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Discordance between Serum Neutralizing Antibody Titers and the Recovery from COVID-19

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The recent pandemic of COVID-19 has caused a tremendous alarm around the world. Details of the infection process in the host have significant bearings on both recovery from the disease and on the correlates of the protection from the future exposures. One of these factors is the presence and titers of neutralizing Abs (NAbs) in infected people. In the current study, we set out to investigate NAbs in the recovered subjects discharged from the hospital in full health. Serum samples from a total of 49 documented consecutive COVID-19 subjects were included in the study. All the subjects were adults, and serum samples collected during the discharge were tested in viral neutralization, enzyme immunoassay (EIA), and Western immunoblot tests against viral Ags. Even though a majority of the recovered subjects had raised significant NAb titers, there is a substantial number of recovered patients (10 out of 49) with no or low titers of NAbs against the virus. In these cohorts as well as in patients with high NAb titers, viral Ag binding Abs were detectable in EIA tests. Both NAb titers and EIA detectable Abs are increased in patients experiencing a severe form of the disease, and in older patients the Ab titers were heightened. The main conclusion is that the recovery from SARS-CoV-2 infection is not solely dependent on high NAb titers in affected subjects, and this recovery process is probably produced by a complex interplay between many factors, including immune response, age of the subjects, and viral pathology. ***The Journal of Immunology*****, 2020, 205: 2719–2725.**

Currently, many aspects of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) are scrutinized. One of the most investigated aspects of this infection is the type of immune response induced during the disease process.

Neutralization of the infecting virus is the holy grail of vaccination efforts (1). Whether attained after a recovered infection or following a vaccination, high titers of neutralizing Abs (NAbs) in circulation are considered to be one of the main requirements of protection from future exposures to viral or bacterial pathogens. As prophylactics or even therapeutics, NAbs could be useful biologicals especially against the infections caused by difficult microorganisms. Regarding COVID-19, a similar conclusion could be drawn that high titer of NAbs could be the biomarker of the survival from clinical infection, and therefore, the presence and titers of NAbs need to be addressed in the recovered subjects.

It could be argued that serum NAb titers may not correlate well with protection from a predominantly respiratory pathogen that utilizes mucosal surfaces as a point of entry where the incoming virus should first be encountering mucosal elements of immunity. We know from other similar respiratory pathogens that presence of NAbs in the serum has a significant beneficial effect on ameliorating the severity of the subsequent infection (2–4). Passively transferred polyclonal or monoclonal NAbs are known to reduce the hospitalization and rate of intensive care admissions in severe respiratory syncytial virus infection for high-risk infants, and this practice has been part of an approved immunoprophylactic regimen (5–7). Therefore, it is imperative to determine serum NAb titers in patients recovered from COVID-19 infection to pinpoint the precise role played by NAbs during recovery from infection. In this study, we addressed this issue and investigated the serum NAb titers of the recovered patients discharged from the hospital seemingly in full health. Furthermore, determination and establishing relevance of these Abs in recovery from COVID-19 infections might have implications of the future vaccine efforts as well as the clinical management of current patients on issues ranging from testing and administration of immune plasma as therapeutic reagent to inducing positive prognostic outcomes.

In cases of viral infections in which viremic phases dominate the pathogenicity, it is well established that serum NAb titers correlate well with the protection. For instance, for measles infections, the goal of vaccination is to provide an adequate titer of virus-specific NAbs in the serum, which will provide protection from subsequent viral exposure (8). Supporting the critical role of NAbs for protection from actual infection in an outbreak setting, it was shown that measles infection is more likely to be experienced by school children if the pre-exposure serum NAb titers are below the certain limits (9).

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Abbreviations used in this article: CI, confidence interval; COVID-19, coronavirus disease 2019; CPE, cytopathic effect; EIA, enzyme immunoassay; NAb, neutralizing Ab; RT-qPCR, RT–quantitative PCR; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2; VNA, virus neutralization assay; WHO, World Health Organization.

In COVID-19 infections, the NAbs are considered to be possible therapeutic agents against this ravaging infection (10). In SARS-CoV infection, however, there are contradictory studies on the role of NAbs. Some suggest spike protein–specific NAbs were protective in the infection process, and others indicate that NAbs further complicate the lung injury or harmful effect studies (11–14). Similar studies need to be conducted in SARS-CoV-2 infection to delineate the precise role of NAbs played during the infection and recovery and upon exposure to the following viral encounter.

