Original Article

Longitudinal neutralizing antibody responses after SARS-CoV-2 infection: A convalescent cohort study in Taiwan

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Abstract   *Background*: Understanding the neutralizing antibody (NAb) titer against COVID-19 over time is important to provide information for vaccine implementation. The longitudinal NAb titer over one year after SARS-CoV-2 infection is still unclear. The purposes of this study are to evaluate the duration of the neutralizing NAb titers in COVID-19 convalescents and factors associated with the titer positive duration.

Methods: A cohort study followed COVID-19 individuals diagnosed between 2020 and 2021 May 15th from the COVID-19 database from the Taiwan Centers for Disease Control. We analyzed NAb titers from convalescent SARS-CoV-2 individuals. We used generalized estimating equations (GEE) and a Cox regression model to summarize the factors associated with NAb titers against COVID-19 decaying in the vaccine-free population.

Results: A total of 203 convalescent subjects with 297 analytic samples were followed for a period of up to 588 days. Our study suggests that convalescent COVID-19 in individuals after more than a year and four months pertains to only 25% of positive titers. The GEE model indicates that longer follow-up duration was associated with a significantly lower NAb titer. The Cox regression model indicated the disease severity with advanced condition was associated with maintaining NAb titers (adjusted hazard ratio: 2.01, 95% CI: 1.11–3.63) and that smoking was also associated with higher risk of negative NAb titers (adjusted hazard ratio: 0.55, 95% CI: 0.33–0.92).

Conclusions: Neutralizing antibody titers diminished after more than a year. The antibody titer response against SARS-CoV-2 in naturally convalescent individuals provides a reference for vaccinations.

Introduction

The COVID-19 pandemic, a global health threat, has created an unprecedented, multidimensional crisis worldwide.[1] Neutralizing antibodies (NAbs) in viral infections play a key role in reducing viral replication and increasing viral clearance.[2–5] According to previous studies, the level of serum NAbs have been correlated with immune protection against SARS-CoV-2 [6]. Understanding the longitudinal dynamics of the NAbs to SARS-CoV-2 is important since it could provide information about protective immunity over time to guide policies for clinical preparedness and vaccine implementation.[7]

Past studies which showed that the follow-up period after infection was 4–6 months of convalescence suggested that the duration of NAbs is likely to be more than 6 months due to the slower trend of decreasing antibody titers.[8–10] Later, several studies reported that most individuals had detectable IgG antibodies against the receptor-binding domain or surrogate NAbs nearly one year after infection.[11,12] A study in Hong Kong showed that NAbs in 73% of serum samples were detectable at days 201–386

with a cutoff of 30% inhibition by the surrogate neutralization test.[13] A study in the USA also showed that anti-SARS-CoV-2 spike antibodies remained detectable for at least 11 months after infection and found that SARS-CoV-2 infection induced long-lived bone marrow plasma cells 8 months after infection in convalescent individuals who experienced mild SARS-CoV-2 infection.[14] These studies showed that the follow-up period after infection was more than half a year of convalescence and rarely measured the neutralizing antibody titers in convalescent COVID-19 individuals. One study pointed out that different SARS-CoV-2 serological assays and the predictive ability of serum neutralization titers deteriorated with time.[15] Therefore, a longer duration of NAbs in convalescent persons after infection has not yet been well established.[4,16] Here, we have a cohort study of 203 confirmed patients who are being longitudinally followed up for a longer period, up to 588 days after confirmation of infection, with 297 serum samples. This study aimed to explore the following: (1) the association between NAb levels and time after COVID-19 and (2) the factors associated with neutralizing antibody decay over time.

Methods

Study design and participants

We conducted a cohort study of convalescent individuals who recovered from COVID-19 in Taiwan. The Taiwan Centers for Disease Control (TCDC) listed COVID-19 as a notifiable disease on January 15, 2020 and asked all reported patients to be hospitalized until SARS-CoV-2 RT−PCR negative before May 2021. The remaining SARS-CoV-2 serum samples collected during the period of hospitalization due to COVID-19 were sent to the TCDC. In the study, participants who were over 20 years of age with prior RT−PCR-diagnosed COVID-19 and were reported to the TCDC between January 2020 and May 15th, 2021 were recruited. We contacted convalescent individuals between April and July 2021. When convalescent individuals agreed to allow us to use their initial serum samples collected in the acute infection stage and prospectively collected convalescent serum samples from the follow-up periods, they gave informed consent. The study was approved by the Institutional Review Board Committee of the National Yang Ming Chiao Tung University (IRB number YM110042E), National Yang Ming Chiao Tung University Hospital (IRB number 2021D002), and Taoyuan General Hospital, Ministry of Health & Welfare (IRB number TYG110026).

Outcome and variable definition

The variables in the TCDC disease databases include patient names, unique personal ID, date of birth, sex, date of diagnosis, disease severity, and medical information regarding COVID-19 symptoms and treatment. All analyses were carried out in individuals older than 20 years old and stratified into two age groups (under 50 years of age vs. 50 years of ages and over).

COVID-19 severity was classified according to the definition of the Taiwan SARS-CoV-2 clinical guideline, which referred to the guidelines of the World Health Organization, National Institutes of Health (NIH), and International Disease Society of America (IDSA).[17−19] There were four categories: mild disease (asymptomatic, or symptomatic without pneumonia and independent), moderate disease (signs with pneumonia, but no signs of severe pneumonia, including SpO2 > 94% on room air), severe disease (signs with severe pneumonia with one of the following breath rates >30 per minute, SpO2 ≤ 94% on room air or infiltration >50% or PaO2/FiO2 ratio <300), and critical disease (acute respiratory distress syndrome (ARDS)). The medical doctors at the TCDC who assessed the clinical information from clinical sites and recorded the data used as variables in this dataset were blinded to the subsequent patient outcomes. Specifically, for the analysis, convalescent individuals were classified into two groups: one group was mild disease, and the other group, advanced disease, was a combination of moderate, severe, and critical disease.

