurements is significant ($p = 0.002$). There is also a significant decrease in the NAb titers of mild patients on the follow-up (average rank of 14.7 vs. average rank of 11.6, $p = 0.004$). However, the decrease of the NAb titers in the moderate/severe group was not statistically significant (average rank of 9.5 vs. average rank of 8.2, $p = 0.119$). In the comparative analysis according to the post-symptom day groups from which the first NAb test was obtained, follow up NAb titers were significantly higher in the mild 4–9 days group (median, 30; range, 0–40) when compared with moderate/severe group (median, 7.5; range, 0–30), $p = 0.019$ (Figure 2B).

Compared to the discharge NAb titers, the follow-up NAb titers significantly decreased in the group of 4–9 days (average rank of 14.0 vs. 10.1, $p = 0.004$) and increased in the group of 10–14 days (average rank of 3.0 vs. 7.2, $p = 0.009$). However, the difference was not

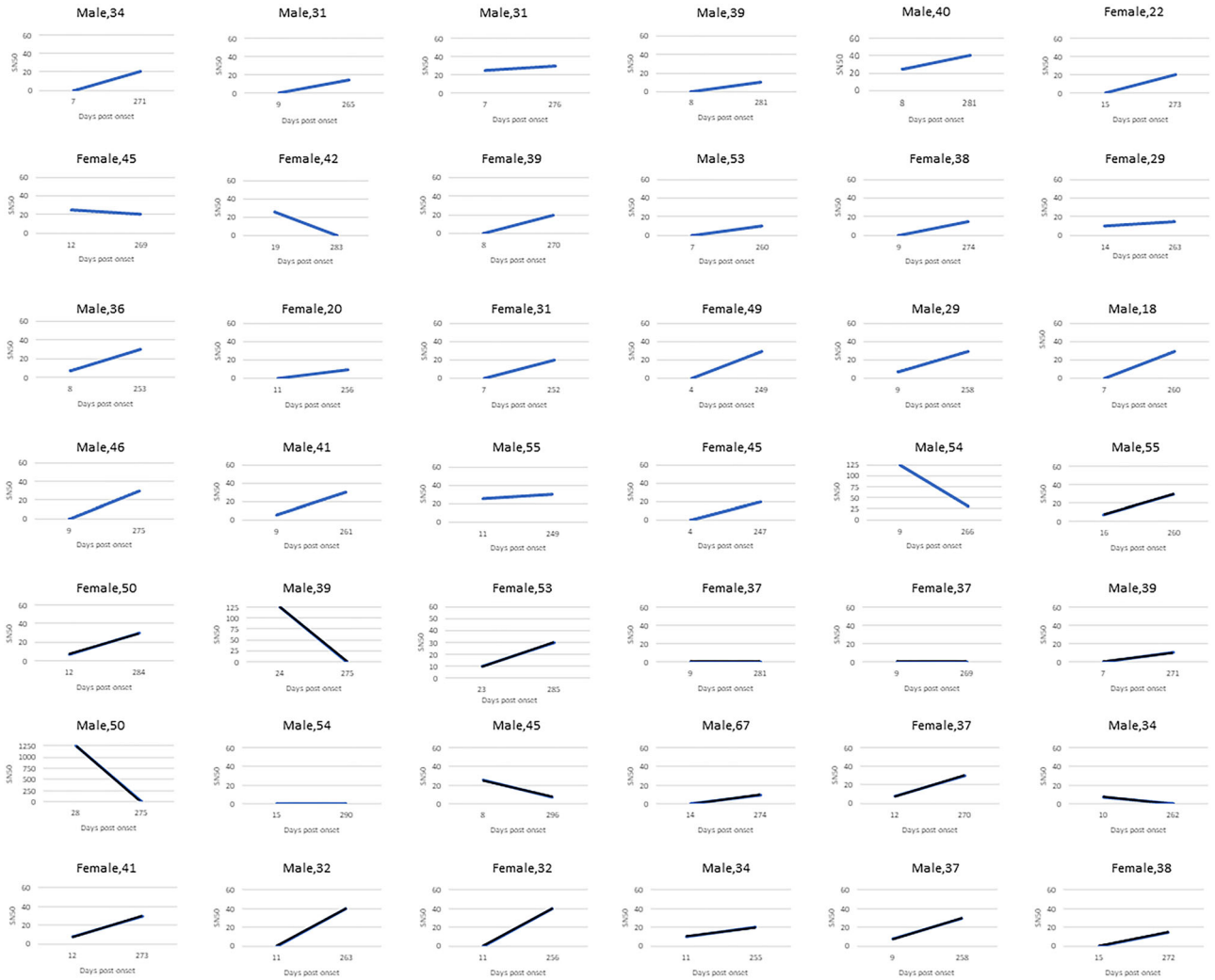

FIGURE 1 The changes in the NAb titers over time and age and gender characteristics of each patient

TABLE 1 Clinical, laboratory, and demographic characteristics of the patients

	Total (n = 42) (%)	Mild (n = 23) (%)	Moderate/severe (n = 19) (%)	p
Age, mean ± *SD*, years	40 ± 10.2	37.7 ± 10.4	42.7 ± 9.6	0.224
Male gender	24 (57.1%)	13 (56.5%)	11 (57.9%)	0.589
PCR confirmation	35 (83.3%)	18 (78.3%)	17 (89.5%)	0.293
NAbs (+)	21 (50.0%)	10 (43.5%)	11 (57.9%)	0.268
Follow-up NAbs (+)	35 (83.3%)	22 (95.7%)	13 (68.4%)	**0.025**
NAbs titer, median (IQR)	2.5 (10.0)	0.0 (25.0)	7.5 (10.0)	0.535
Follow-up NAbs titer, median (IQR)	20.0 (20.0)	20.0 (15.0)	15.0 (30.0)	0.823
Comorbidity	9 (21.4%)	1 (4.3%)	8 (42.1%)	**0.004**