Abs that bind directly to the receptor-binding site of the virus could have an immediate neutralizing effect on the virus, and most in vitro neutralization assays based on cell culture are designed to test such bindings, and therefore, anti–receptor-binding site Abs are probed. It might be possible that Abs that do not bind specifically to the receptor-binding domain of the spike protein of SARS-CoV-2 but somehow prevent the fusion process between the viral and cellular membranes or inhibit uncoating could also demonstrate neutralizing effect detected in vitro. In the study, an in vitro neutralization assay on cell cultures is performed, therefore, all these types of Abs should be detected in the process. How virus neutralization proceeds in vivo is probably more complex than what could develop in in vitro cell culture infection models. It is possible that complement factors might be involved in some Ab–virus interactions.

In this study, we wanted to assess the presence of NAbs in COVID-19–recovered cohorts. It is essential to delineate the role of humoral immunity, whether it be neutralizing or nonneutralizing, in the overall viral infection process. The information gained from this type of scrutiny might have significant implications for vaccine attempts and therapeutic initiatives.

Materials and Methods

Study subjects and patient samples

The institutional review board approval for the study was granted by the Bezmialem Vakif University Institutional Review Board and by the Turkish Ministry of Health. The patient samples were the archived samples submitted to clinical laboratories of Bezmialem Vakif University Hospital. The subject had given informed consent for the banking and future use of their samples for biochemical, virological, and serological testing.

The samples were from consecutively discharged patients from the hospital after a course of hospitalizations for COVID-19 disease. Discharge decisions were made upon clinical improvements and two negative RT–quantitative PCR (RT-qPCR) tests from nasopharyngeal samples. The samples were collected during the discharge and deidentified as mandated by the institutional review board protocol, and serum samples were kept at −80°C until tested.

A total of 49 samples from documented COVID-19 patients were included in the study. The documentation of COVID-19 infection was based on positive RT-qPCR test or chest computed tomography results as well as SARS-CoV-2–specific Ab tests. The demographic data on the patients are provided in the *Results* section.

The clinical severity of the patients was assessed as per the World Health Organization (WHO) guidelines, *Clinical Management of COVID-19 Patients-Interim Guidance* (15). Accordingly, the clinical severity was assessed as 1) mild, 2) moderate, 3) severe, or 4) critical.

Cells and the virus

In the experiments, Vero E6 cells were used. Vero E6 cells were grown in DMEM (Sigma-Aldrich, Taufkirchen, Germany) supplemented with 10% FBS (Life Technologies, Thermo Fisher Scientific, Waltham, MA), 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml amphotericin B (Thermo Fisher Scientific).

The virus strain used in the experiments was isolated from nasopharyngeal specimens submitted to the Clinical Microbiology Laboratory of Bezmialem Vakif University Hospital for COVID-19 diagnosis from a 2-y-old girl. The virus strain (hCoV-19/Turkey/Istanbul-BezmCoV1/2020, GISAID accession number EPI_ISL_457824, https://gisaid.ibg.edu.tr/) was grown on Vero E6 cells. For virus isolation, nasopharyngeal specimen of the COVID-19–confirmed patient was used to infect cells. After 3 d postinfection, virus-containing supernatants were harvested by centrifugation and used for identification and passage of the virus. After the passage, the virus titration was determined by 50% tissue culture–infective dose experiments on 96-well plates on experiments on Vero E6 cells and confirmed by RT-qPCR.

All the viral tests and neutralization assays were performed in the Bezmialem Vakif University Hospital Tuberculosis Laboratory with biosafety level 3 conditions.

RT-qPCR

The principal diagnostic means of COVID-19 infection was RT-qPCR assay as described by WHO interim guidelines on laboratory detection of suspected human cases (16). For the assay, nasopharyngeal and oropharyngeal swab samples were collected from suspected patients, and RT-qPCR assays (Bio-Speedy SARS-COV2-2019-nCoV-qPCR Detection Kit; Bioeksen R&D Technologies, Istanbul, Turkey) were performed at Bezmialem Vakif University Clinical Microbiology Laboratories on Rotor-Gene Q Series run on software 2.1.0-build 9 (Qiagen N.V., Venlo, the Netherlands). The assay was directed to RNA-dependent RNA polymerase gene of Wuhan strain of SARS-CoV-2. The performance criteria for the assay were established as 3.8 copy viral RNA per reaction by the Public Health Central Laboratories of Turkish Health Ministry.

Enzyme immunoassays

For enzyme immunoassay (EIA), hCoV-19/Turkey/Istanbul-BezmCoV1/2020 virus strain–infected Vero E6 cell lysate was used as solid phase Ag. For this purpose, Vero E6 cells infected with 0.001 multiplicity of infection were collected after full cytopathic effect (CPE) was observed. The cell lysate was inactivated at 60°C for 1 h and centrifuged at 3000 × *g* for 10 min, and the pellet was used as the solid phase Ag in EIA.