The major outcomes were the trend of NAb titers in convalescent COVID-19 individuals over time and factors associated with positive titers in the NAb-positive cohort. Our study defined "positive titers" as a NAb titer higher than 80-fold (45.97 IU/ml).[20−22] The time interval was calculated as the date of reported COVID-19 to the last recent collection date of convalescent serum.

SARS-CoV-2 neutralization assays

To measure SARS-CoV-2 neutralizing antibody titers, Taiwan CDC strain number 4 (hCoV-19/Taiwan/4/2020; Global Initiative on Sharing All Influenza Data accession ID EPI_-ISL_411927) was titrated to calculate the median tissue culture infectious dose (TCID50). Serum samples were titrated in 8 twofold serial dilutions (starting dilution 1:80 to a final dilution of 1:10240). Each serum dilution (50 μL) and medium (50 μL) containing 100 TCID50 of the virus were mixed and incubated at 37 °C for 1 h. Subsequently, 96-well tissue culture plates (1.2×10^4 cells per well) with Vero E6 cells were infected with 100μL/well of virus/serum mixtures and incubated at 37 °C and 5% CO_2 for 4−5 days. The NAb titers were defined as the reciprocal of the highest dilution capable of inhibiting 50% of the cytopathic effect. The NAb results were derived from quadruplicated tests and calculated according to the Reed-Muench method.

To facilitate the conversion of geometric mean titers (GMTs) to international units (IUs/ml), we purchased the WHO international standard reference panels (20/136, 20/268 and reference samples, 20/150, 20/148, 20/144, and 20/140) from the National Institute for Biological Standards and Control (NIBSC; Potters Bar, UK) for comparison. A neutralizing assay was first developed to calculate the GMTs of the NIBSC serum samples. The function was then developed through the conversion between the GMTs and the assigned IU/ml of each standardized NIBSC serum sample,

$$y = 0.4964x^{1.0334}$$

where y is the titer value of IU/mL and x is the titer value of the GMT.

Statistical analysis

To evaluate the association of follow-up duration and neutralizing antibody titers, we applied a generalized estimating equations (GEE) for repeated measures on NAb titers. We also adjusted for participant's age, sex, living abroad, and body mass index (BMI) in the equations.

A Cox proportional hazards model was used to derive the hazard ratio (HR) and 95% CIs for the association between disease severity and neutralizing antibody titers during the follow-up period. This model, with calendar day as the timescale, eliminates the need to model the underlying temporal trends, which are estimated as the baseline hazard. We also adjusted for participant age, sex, smoking, living abroad, and BMI in the model. The parameters were adjusted by stepwise approaches before inclusion in the Cox model and exclusion by collinearity tests.

The geometric mean of neutralization titers (GMT) and median titers were used in the correlation and stratification analyses for other measurements. Neutralization titers below the limit of detection (negative NAb titer ≤ 45.97 IU/ml) were assigned an arbitrary value of 45.97 IU/ml prior to calculating the geometric mean for the purposes of the correlation and regression model. In the summary statistics, we stratified the follow-up period of each individual

into four levels: 0—49 days, 50—180 days, 181—365 days and over on year (>365 days). In each follow-up group, the geometric mean of neutralization titers (GMT) and median titers were calculated.

All analyses were carried out in R (version 4.2.2) and SAS 9.4.1.

Results

Study population

There were more than a thousand COVID-19 cases reported to the TCDC between January 2020 and May 15th in 2021 in Taiwan. We excluded some cases with conditions such as no phone call, passed away, foreigners and then contacted 841 individuals. A total of 434 convalescent individuals were contacted successfully, and 236 individuals participated in this study. In this study, 33 convalescent individuals whose serum was collected after COVID-19 vaccination were excluded, and a total of 203 convalescent individuals with 297 serum samples with a follow-up period of up to 588 days were available for the final analysis, as shown in Fig. 1. All sera in this study were collected before any of these individuals received any COVID-19 vaccine.

The cohort of 203 convalescent individuals included 114 (56.16%) males and 89 (43.84%) females. One-third of the subjects lived abroad (75 subjects, 36.95%) for a long time and were diagnosed during the quarantine period. The mean age at the date of diagnosis was 24.04 years old (standard deviation: 3.92). A total of 40 subjects (19.70%) were smokers. Overall, 158 subjects (77.83%) were classified with mild COVID-19 disease status during the hospitalization period, and 45 subjects were assigned to the advanced disease group for the following Cox regression analysis (29 cases of moderate disease, 14 cases of severe

disease, and 2 cases of critical disease). The demographic characteristics are listed in Table 1.

Neutralizing antibody titer and follow-up duration

A total of 297 serum samples tested by the NAb were collected between days 26 and 588 after COVID-19 was reported, with samples nominally denoted as acute (<50 days), early (50—180 days), intermediate (181—365 days), and late (>365 days) convalescence. Each follow-up period of the subjects is presented in Fig. 2(A). During convalescence, all NAb measurements showed a general decreasing trend, as shown in Fig. 2(B). The correlation coefficient between NAb titers and follow-up days was −0.33385 with p value < 0.0001. By using Kaplan—Meier survival analysis, our cohort showed that the portion of convalescent COVID-19 individuals who remained positive for NAb titers decreased rapidly from 50% on the 492nd day to 25% on the 512nd day (Fig. 2(C)).

Negative sera were defined by a NAb titer ≤ 45.97 IU/ml. Neutralization titers below the limit of detection (negative NAb titer ≤ 45.97 IU/ml) were assigned an arbitrary value of 45.97 IU/ml. Among 57 individuals within the acute phase, 45.61% showed negative results. We defined the acute phase as < 50 days after onset and the NAb developed and rose 14—20 days after onset. In the acute phase, the subgroups were divided to 0—14days and 15—20 days after onset. The proportion of the negative results in the 0—14 days subgroup was 46.15%, then decrease to 22.73% in the 15—50 days subgroup (Supplementary Table 1).