Hypertension	5 (11.9%)	0 (0.0%)	5 (26.3%)	**0.015**
Cardiovascular disease	2 (4.8%)	0 (0.0%)	2 (10.5%)	0.199
COPD	2 (4.8%)	1 (4.3%)	1 (5.3%)	0.706
Fever	18 (42.9%)	6 (26.1%)	12 (63.2%)	**0.017**
Cough	29 (69.0%)	14 (60.9%)	15 (78.9%)	0.11
Dyspnea	8 (19.0%)	3 (13.0%)	5 (26.3%)	0.243
Sore throat	7 (16.7%)	4 (17.4%)	3 (15.8%)	0.612
Diarrhea	3 (7.1%)	1 (4.3%)	2 (10.5%)	0.427
Myalgia	12 (28.6%)	7 (30.4%)	5 (26.3%)	0.521
Tachypnea	16 (38.1%)	5 (21.7%)	11 (57.9%)	**0.018**
Length of hospital stay, mean ± *SD*, day	8.1 ± 4.3	6.8 ± 3.0	9.7 ± 5.1	**0.036**
ICU requirement	3 (7.1%)	0 (0.0%)	3 (15.8%)	0.084

Note: Bold values are statistically significant.

Abbreviations: COPD, chronic obstructive pulmonary disease; ICU, intensive care unit; IQR, interquartile range.

significant in the group of 15–28 days (average rank of 2.5 vs. 6.0, p = 0.498). Univariate GEE analyses revealed that age, gender, and disease severity had no effect on the change of antibody titers over time. The only significant factor between the change of antibody titers over time was the post-onset sampling time of the discharge test. In other words, when the sampling time of the discharge NAb test was compared (4–9 days vs. 15–28 days post-onset), there was a significant increase in the long term NAb response compared to the baseline level in days 4–9 group in univariate analyses (p = 0.015). Multivariate analyses revealed that no significant parameter had an impact on the difference between baseline and follow-up NAb titers (Table 3). The distribution of the number of patients for the discharge and follow-up NAb titers according to time post-onset is given in Figure 3A,B.