For negative Ag controls, uninfected Vero E6 cells scraped off the flasks were also treated as described for virus-infected cells. The amounts of virus-specific Ags present in the cell lysate were ND. In the validation of EIA and throughout testing, uninfected Vero E6 cell lysate with the same amount of protein concentrations was tested against each serum dilution, and these readings were evaluated as negative controls.

Flat-bottom polystyrene 96-well microtiter plates (Immulon1B; Dutscher Scientific UK, West Thurrock, Essex, U.K.) were coated overnight at 4°C with 5 μg/well infected Vero E6 cell lysate and blocked with 200 μl of 5% nonfat dairy milk (Sigma-Aldrich) in PBS with 0.2% Tween 20 (PBST; pH 7.2) for 2 h at room temperature. The plates were then aspirated and incubated for 1 h at 37°C with 100 μl of 100-fold dilution of the confirmed patient sera in 5% nonfat dairy milk in PBST. As negative controls, Ab-negative sera from healthy human volunteers were used at the same dilutions. For this aim, 49 SARS-CoV-2 Ab-positive and 10 SARS-CoV-2 Ab-negative human sera were tested. All reactions were performed in duplicate. The plates were washed for three cycles with PBST and incubated for 1 h at 37°C with 100 μl of a 10,000-fold dilution of HRP-conjugated goat anti-human IgG (1:10,000, 5172-2504; BioRad, Hercules, CA). The plates were washed three cycles with PBST, and 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB; Abcam, Cambridge, U.K.) substrate was added per well. The plates were incubated in the dark at 37°C for 10 min, and 100 μl of 2 N H_2SO_4 was added to each well to stop reaction. Absorbance of samples was measured in iMark microplate reader (BioRad) at 415 nm. All washing steps were performed using Wellwash Versa Microplate Washer (Thermo Fisher Scientific). The cut off value between the positive and negative samples was determined after reading the OD values of duplicate negative samples, applying the formula mean OD of negative samples + 2 SD = 0.407. The in-house–developed EIA was validated by performing a side-by-side comparison of negative and positive samples with low, medium, and high Ab titers on a Food and Drug Administration–approved commercial chemiluminescent microparticle SARS-CoV-2 IgG assay (Abbott, Abbott Park, IL). The commercial test has an Emergency Use Authorization from the U.S. Food and Drug Administration.

Virus neutralization assay

Previously described virus neutralization assay (VNA) procedures were essentially followed (17). Vero E6 cells (3×10^4 per well) were seeded 24 h before the infection in a 96-well plate (TPP Techno Plastic Products AG, Trasadingen, Switzerland). On the day of infection, the cells were washed twice. Serum samples from patients were incubated at 56°C for 1 h and then diluted 4-fold in cell culture medium. Aliquots (100 μl) of diluted serum samples (from 4-fold to 1024-fold) were added to 100 μl of cell culture medium containing 50% tissue culture–infective dose of the virus (hCoV-19/Turkey/Istanbul-BezmCoV1/2020, GISAID accession number EPI_ISL_457824, https://gisaid.ibg.edu.tr/) on a 96-well plate and incubated at 37°C for 1 h in 5% CO_2. Two hundred microliters of virus Ab mix was then added to cells in 96-well plates, and plates were kept at 37°C

for 3-d incubation for development of a full CPE as determined by microscopic examination. The highest dilution of serum that showed full inhibition of CPE was recorded as NAb titer. Assays were performed in duplicates with four negative control samples from Ab-negative healthy volunteer subjects. All the samples were tested in two occasions, and on each time, all the samples were tested in duplicates. On each 96-well plate, Ab-positive and -negative serum samples and no-serum-added wells as well as no-virus-added wells, all in duplicate, were included. The tests were done in blinded fashion in which clinical data on the patients were not provided to the researchers performing VNA, EIA, and Western blot tests. During the initial validation phase, the serum samples at different dilutions were added to wells without virus to assess the toxicity of the serum samples on cells alone. Only at 1/2 serum dilutions were the samples toxic to cells, and in the remainder of the assays, the samples were diluted starting at 1/4 dilutions.