Then, among individuals who had recovered within 50—180 days, only 33.33% had a NAb titer ≤ 45.97 IU/ml. While both intermediate- and late-phase individuals, 56.41% and 58.87% comprised those with negative results, respectively (Table 2). Neutralization activity (NAb) was still present during follow-up, with a median serological titer of 192.6 IU/ml in the acute group (<50 days), which then
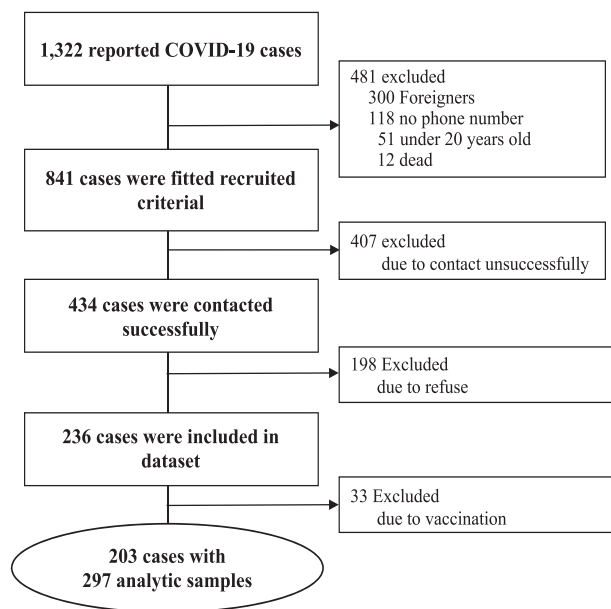

Fig. 1. Cohort screening workflow in the study of unvaccinated convalescent COVID-19 individuals in Taiwan.

Table 1 Demographic data for the convalescent individuals (N = 203).

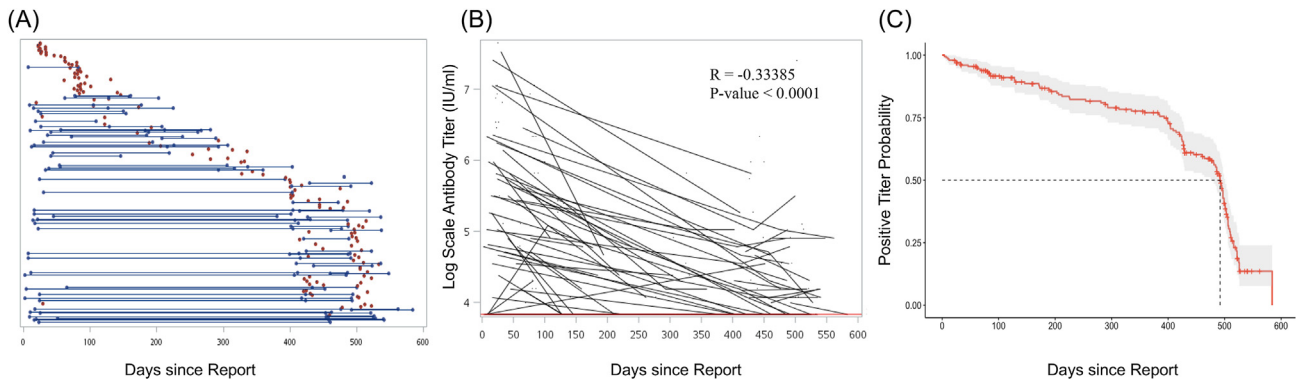| Characteristics	N (%)
Age, mean [range], yr	37.73 [20—88]
< 50 yr	184 (90.64)
≥ 50 yr	19 (9.46)
Sex	
Male	114 (56.16)
Female	89 (43.84)
BMI, mean ± SD	24.04 ± 3.92
Disease Severity	
Mild	158 (77.83)
Moderate	29 (14.29)
Severe	14 (6.90)
Critical	2 (1.00)
Smoking Habit	
Smoking	40 (19.70)
Nonsmoking	163 (80.30)
Living Abroad	
Yes	75 (36.95)
No	128 (63.05)

(A)

(B)

(C)

Fig. 2. (**A**) Individual follow-up period from the COVID-19 official report date. In our cohort, the longest follow-up period was 588 days from the official report date. (**B**) The trends of neutralizing antibody titer on the log 2 scale along with follow-ups. The red line represents a detection limit of 45.97 IU/ml. (**C**) Kaplan—Meier survival analysis for positive NAbs titers of convalescent COVID-19 individuals. The KM plot demonstrated a deceasing trend over time, and the median probability (50%) of positive NAbs occurred on the 492nd day. The population remaining positive for NAbs rapidly decreased to 25% of individuals on the 512nd day.

Table 2 Individual distribution and geometric mean titer are classified by diesease severity (297 samples in 203 individuals).