4 | DISCUSSION

NAbs have been expected as predictors of antibody-mediated immunity.[8] It is supposed that it will either eliminate the risk of re-infection or in case of re-infection, at least NAbs may decrease the severity of infection. However, it is difficult to present the presence of NAbs as conclusive evidence for preventing reinfection.[9] Although the exact threshold for the NAb titer that will be more probable to prevent reinfection is unknown, higher titers are more likely to have a chance for prevention. Another important issue that needs to be clarified is how long antibody-mediated immunity is elicited after natural infection will last. Its importance derives from its contribution to the development of herd immunity and on the decision of the length of time for using "immunity passports."[10,11] This is also necessary for determining whether a booster dose of vaccination is necessary for recovered patients and if so when it is supposed to be administered.[8,12]

Humoral immunity via primary infection of other coronaviruses lasts for several months although antibody titers wane over time.[13–15] In terms of SARS-CoV-2 infection, there are several studies conducted in acute and early convalescence periods and they have reported that NAb titers vary depending on the severity of the primary infection and the length of time from onset.[2,5,12] In addition, Legros et al.[16] reported a rapid decline in NAb titers elicited via SARS CoV-2 infections after recovery compared with other coronaviruses infections.

TABLE 2 The median NAbs titers in relation with time post-onset

Sampling time after onset for discharge antibody tests	Mild patients, SN50 (n = 23)				Moderate/severe patients, SN50 (n = 19)			
	Discharge Median (min–max)	95% CI	Follow-up Median (min–max)	95% CI	Discharge Median (min–max)	95% CI	Follow-up Median (min–max)	95% CI
4–9 days	0 (0–125)	−4.2 to 27.1	30 (10–40)	19.7–28.5	0 (0–25)	−7.0 to 20.0	7.5 (0– 30)	−5.8 to 24.8
10–14 days	17.5 (0– 25)	−4.5 to 34.5	17.5 (10–30)	5.2–32.3	7.5 (0–10)	1.5–8.5	30 (0–40)	13.2–36.8
15–28 days	12.5 (0– 25)	−146.3 to 171.3	10 (0–20)	−117.1 to 137.1	8.75 (0–1250)	−293.7 to 757.9	7.5 (0–30)	−3.0 to 28.0
Total	0 (0–125)	0.7–23.7	20 (0–40)	17.8–26.1	7.5 (0–1250)	−60.5 to 214.7	15 (0–40)	9.8–24.2