Western blotting

Uninfected and SARS-CoV-2–infected Vero E6 cell lysates were mixed with Laemmli Buffer (4% SDS, 10% 2-ME, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris-HCl) in 2:1, boiled for 5 min at 95°C, and centrifuged at 10,000 × *g* for 5 min. Supernatants containing equal amount of protein (32.5 μg/well) were loaded on 15–10% polyacrylamide gel and run on Mini-Protean Tetra-Cell (BioRad). Following separation, proteins were transferred to nitrocellulose membrane in transfer buffer (24 mM Tris, 192 mM glycine, and 20% [v/v] methanol) at 25 V for 7 min on semidry blotter (Trans-Blot Turbo; BioRad). The membranes were blocked with blocking buffer (10 mM Tris pH 7.4, 0.9% NaCl, 0.05% Tween-20, 5% skim milk) for 2 h at room temperature and incubated overnight at 4°C either with SARS-CoV-2–infected or uninfected human sera diluted to 1:100 in blocking buffer. After incubation with goat anti-human IgG Ab (1:10,000, 5172-2504; BioRad) for 1 h at room temperature, membranes were processed for chemiluminescence detection (WesternBright Sirius Chemiluminescent Detection Kit; Advansta, San Jose, CA), according to the manufacturer's instructions on Fusion FX (Vilber, Collegien, France).

Statistical analyses

The statistical analyses were made by Prism 8 for OS X Version 8.4.2 (GraphPad Software, San Diego, CA) program. The correlations were analyzed using Pearson and Spearman tests in Prism 8.0 (GraphPad). Grouped data are compared by Wilcoxon or Mann–Whitney *U* tests using Prism 8.0 (GraphPad) and are generally demonstrated as median ± interquartile range. Ninety five percent confidence intervals (CI) and *p* values were also determined. To delineate the influence of factors such as age, clinical severity, and EIA results on NAb titers, a multiple linear regression analysis was performed.

Results

A total of 49 patients, 23 females and 26 males, were included in the study. The average age of the subjects was 50.96 y old, with ages ranging from 20 to 80 y. Demographic and clinical characteristics of patients with COVID-19 and a control cohort of 10 adults are reported in Table I. The severity of the clinical symptoms ranged from mild to critical conditions as determined by WHO interim guidelines (15).

A total of 39 (79.59%) out of 49 COVID-19–recovered subjects had serum NAb titers of 1/32 or above as determined by cell culture neutralization assay (Fig. 1). Three patients (6.12%) had no detectable serum NAb titers. One (2%) had 1/4, three (6.12%) had 1/8, and three (6.12%) had 1/16 NAb titers. Overall, the 10 patients (20.36%) out of 49 had 1/16 or less NAb titers.

Next, we looked at the mean clinical severity scores of patients in each serum neutralization group and searched for a possible correlation between the titers of NAbs and the mean severity of clinical symptoms (Fig. 2). A significant correlation was noted between two variables (r = 0.73, p = 0.016, 95% CI = 0.1831–0.9311), indicating a relationship between the titers of NAbs and the disease severity.

In an in-house–developed EIA assay, serum samples from all the recovered subjects were tested, and accordingly, all had detectable Ab responses to viral Ags at 1:100 dilutions (Fig. 3A).

Table I. Demographics and clinical characteristics of COVID-19 patients and healthy control subjects

Characteristic	Median	IQR
Characteristics of 49 subjects recovered from COVID-19		
Age, median (y)	52	59–42
Male sex (%)	53	
Time since symptom onset (d)	15	21–13
Time since positive nasal swab SARS-CoV-2 PCR (d)	14	14–9
Illness severity[a]		
Mild (%)	18	
Moderate (%)	55	
Severe (%)	18	
Critical (%)	8	
Age and sex of 10 healthy control sera		
Age (y)	41	51, 25–36
Male sex (%)	50	

[a]The severity of the clinical symptoms ranged from mild to critical conditions as determined by WHO interim guidelines.
IQR, interquartile range.

The patients who did not have detectable NAbs were also positive for viral Ag binding Abs even over several dilutions (Fig. 3B). The Abs primarily reacted to the viral protein at ∼48 kDa, presumably the nucleocapsid protein of SARS-CoV-2 (18, 19) as determined by Western immunoblot experiments (Fig. 3C).

Next, we evaluated the relationship between NAb titers and EIA results (Fig. 4A). There was a positive correlation between titers of NAbs and OD readings at 415 nm on EIA assay (r = 0.47, p = 0.0006, 95% CI = 0.2225–0.6664). This correlation was significant. Similarly, there was a significant correlation (r = 0.31, p = 0.0316, 95% CI = 0.02873–0.5418) between OD readings on EIA assay and the clinical severity of the disease (Fig. 4B).