		Follow-up Time		All Samples			≤45.97 IU/ml		p value
		Mean (Days)	P-value	N	GMT	Median	n	%	
Acute (0—49 days)	Mild	23.86	0.0742	44	178.48	178.63	23	52.27	0 0.2443
	Advanced	17.69		13	247.36	244.54	3	23.07	
	Subtotal			57	192.27	192.60	26	45.61	
Early (50—180 days)	Mild	98.76	0.5901	41	75.31	65.77	19	46.34	0.0043
	Advanced	93.58		19	89.50	192.60	1	5.26	
	Subtotal			60	114.42	94.10	20	33.33	
Intermediate (181—365 days)	Mild	258.75	0.1210	32	58.87	45.97	21	65.63	0.0289
	Advanced	295.14		7	89.17	79.76	1	14.29	
	Subtotal			39	63.55	45.97	22	56.41	
Late (> 365 days)	Mild	462.60	0.2907	106	63.82	45.97	68	64.15	0.7672
	Advanced	472.09		35	76.94	58.37	15	42.86	
	Subtotal			141	66.88	45.97	83	58.87	

The value 45.97 IU/ml represented the detection limit of the neutralization titer. The statistical significance between mild disease and advanced disease for each time interval was compared by a two-tailed unpaired t test, and a p value < 0.05 was considered to reach statistical significance.

decreased to 94.10 IU/ml for early phase cases (50—180 days). After 181 days, the persistent downward trend signified a negative neutralization activity, which was below 45.97 IU/ml (Table 2). The NAb waning rates between the time intervals were calculated by mean follow-up time and GMT. The GMT in the advance disease group waned faster than the mild group during the first 180 days from acute stage to early stage (Supplementary Table 2).

The GEE model indicates that longer follow-up duration was associated with a significantly lower NAb titer among the study cohort (Table 3). Sex, age and living abroad were not associated significantly with NAb titer. However,

Table 3 The associations of the factors of NAb titers analyzed by generalized estimated equations (GEE).

Variable	Reference	Estimate	S.E.	95% Confidence interval (CI)	Z-value	P-value
Intercept		5.30	0.17	[4.962, 5.646]	30.42	<0.0001*
Disease Severity	Advance	−0.26	0.13	[−0.510, −0.011]	−2.05	0.0404*
Living Abroad	N	−0.14	0.11	[−0.357, 0.068]	−1.33	0.183
Sex	Female	0.08	0.11	[−0.134, 0.298]	0.75	0.4562
Age Group	<50 yr	0.35	0.21	[−0.069, 0.759]	1.64	0.102
Time(Days)		−0.002	0.0003	[−0.003, −0.002]	−7.63	<0.0001*
Smoking	N	−0.32	0.16	[−0.627, −0.018]	−2.08	0.0377*

disease severity with mild condition and smoking were at increased probability of lower NAb titer (Table 3).

Disease severity and NAb

Individuals with mild symptoms persistently had a greater proportion of NAbs lower than the detectable level (≤45.97 IU/ml) throughout every phase. In the intermediate and late phases, with almost a hundred mild participants, more than 60% had negative results when advanced cases after 181 days had 14.29% (n = 1) and 42.86% (n = 15) negative outcomes, respectively.

The neutralizing antibody levels showed a strong association according to disease severity. Participants with advanced COVID-19 had higher neutralizing titers (moderate/severe/critical GMT 247.36 IU/ml) compared to the 23 participants with mild COVID-19 (GMT 178.48 IU/ml), but the results of the two-tailed unpaired t test did not reach statistical significance (p value = 0.2443), as illustrated in Table 2. The increase in neutralization titer was similar across all phases, with individuals with advanced disease maintaining higher titers at early phase (50—180 days) (GMT 89.50 IU/ml in advanced disease vs. GMT 75.31 IU/ml in mild disease participants) and reached statistical significance (p = 0.0043). Furthermore, the intermediate and late phases had a similar trend when comparing disease severity to NAb titers, and higher median titers were observed in the advanced disease group (median titer: 79.76 IU/ml in the intermediate phase; 58.37 IU/ml in the late phase) than in the mild group (median titer: 45.97 IU/ml).

The Kaplan—Meier survival analysis of the maintenance of high neutralizing antibody titers among individuals with mild symptoms and advanced symptoms (log-rank test p value = 0.0057) is shown in Fig. 3(A). The cumulative hazard indicated that advanced disease was associated with significantly higher positive NAb titers (hazard ratio: 2.01, 95% CI: 1.11—3.63), as shown in Fig. 3(B).

Smoking habit and NAb

The neutralizing antibody responses demonstrated higher NAbs titers in the smoking groups (median 69.94 IU/ml) than in the nonsmoking group (median 58.37 IU/ml) during the acute phase (0—49 days). The neutralization titer levels were the opposite across the early phase at 50—180 days, and the nonsmoking group showed higher titers (median titer 94.10 IU/ml) than the smoking group (median titer 62.07 IU/ml). After 181 days, the majority of the mild
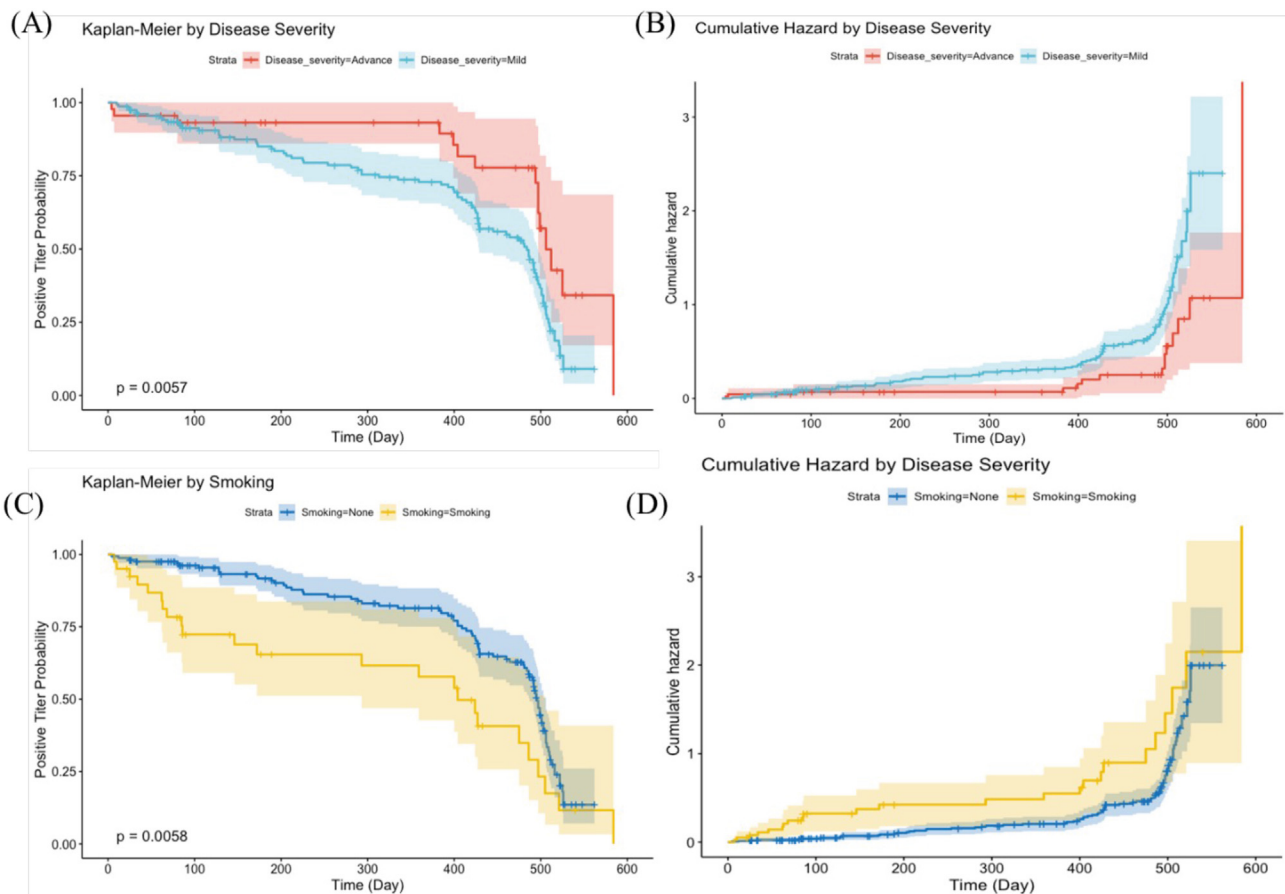