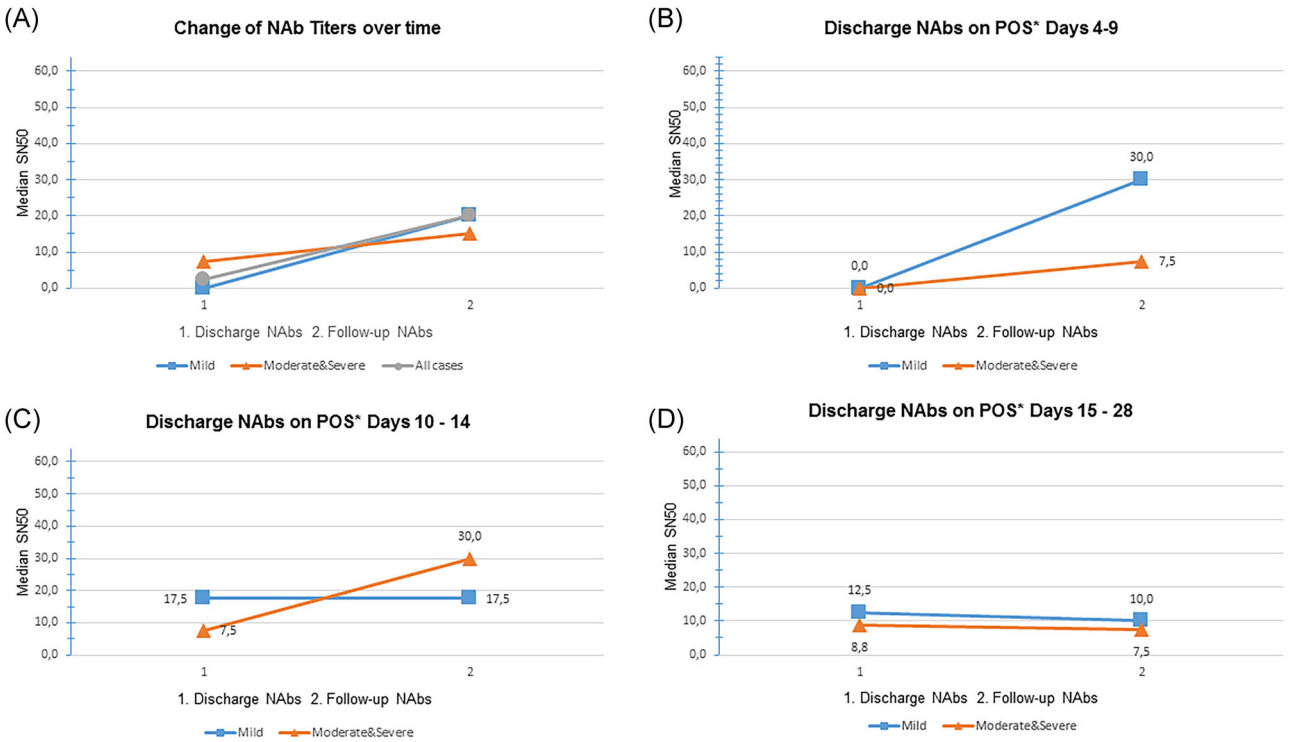

FIGURE 2 (A–D) The changes in median levels of NAb titers over time. *POS, post-onset of symptoms

In the present study, the presence and titers of long-term NAb response elicited by the primary infection were investigated. Baseline and the follow-up serum samples were investigated to clarify the persistence of NAbs and to determine the change of NAb levels compared to baseline over time up to 10 months after primary infection. As it was the gold standard, a neutralization assay based on authentic SARS-CoV-2 was performed in a BSL3+ laboratory.[6] The importance of the present study is that it shows detectable NAbs in 83% of the recovered patients up to nearly 10 months after onset. A previous study reported that the majority of the recovered patients (84.3%) had detectable NAbs at the median of 104 days after symptom onset and a 3.8-fold decrease was observed in the NAb titers compared to 30-day post-onset values. They concluded that there is a predefined rapid decline in the first 3 months and the rate of decrease slows down thereafter.[5]

Of the patients who had a negative discharge neutralization test, only three (7.1%) still had negative test results in the convalescent phase. It is noteworthy that in two patients with severe COVID-19 and strong NAb response (NAb titers of 1:1250 and, 1:125) in the acute phase of infection, the antibody titers became negative within the 10 months after onset. These findings agree with previous reports suggesting that the severe infection leads to a strong reaction in the antibody response via short-lived plasmablast, and then wanes more rapidly.[5] However, further studies with large cohorts are needed to define this issue more precisely.

In the present study, milder infections were found as the only predictor of long-term detectable NAb response. Other than this, age, gender, and comorbidity did not have an effect. In contrast, the severe infection was reported to lead to a higher antibody response in the early phase of the disease.[8]

TABLE 3 Generalized estimating equation analysis of factors with an impact on the difference between discharge and follow-up NAb titers

Characteristics	Univariate		Multivariate		
	p	*β* coefficient	Standard error	95% CI	*p*
Gender					
Male versus female	0.047	1.159	0.5401	(−0.325 to 1.792)	0.175
Age, years					
36–50 versus 18–35	0.071	1.183	0.6852	(−1.371 to 1.314)	0.967
≥51 versus 18–35	0.272	0.393	0.3462	(−0.320 to 1.037)	0.301
Disease severity					
Moderate/severe versus mild	0.103	1.151	0.2584	(−0.657 to 0.356)	0.56
Days since onset					
15–30 versus 4–9	0.015	1.955	1.0996	(−0.289 to 4.021)	0.090
10–14 versus 4–9	0.303	−0.208	0.4182	(−0.578 to 1.061)	0.564