In reviewing the data, we noticed that the age of the subjects seems to have influence on the parameters evaluated. Therefore, the correlation between ages of the subjects and NAb titers (Fig. 5A), EIA results (Fig. 5B), and the severity of the clinical symptoms (Fig. 5C) was determined. Accordingly, the ages of the subjects positively correlated with NAb titers (r = 0.29, p = 0.04,

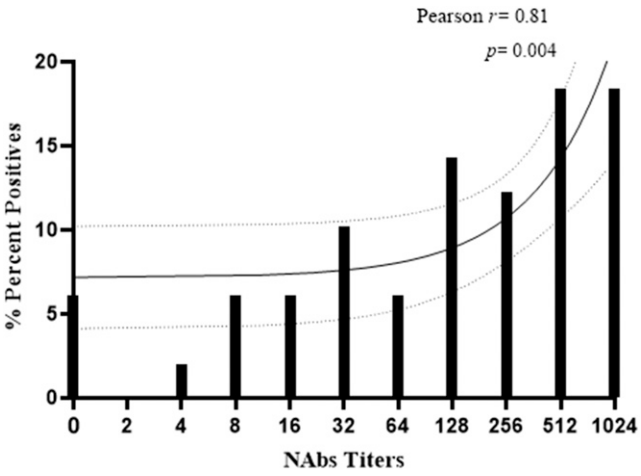

FIGURE 1. Percentage of patients with different titers of NAbs. Spearman correlation (two-tailed) of the serum neutralization activity with percentage of recovered patients (n = 49) is presented. All the samples were tested in two occasions, and on each time, all the samples were tested in duplicate. On each 96-well plate, Ab-positive and -negative serum samples and no-serum-added wells as well as no-virus-added wells, all in duplicate, were included.

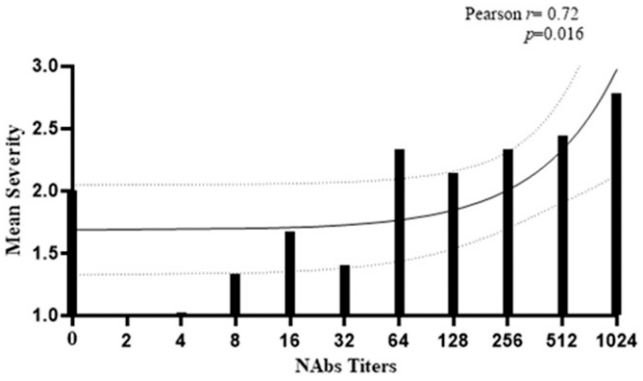

FIGURE 2. The relationship between the NAb titers and mean score of clinical severity. Spearman correlation (two-tailed) of the serum neutralization activity with mean score of clinical severity ($n = 49$) is performed. All the samples were tested in two occasions, and each time, all the samples were tested in duplicate. On each 96-well plate, Ab-positive and -negative serum samples and no-serum-added wells as well as no-virus-added wells, all in duplicate, were included.

95% CI = 0.01213–0.53), EIA readings ($r = 0.38$, $p = 0.006$, 95% CI = 0.1122–0.5984), and clinical severity assessments ($r = 0.38$, $p = 0.007$, 95% CI = 0.1093–0.5965).

Multiple linear regression modeling with serum NAb titers as the outcome variable demonstrated that the overall model was significant ($p < 0.0001$). EIA results were the most significant factor associated with serum NAb titers (Table II). Clinical severity of COVID-19 disease was the second most significant factor related to serum NAb data. The age of the patients was also effective, however it did not reach a significance level as those of the previous factors.

Discussion

Our data underline several important features of the recovery from COVID-19 infection. First, 20% of the tested subjects had neutralization titer of 1/16 or below. These data indicate that a substantial number of the recovered patients do not have a significant titer of NAbs against SARS-CoV-2 virus as demonstrated in cell culture–based neutralization assay. It has generally been assumed that the Abs capable of inhibiting the viral infectivity are the cardinal arm of immune protection from viral infections. However, our data indicate that even though a majority of the recovered patients raised high titers of NAbs specific for infecting virus, there are still a substantial number of patients who did not mount a significant Ab-based response, which could inhibit viral growth by themselves. These NAb-negative patients, however, produced Abs that could bind to viral Ags as demonstrated in EIA. Apparently, these viral Ag binding Abs were not capable of inhibiting the viral growth in vitro. It is probable that these Abs do contribute to the recovery from COVID-19 disease in vivo by a myriad of mechanisms as demonstrated in other viral infections. These mechanisms might include a complement-mediated viral neutralization, Ab-mediated cellular cytotoxicity, or bringing in the element of phagocytic cells into the picture. In cell culture–based viral neutralization assays, unfortunately, there is no universally accepted method to test the role of these additional immune elements in the inhibition of viral infection. One can envision that in the patients who did not have significantly detectable NAbs in their sera, there are Abs that bind to the surface proteins of SARS-CoV-2, but apparently those Abs were not able to prevent the virus to get into the cells and establish a productive viral infection. The full mechanistic details of virus neutralization by Abs are largely unknown. It could be speculated that prevention viral binding to the cellular receptor is probably the most important mechanism of viral neutralization (20). However, in other viral systems, it has been known that Abs that inhibit the fusion between the viral membranes and membranes of vesicles in the cytoplasm could also induce viral neutralization (21). Abs preventing the viral uncoating or viral release from the infected cells would also produce