Fig. 3. Kaplan—Meier survival analysis for a NAb titer response during convalescence. (**A**) Kaplan—Meier survival curves were stratified by disease severity. The log-rank test reached statistical significance between advanced disease and mild disease (p = 0.0057) (**B**) The cumulative hazard of decreasing neutralization response by disease severity. The log-rank test reached statistical significance between ever smokers and never smokers (p = 0.0058). (**C**) The Kaplan—Meier survival curves were stratified by smoking habit. (**D**) The cumulative hazard of decreasing neutralization response by smoking habit.

Table 4 Individual distribution and geometric mean titer classified by smoking.

		All Samples			≤45.97 (IU/ml)		P value
		N	GMT	Median	n	%	
Acute (0—49 days)	Non-Smoking	41	89.69	58.37	19	46.34	0.3377
	Smoking	16	124.08	69.94	7	43.75	
	Subtotal	57	192.27	192.60	26	45.61	
Early (50—180 days)	Non-Smoking	44	109.33	94.10	13	29.55	0.0728
	Smoking	16	67.41	62.07	7	43.75	
	Subtotal	60	114.42	94.10	20	33.33	
Intermediate (181—365 days)	Non-Smoking	33	61.28	45.97	18	54.55	0.8637
	Smoking	6	61.49	45.97	4	66.67	
	Subtotal	39	63.55	45.97	22	56.41	
Late (> 365 days)	Non-Smoking	122	66.45	45.97	70	57.38	0.5229
	Smoking	19	62.15	45.97	13	68.42	
	Subtotal	141	66.88	45.97	83	58.87	

The statistical significance between smoking and nonsmoking for each time interval was compared by a two-tailed unpaired t test, and a p value < 0.05 was considered to reach statistical significance.

participants had NAb levels lower than the detectable level (≤45.97 IU/ml), but for nonsmoking cases, less than 58% were detectable. In the intermediate and late phases, the median NAb values for both the smoking and nonsmoking groups were 45.97 IU/ml, as shown in Table 4.

The Kaplan—Meier survival analysis (log-rank test, p value = 0.0058) illustrated that nonsmoking group could keep the positive NAb titers longer compared to the smoking group (Fig. 3(B)). The cumulative hazard indicated that the smoking group had a higher risk of negative NAbs (hazard ratio: 0.55, 95% CI: 0.33—0.92), as shown in Fig. 3(D).

Cox regression model for maintaining neutralizing antibody titer

The Cox regression model indicated that disease severity with advanced condition was most strongly associated with maintaining positive NAbs (adjusted odds ratio: 2.01, 95% CI: 1.11—3.63, p = 0.0209), as shown in Fig. 4. In addition, the positive neutralizing titers among participants who reported being an ever-smoker were significantly different from those of participants who had never smoked (adjusted odds ratio: 0.55, 95% CI: 0.33—0.92 p = 0.0217). Sex, living abroad, BMI, and age were not significant cofactors. Multivariable Cox regression models examined the effects of parameters in predicting positive NAbs in our cohort, and the model performance was determined by global p value (log-rank test P value = 0.0087) and Akaike information criteria (AIC = 894.15), as shown in Fig. 4.

Discussion

We measured real-world data on serum neutralizing antibody titers for an extended period, up to 588 days after confirmation of SARS-CoV-2 infection in convalescent COVID-19 cohorts, to calculate the long-term trend of SARS-CoV-2 immunity. The SARS-CoV-2 neutralizing responses in convalescent COVID-19 individuals are known to show a decreasing trend with time after diagnosis.[3,7,21,23—26] The

titers of neutralizing antibodies decreased modestly, with the titers at 3—4 months after diagnosis generally being approximately 4-fold lower than those at 1 month.[13,15] Our study suggests that convalescent COVID-19 individuals, after more than a year and four months, account for up to only 25% of positive NAbs on the 512nd day.