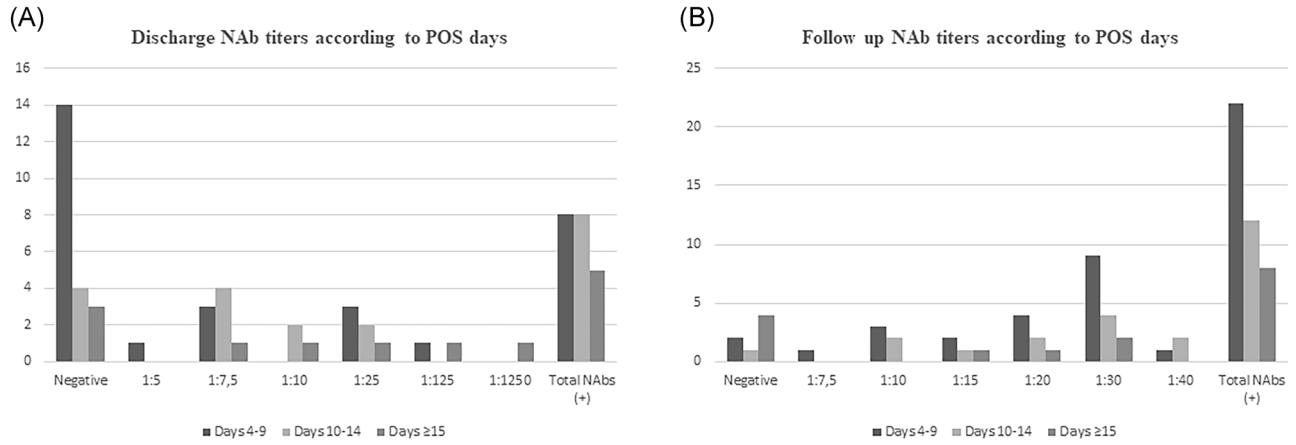

FIGURE 3 (A, B) The distribution of the number of patients by time post-onset based on discharge (A) and follow-up (B) NAb titers. *POS, post-onset of symptoms

Factors that may affect the changing dynamics of long-term NAb titers in comparison to the baseline values were also evaluated in the present study. Crawford et al.[5] reported that the severity of the disease did not have an effect on NAb titers, which were measured 3 months after the disease onset. Consistent with this study, we found no significant effect of disease severity on changing titers of long-term NAbs, which were measured at approximately 10 months after the disease onset, in comparison to the baseline values. Besides this, age and gender did not have an effect, as well. Wang et al.[8] reported similar findings for gender and severity but other than age in convalescent patients at approximately 2 months after onset. They concluded that 61–84 years old patients had significantly higher NAb titers.[8] The mean age of the present study was not so high, which may be the reason for the different outcomes. Further studies are needed to clarify this issue.

The present study had some limitations. First, this is a single-center study with a small study population with a low number of severe patients. Second, further studies are needed to investigate long-term cellular immunity in addition to a humoral antibody response. Finally, the presence of low-level NAbs does not mean conclusively that it is enough to prevent re-infection.

In conclusion, despite its limitations, this study reveals a significant decrease in terms of NAb titers over time. However, NAb response was still detectable in most patients in approximately 10 months postinfection, in line with the previous reports for other coronaviruses.[17] Patients with milder clinical manifestations have a greater chance of having a detectable antibody response in the long-term convalescence.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Conception and design of the work: Aliye Bastug, Hurrem Bodur, and Aykut Ozkul. *Investigation, data collection*: Aliye Bastug, Omer Aydos, and Nazlican Filazi. *The acquisition, analysis, or interpretation of data for the work*: Aliye Bastug, Hurrem Bodur, Aykut Ozkul, and Ergun Oksuz. *Writing original draft*: Aliye Bastug. *Review & Editing draft*: all authors. *Final approval of the version to be published*: All authors.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Aliye Bastug http://orcid.org/0000-0002-8831-4877

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