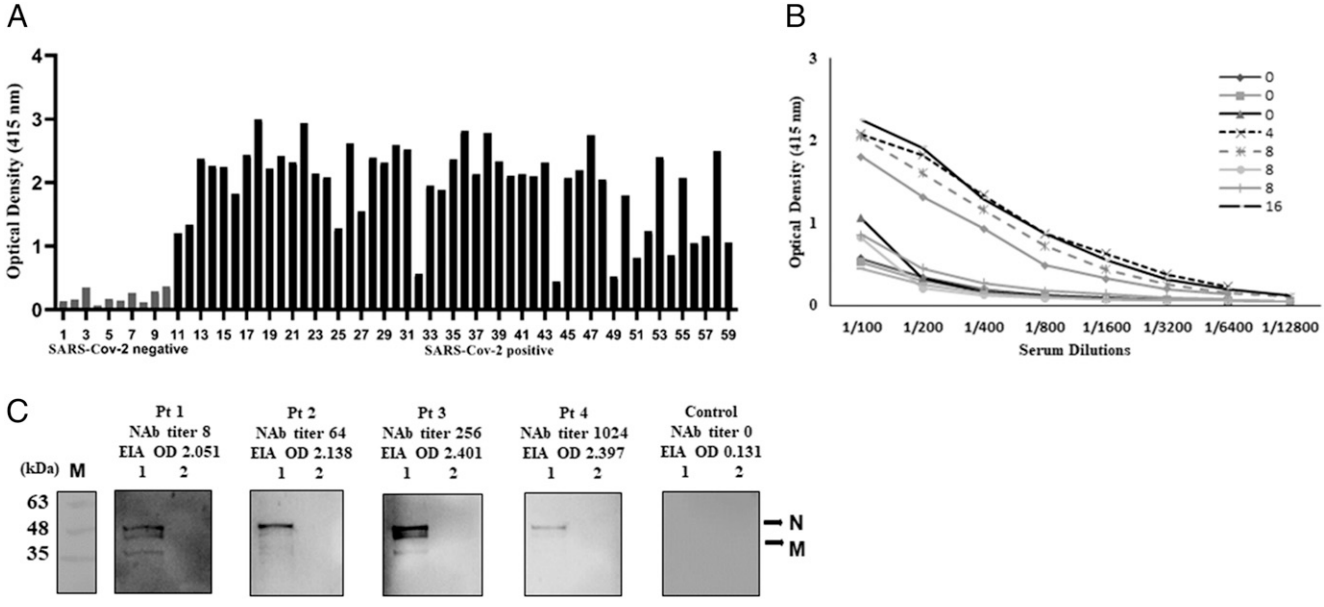

FIGURE 3. EIA readings (**A** and **B**) and Western blotting (**C**) experiments are presented. EIA results of serum samples (1:100 dilutions) from all recovered subjects (A) are plotted against the NAb titers. EIA data are compared by Mann–Whitney *U* test ($p = 0.002$). For EIA, 49 SARS-CoV-2 Ab-positive and 10 SARS-CoV-2 Ab-negative human sera were tested. All reactions were performed in duplicate. (B) EIA results from the samples with no or low titers of NAbs (0, 4, 8, 16) at increasing dilutions are presented. (C) Western blot results from four different serum samples. Lane 1, virus infected; lane 2, uninfected cell lysates. Membranes were incubated with serum samples with four different NAb titers and one healthy serum sample.