Our study found that the positive duration of SARS-CoV-2 neutralization titers was correlated with the disease severity of COVID-19 in all phases of onset. Individuals with more severe symptoms tended to have higher antibody responses in the acute phase after infection and maintained a higher proportion of titers with a longer duration in the first year after diagnosis. Previous studies have proven that neutralizing antibodies against SARS-CoV-2 have a positive correlation with the severity of the disease in cross-sectional analysis.[2,4,6,21,22,27] These longitudinal studies also showed the duration of the immune response with regard to disease severity. Furthermore, our study showed that individuals with advanced disease generally presented a longer positive duration of neutralizing antibody titers than individuals with mild symptoms after symptom onset or even asymptomatic infection. This result remains consistent with another similar study that reported enhanced long-term adaptive immunity in individuals with advanced disease compared with those with mild disease.[4,13,28]

Based on the Cox regression model, smoking was a risk factor for a decline in positive neutralizing antibody titers over the follow-up period. Previous studies focusing on age and smoking that predicted antibody titers 3 months after the second dose of the BNT vaccine in a Japanese population also found that smoking was a risk factor for low antibody titers.[15,29—32] Our study showed that the adjusted odds ratio for individuals with a smoking habit was 0.547 for maintaining neutralizing antibody protection, which was significant (p value = 0.0217).

The detection threshold of the NAb in this study was based on the FDA-recommended criteria for emergency use authorization (EUA). The plasma, which was screened by ELISA, was from a convalescent individual with a neutralizing antibody titer over 1:160 or higher. This optimized threshold was utilized in our determination of antibody

Hazard ratio of Positive of Neutralizing Antibody Titer

Group		Hazard Ratio		P-value
Severity	Mild (N=158)	Reference		
	Advanced (N =45)	2.01 (1.11 - 3.63)		0.021 *
Living_Abroad	No (N=128)	Reference		
	Yes (N=75)	0.77 (0.50 - 1.18)		0.227
Age_Group	<50yr (N=184)	Reference		
	>=50yr (N=19)	1.44 (0.51 - 4.02)		0.488
Smoking	None (N=163)	Reference		
	Smoking (N=40)	0.55 (0.33 - 0.92)		0.022 *
BMI	(N=203)	1.00 (0.95 - 1.06)		0.918
Sex	Female (N=89)	Reference		
	Male (N=114)	0.85 (0.55 - 1.30)		0.447

Events; 105; Global p-value (Log-Rank): 0.0087392
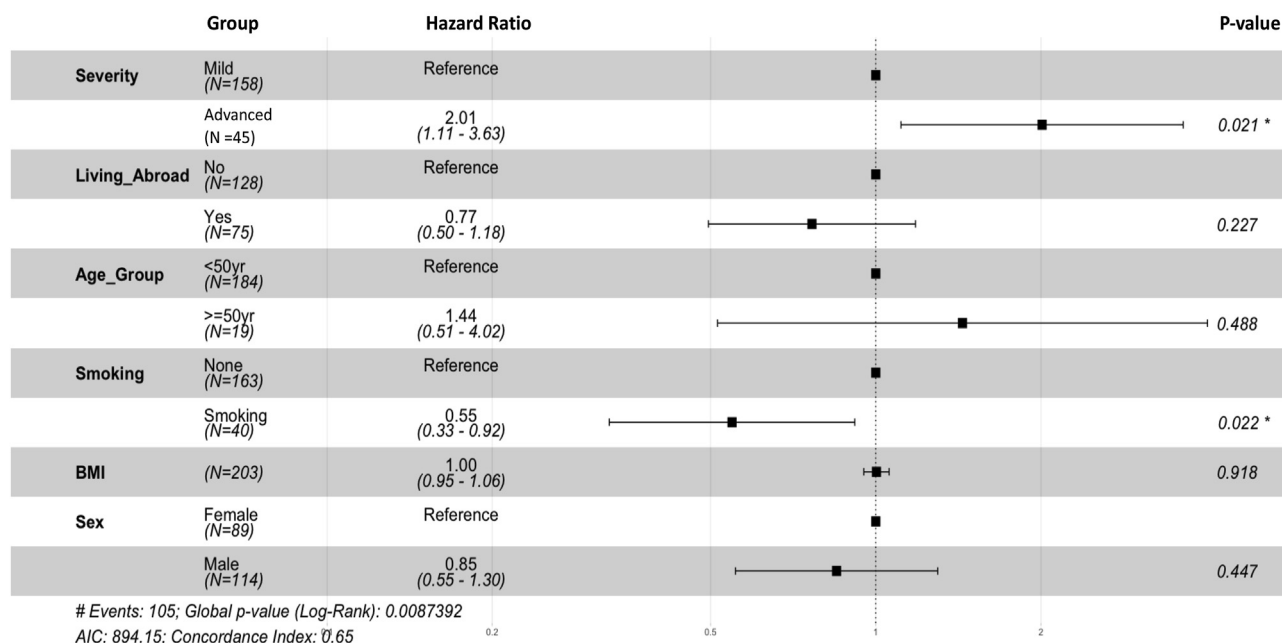AIC: 894.15; Concordance Index: 0.65

Fig. 4. Forest plot of Cox proportional hazards model for the positive neutralizing antibody titer. The figure provides a forest plot reporting the hazard ratio (HR) and the 95% confidence intervals of the HR for each covariate included in the Cox proportional hazards model. The best fitting Cox model was determined by Akaike information criteria (AIC = 894.15) and log-rank test (P value = 0.0087).

protection as 1:80.[33,34] Based on one study that they determined NAb cutoff titer (titer$\geq$80) as suitable for comparing immunoassays and the other study estimated the 50% protective neutralization level as 54 IU/ml (95% CI 30—96 IU/ml), we set the cutoff titer as titer$\geq$80 and $\geq$ 45.97 IU/m for the virus-live neutralizing assay.[35,36] To assure our lab results performance, a subset of samples was tested by using other platforms of GenScript cPass™ sVNT, indicating that two platforms robustly correlated with specific titers of neutralizing antibodies for samples from acute phase. High sensitivity (85.22%), specificity (88.89%), positive predictive value (97.03%) and negative predictive value (58.54%) were evident. The reliability (Cohen's kappa coefficients = 0.62, 95% C.I. = [0.47—0.77]) indicated fair agreement between GenScript cPass and reported method.

In this study, several constraints limited the predictive ability of the Cox regression model. The 181- to 365-day interval had fewer serum samples from individuals with advanced disease, which limited our ability to make a reliable comparison of the protective duration among different phases. Therefore, the comprehensive change in the antibody response during the late phase might be overestimated due to a delayed cutoff point (>365 days). The NAb titers ranges fall within 320-fold and we didn't use a lower dilution factor (e.g. 20-fold) to conduct the neutralizing assay due to the limited volume of each serum. However, the definition of "positive titers" as a NAb titer higher than 80-fold (45.97 IU/ml) was still confidence according to the past studies.[35,36] Despite this potential limitations, the strengths of our study include successful prospective follow-up of a cohort without COVID-19

vaccination for more than one year and the use of objective biologic markers, the plasma neutralizing antibody titer (NAb).

In this study, we focused on the positive titers of the plasma neutralizing antibody response as a predictive parameter over the follow-up period. Disease severity altered the consequences of the positive titers in naturally convalescent individuals. Understanding the long-term antibody-based protection of SARS-CoV-2 neutralization will provide insights into the nature of protective antibody responses.[37]

Declaration of competing interest

The authors have no conflicts of interest.

Acknowledgments

References

1. Gao GF. From "A"IV to "Z"IKV: attacks from emerging and re-emerging pathogens. *Cell* 2018;**172**:1157—9.

2. Duan K, Liu B, Li C, Zhang H, Yu T, Qu J, et al. Effectiveness of convalescent plasma therapy in severe COVID-19 patients. *Proc Natl Acad Sci U S A* 2020;**117**:9490—6.

3. Shen C, Wang Z, Zhao F, Yang Y, Li J, Yuan J, et al. Treatment of 5 critically ill patients with COVID-19 with convalescent plasma. *JAMA* 2020;**323**:1582—9.

4. Arkhipova-Jenkins I, Helfand M, Armstrong C, Gean E, Anderson J, Paynter RA, et al. Antibody response after SARS-CoV-2 infection and implications for immunity: a rapid living review. *Ann Intern Med* 2021;**174**:811—21.

5. Lin Q, Zhu L, Ni Z, Meng H, You L. Duration of serum neutralizing antibodies for SARS-CoV-2: lessons from SARS-CoV infection. *J Microbiol Immunol Infect* 2020;**53**:821—2.

6. Khoury DS, Cromer D, Reynaldi A, Schlub TE, Wheatley AK, Juno JA, et al. Neutralizing antibody levels are highly predictive of immune protection from symptomatic SARS-CoV-2 infection. *Nat Med* 2021;**27**:1205—11.

7. Chvatal-Medina M, Mendez-Cortina Y, Patino PJ, Velilla PA, Rugeles MT. Antibody responses in COVID-19: a review. *Front Immunol* 2021;**12**:633184.

8. He Z, Ren L, Yang J, Guo L, Feng L, Ma C, et al. Seroprevalence and humoral immune durability of anti-SARS-CoV-2 antibodies in Wuhan, China: a longitudinal, population-level, cross-sectional study. *Lancet* 2021;**397**:1075—84.

9. Peghin M, De Martino M, Fabris M, Palese A, Visintini E, Graziano E, et al. The fall in antibody response to SARS-CoV-2: a longitudinal study of asymptomatic to critically ill patients up to 10 Months after recovery. *J Clin Microbiol* 2021;**59**: e0113821.

10. Varona JF, Madurga R, Penalver F, Abarca E, Almirall C, Cruz M, et al. Kinetics of anti-SARS-CoV-2 antibodies over time. Results of 10 month follow up in over 300 seropositive health care workers. *Eur J Intern Med* 2021;**89**:97—103.

11. Westmeier J, Paniskaki K, Karakose Z, Werner T, Sutter K, Dolff S, et al. Erratum for Westmeier et al., "Impaired cytotoxic CD8$^+$ T cell response in elderly COVID-19 patients. *mBio* 2020;**11**. e02805—02820.

12. Ganji A, Farahani I, Khansarinejad B, Ghazavi A, Mosayebi G. Increased expression of CD8 marker on T-cells in COVID-19 patients. *Blood Cells Mol Dis* 2020;**83**:102437.

13. Lau EH, Hui DS, Tsang OT, Chan WH, Kwan MY, Chiu SS, et al. Long-term persistence of SARS-CoV-2 neutralizing antibody responses after infection and estimates of the duration of protection. *EClinicalMedicine* 2021;**41**:101174.

14. Turner JS, Kim W, Kalaidina E, Goss CW, Rauseo AM, Schmitz AJ, et al. SARS-CoV-2 infection induces long-lived bone marrow plasma cells in humans. *Nature* 2021;**595**:421—5.

15. Muecksch F, Wise H, Batchelor B, Squires M, Semple E, Richardson C, et al. Longitudinal serological analysis and neutralizing antibody levels in coronavirus disease 2019 convalescent patients. *J Infect Dis* 2021;**223**:389—98.

16. Fu Y, Li Y, Guo E, He L, Liu J, Yang B, et al. Dynamics and correlation among viral positivity, seroconversion, and disease severity in COVID-19: a retrospective study. *Ann Intern Med* 2021;**174**:453—61.