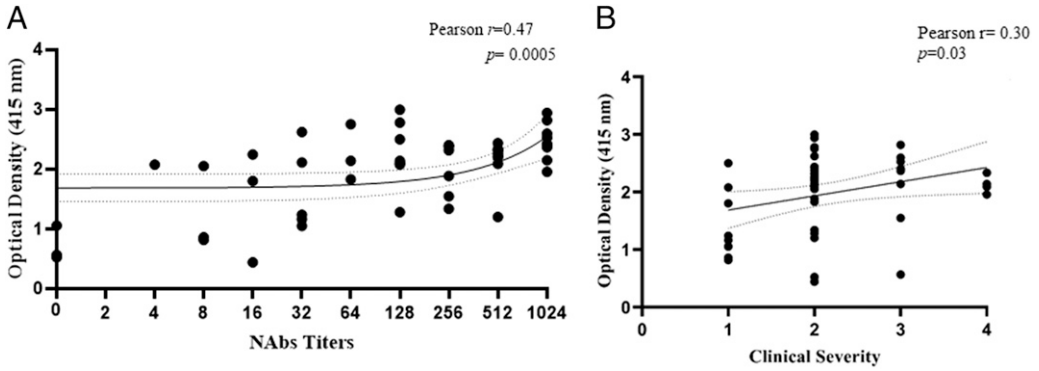

FIGURE 4. The relationships between EIA results and NAb titers (**A**) and between EIA results and clinical severity (**B**) are presented. Spearman correlation (two-tailed) of whole virus-specific Ab titer of recovered patients with the serum neutralization activity and with clinical severity (n = 49) are presented. For EIA, 49 SARS-CoV-2 Ab-positive and 10 SARS-CoV-2 Ab-negative human sera were tested. All reactions were performed in duplicate. For VNA, all the samples were tested in two occasions, and each time, all the samples were tested in duplicate. On each 96-well plate, Ab-positive and -negative serum samples and no-serum-added wells as well as no-virus-added wells, all in duplicate, were included.

inhibition of viral infectivity, and it remains to be investigated if any of these mechanistic details are functional in COVID-19 disease.

All the recovered patients had viral Ag binding Abs in their serum as demonstrated by EIA. In some patients, these Abs were not inhibiting viral infection of cells in the culture, but nevertheless these cohorts fully recovered from COVID-19 infection. These observations indicate that in vaccine efforts it may be reasonable to include Ags capable of inducing such Abs. These vaccine-candidate Ags could be internal viral proteins such as nucleocapsid protein or the structures outside of the receptor-binding domain of spike protein. Indeed, in the Western blot experiment of the study, the presence of nucleocapsid-specific Abs in all the serum samples tested, including the samples with low NAb titers, was demonstrated (Fig. 3C). It has been established in SARS-CoV infection that during even the early phases of disease, Abs capable of binding to this abundantly expressed viral nucleocapsid are the most frequently detected viral-specific Abs in recovered patients (22). When designing such vaccines that are effective in all the subjects receiving the vaccine, it is reasonable to take into account in the data presented in this report and by others that all the protection from COVID-19 infection is not afforded by NAbs alone, and therefore a consideration to the stimulation of non-NAbs and possibly of the other arms of immunity (namely the cellular immunity) should be given.