17. World Health Organization. *Living guidance for clinical management of COVID-19: living guidance, 23 November 2021.* World Health Organization; 2021.

18. Bhimraj A, Morgan RL, Shumaker AH, Lavergne V, Baden L, Cheng VC, et al. Infectious diseases society of America guidelines on the treatment and management of patients with COVID-19. *Clin Infect Dis* 2020:ciaa478.

19. Taiwan Centers for Disease Control. *Clinidal treatment guidelines on SARS-CoV-2 infection.* 2022. https://www.cdc.gov.tw/Category/Page/xCSwc5oznwcqunujPc-qmQ.

20. Boonyaratanakornkit J, Morishima C, Selke S, Zamora D, McGuffin S, Shapiro AE, et al. Clinical, laboratory, and temporal predictors of neutralizing antibodies against SARS-CoV-2 among COVID-19 convalescent plasma donor candidates. *J Clin Invest* 2021;**131**:e144930.

21. Chen W, Zhang J, Qin X, Wang W, Xu M, Wang LF, et al. SARS-CoV-2 neutralizing antibody levels are correlated with severity of COVID-19 pneumonia. *Biomed Pharmacother* 2020;**130**:110629.

22. Klein SL, Pekosz A, Park HS, Ursin RL, Shapiro JR, Benner SE, et al. Sex, age, and hospitalization drive antibody responses in a COVID-19 convalescent plasma donor population. *J Clin Invest* 2020;**130**:6141—50.

23. Chen CY, Liu KT, Shih SR, Ye JJ, Chen YT, Pan HC, et al. Neutralization assessments reveal high cardiothoracic ratio and old age as independent predictors of low neutralizing antibody titers in hemodialysis patients receiving a single dose of COVID-19 vaccine. *J Personalized Med* 2022;**12**:68.

24. Chen YC, Kung YA, Janapatla RP, Hsu MH, Lien R, Chen JC, et al. Neutralization antibody titers against SARS-CoV-2 in an infant born to a mother with COVID-19. *Pediatr Neonatol* 2021; **62**:661—3.

25. Oguntuyo KY, Stevens CS, Hung CT, Ikegame S, Acklin JA, Kowdle SS, et al. Quantifying absolute neutralization titers against SARS-CoV-2 by a standardized virus neutralization assay allows for cross-cohort comparisons of COVID-19 sera. *mBio* 2021;**12**. e02492—02420.

26. Solund C, Underwood AP, Fernandez-Antunez C, Bollerup S, Mikkelsen LS, Villadsen SL, et al. Analysis of neutralization titers against SARS-CoV-2 in health-care workers vaccinated with prime-boost mRNA-mRNA or vector-mRNA COVID-19 vaccines. *Vaccines (Basel)* 2022;**10**:75.

27. Park JH, Cha MJ, Choi H, Kim MC, Chung JW, Lee KS, et al. Relationship between SARS-CoV-2 antibody titer and the severity of COVID-19. *J Microbiol Immunol Infect* 2022;**55**(6):1094—100.

28. Imai K, Kitagawa Y, Tabata S, Kubota K, Nagura-Ikeda M, Matsuoka M, et al. Antibody response patterns in COVID-19 patients with different levels of disease severity in Japan. *J Med Virol* 2021;**93**:3211—8.

29. Sarich P, Cabasag CJ, Liebermann E, Vaneckova P, Carle C, Hughes S, et al. Tobacco smoking changes during the first prevaccination phases of the COVID-19 pandemic: a systematic review and meta-analysis. *EClinicalMedicine* 2022;**47**:101375.

30. Mejia MC, Zoorob R, Levine RS, Huang X, Hennekens CH. Cross-sectional survey of smoking patterns during the COVID-19 pandemic in a tobacco cessation and lung cancer screening program. *Ochsner J* 2022;**22**:48—60.

31. Nomura Y, Sawahata M, Nakamura Y, Koike R, Katsube O, Hagiwara K, et al. Attenuation of antibody titers from 3 to 6 Months after the second dose of the BNT162b2 vaccine depends on sex, with age and smoking risk factors for lower antibody titers at 6 months. *Vaccines (Basel)* 2021;**9**:1500.

32. Nomura Y, Sawahata M, Nakamura Y, Kurihara M, Koike R, Katsube O, et al. Age and smoking predict antibody titres at 3 Months after the second dose of the BNT162b2 COVID-19 vaccine. *Vaccines (Basel)* 2021;**9**:1042.

33. Bloch EM, Shoham S, Casadevall A, Sachais BS, Shaz B, Winters JL, et al. Deployment of convalescent plasma for the prevention and treatment of COVID-19. *J Clin Invest* 2020;**130**: 2757—65.

34. Stadlbauer D, Amanat F, Chromikova V, Jiang K, Strohmeier S, Arunkumar GA, et al. SARS-CoV-2 seroconversion in humans: a detailed protocol for a serological assay, antigen production, and test setup. *Curr Protoc Microbiol* 2020;**57**:e100.

35. Graninger M, Jani CM, Reuberger E, Pruger K, Gaspar P, Springer DN, et al. Comprehensive comparison of seven SARS-CoV-2-specific surrogate virus neutralization and anti-spike IgG antibody assays using a live-virus neutralization assay as a reference. *Microbiol Spectr* 2023;**11**:e0231422.

36. Khoury DS, Cromer D, Reynaldi A, Schlub TE, Wheatley AK, Juno JA, et al. Neutralizing antibody levels are highly predictive of immune protection from symptomatic SARS-CoV-2 infection. *Nat Med* 2021;**27**:1205–11.

37. Sterlin D, Mathian A, Miyara M, Mohr A, Anna F, Claer L, et al. IgA dominates the early neutralizing antibody response to SARS-CoV-2. *Sci Transl Med* 2021;**13**:eabd2223.

Appendix A. Supplementary data

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