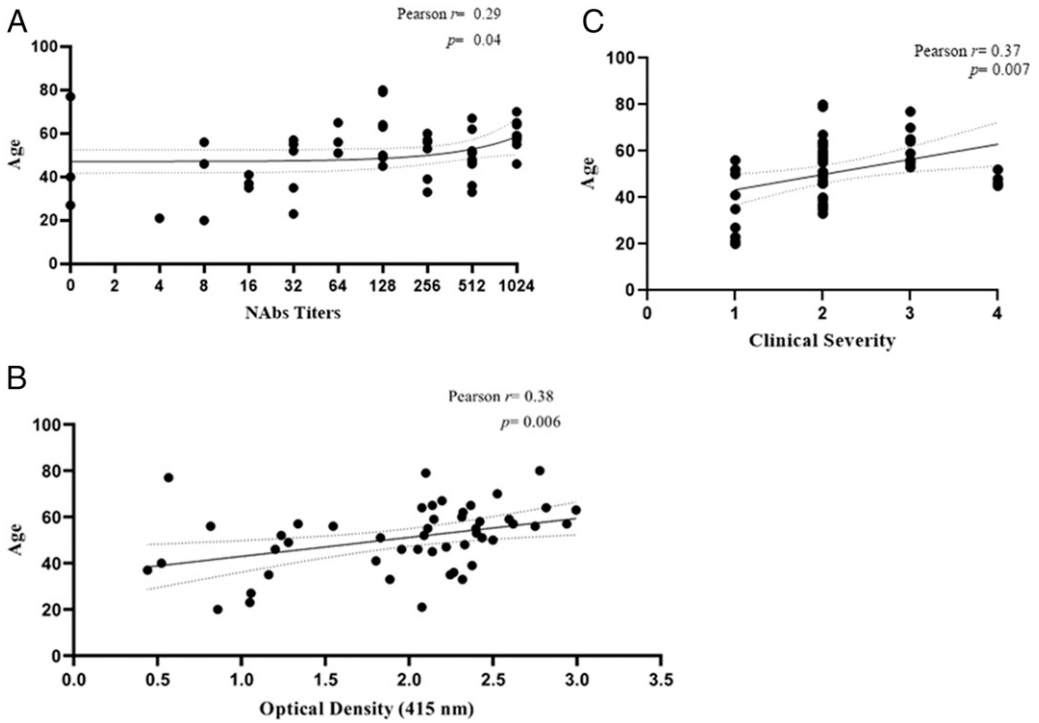

FIGURE 5. The relationship between the age of the subjects and NAb titers (**A**), EIA readings at OD 415 nm (**B**), and clinical severity of the disease (**C**) on individual cohorts are depicted. Spearman correlation (two-tailed) of the age of the recovered patients with neutralizing activity with whole virus-specific Ab titer and with the clinical severity (n = 49) is presented. For EIA, 49 SARS-CoV-2 Ab-positive and 10 SARS-CoV-2 Ab-negative human sera were tested. All reactions were performed in duplicate. For VNA, all the samples were tested in two occasions, and each time, all the samples were tested in duplicate. On each 96-well plate, Ab-positive and -negative serum samples and no-serum-added wells as well as no-virus-added wells, all in duplicate, were included.

Table II. Multiple linear regression analysis of factors associated with NAb titers

Variable	Coefficiency (SE)	*p* Value
Age	0.51 (3.57)	0.89
Clinical severity	163.54 (59.72)	0.01
EIA results	199.88 (74.67)	0.01

SE and *p* value are from a multiple imputation linear regression model.

In a longitudinal study in SARS patients (a closely related coronavirus infection that caused a small-scale pandemic two decades earlier), the detection of NAbs was possible after 15 d of the symptomatic period and reached peak level on day 30. In an 18-patient series, the authors performed consecutive samplings and demonstrated NAbs up to 720 d and concluded that the protection by these Abs would be afforded up to 2 y (14). However, in an experimental macaque model infection of SARS-CoV, Liu et al. (13) ascribed NAbs a role in the promotion of severe acute lung injury and indicated that unlike nucleocapsid-specific Abs, spike protein–specific Abs have roles in the development of severe disease (13, 23).

When we looked at the severity of the clinical symptoms in relation to NAb titers, we noticed a positive relationship between the mean severity of the disease and the titers of the NAbs (Fig. 2). These two sets of observations were not in perfect correlation, but nevertheless, an undeniable relationship between the NAb titers and mean clinical severity existed. Our data on the relationship between the severity of the disease and the titers of NAbs are in support of the recently published study on COVID-19 patients (24). In their study, the authors also noticed a higher titer of Abs in cohorts experiencing a severe clinical disease.

A similar relationship was observed between Ab titers in EIA and clinical severity (Fig. 4A). It is possible that in patients with serious pathologies, viral Ags are more efficiently introduced to humoral immune elements, and therefore, a higher titer of specific Abs are raised. It remains to be established how much of these responses are helping the host in eliminating the virus or in developing severe symptoms. Therefore, it would be premature to conclude that Abs neutralizing or otherwise have a role in the severity of the disease, and this point deserves to be addressed in a larger cohort with much more controlled variables.

Viral Ag binding but neutralization-incapable Abs are shown to be key players in the protection from infection in other viral models (25). Similar phenomenon could be at play in our study with recovered COVID-19 subjects. The subjects with no or low NAbs were positive in the EIA experiment (Fig. 3B), indicating that viral Ags were introduced to the immune system and the patient was able to recover without significant help from NAbs. It is also possible that Ab-independent immune elements could have produced a positive outcome for those subjects. The cellular arm of the immune system also should be investigated in these cohorts.

Another significant finding of our study is that the age of the patients seems to affect the titers of NAbs, EIA results, and the clinical severity of the disease. Wang et al. (24) also noticed a similar trend. It is likely that there is a complex interplay between the age of the infected, immune elements, and the pathology induced by the virus.

It is understandable that the novelty of the results presented in this manuscript is decreasing, and there is a growing body of the data on this subject. However, the lack of NAb response in a portion of recovered subjects is an important issue, and confirmatory data on this subject have merits.

Our results are largely in agreement with the previously reported results of Wu et al. (26). The main difference of this study from that of Wu et al. (26) is that in COVID-19–recovered patients who are consecutively discharged from the hospital, it was noticed that almost one out five patients does not mount a significant NAb response, and unfortunately, earlier study does not address this issue specifically. Another difference between this study and that of Wu et al. (26) is that in the current study, patients with all clinical spectrums were included. In the earlier study, patients with severe symptoms were not accepted into the cohorts because of the concerns related to the immune plasma therapy that was practiced in those patients.

After a short period following the onset of the COVID-19 pandemic, there was a burst of detailed investigations on this disease, and the scope and details of these studies are unparalleled in the history. It is likely that the factors at play during the SARS-CoV-2 infection, including the precise role of NAbs and other elements, will be addressed in much more detail than previously was possible.

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Disclosures

The authors have no financial conflicts of interest.

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