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Research paper

SARS-CoV-2 antibody kinetics eight months from COVID-19 onset: Persistence of spike antibodies but loss of neutralizing antibodies in 24% of convalescent plasma donors

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ABSTRACT

Elucidating the characteristics of human immune response against SARS-CoV-2 is of high priority and relevant for determining vaccine strategies. We report the results of a follow-up evaluation of anti-SARS-CoV-2 antibodies in 148 convalescent plasma donors who participated in a phase 2 study at a median of 8.3 months (range 6.8–10.5 months) post first symptom onset. Monitoring responses over time, we found contraction of antibody responses for all four antigens tested, with Spike antibodies showing higher persistence than Nucleocapsid antibodies. A piecewise linear random-effects multivariate regression analysis showed a bi-phasic antibody decay with a more pronounced decrease during the first 6 months post symptoms onset by analysis of two intervals. Interestingly, antibodies to Spike showed better longevity whereas their neutralization ability contracted faster. As a result, neutralizing antibodies were detected in only 76% of patients at the last time point. In a multivariate analysis, older age and hospitalization were independently associated with higher Spike, Spike-RBD, Nucleocapsid, N-RBD antibodies and neutralizing antibody levels. Results on persistence and neutralizing ability of anti-SARS-CoV-2 antibodies, especially against Spike and Spike-RBD, should be considered in the design of future vaccination strategies.

1. Introduction

The duration of antibody responses against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an area of extensive research in light of the new approvals of vaccines against SARS-CoV-2 and the use of convalescent plasma for patients with coronavirus disease 2019 (COVID-19) [1-4]. Production of anti-SARS-CoV-2 antibodies is observed in almost all symptomatic patients with COVID-19 and the elevated titers correlate with the severity of the symptoms [5-9]. Several observational studies have reported that asymptomatic individuals have also developed anti-SARS-CoV-2 antibodies [10-13]. The frequency of asymptomatic COVID-19 carriers who develop SARS-CoV-2-specific

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antibodies is much lower compared to symptomatic patients [9,14-16] and this possibly correlates with the viral load [17].

Several studies have shown a continuous decrease in antibody titers against SARS-CoV-2 observed at one to three months after symptoms onset [18-20], while others, including a large study in the Icelandic population, did not confirm reduction of these antibodies 3–4 months after infection [21,22]. A possible reduction in anti-SARS-CoV-2 antibodies may affect immunity against the virus, although cases with COVID-19 re-infection are rare to-date [23,24]. A previous COVID-19 infection provides 90% protection against symptomatic reinfection over a 5 month period; a study by Public Health England of 20,787 healthcare workers showed that 6614 out of 20,787 had antibodies after infection and this group developed 44 reinfections [25]. Furthermore, kinetics of antibodies against SARS-CoV-2 is of great importance after the introduction of the new vaccines as it will determine the time of possible re-vaccinations.

The aim of this study was to determine the levels of anti-SARS-CoV-2 antibodies after 6–8 months post COVID-19 disease in paired samples from donors who participated in a phase 2 clinical study of convalescent plasma for the treatment of COVID-19.

2. Materials and methods

Study Design: This study included plasma donors who participated in an ongoing phase 2 study (NCT04408209) for the use of convalescent plasma for the treatment of COVID-19 infection, started in Greece on April 28, 2020. Continued measurements of anti-SARS-CoV-2 binding antibodies and neutralizing antibodies (NAb) is a secondary endpoint of the phase 2 study. Donors gave informed consent, as previously described [9].

The study design for plasma donors and results of the screening for these donors at a median of 2.1 months post symptom onset or PCR positivity have been previously reported [9], defined as time of infection. The present analysis reports the results regarding the continued presence of anti-SARS-CoV-2 antibodies in samples collected longitudinally at a median of 5.9 months ($n = 135$) and 8.3 months ($n = 94$) post infection. Analysis of the 8-month time point included patients ($N = 81$) analyzed at all three timepoints (median 2.1, 5.6 and 8.4 months).

Inclusion criteria for the plasma donors: All inclusion criteria for the plasma donors have been previously described [9]. In summary, the main inclusion criteria included: (i) signed informed consent; (ii) confirmed SARS-CoV-2 infection by PCR of the nasal and/or pharyngeal swab; (iii) interval of at least 14 days after complete recovery from a SARS-CoV-2 infection (no symptoms, complete resolution of organ dysfunction which was caused by SARS-CoV-2); (iv) anti-SARS-CoV-2 immune response with anti-SARS-CoV-2 antibodies; (v) two negative SARS-CoV-2 PCR results (nasal and/or pharyngeal swab); the time interval between the two negative tests should be at least 7 days.

Plasma Donors Enrollment: Volunteer donors were tested for the presence of anti-SARS-CoV-2 in the period April 28, 2020 to July 30, 2020 in four centers in Greece and, after their initial screening, in the same centers. All study procedures were carried out in accordance with the declaration of Helsinki (18th World Medical Association Assembly), its subsequent amendments, the Greek regulations and guidelines, as well as the Good Clinical practice Guidelines (GCP) as defined by the International Conference of Harmonization. The study was approved by the local ethics committees of all participating hospitals.

Detection of anti-SARS-CoV2 antibodies: For the detection of anti-SARS-CoV-2 antibodies, we used four in-house ELISA assays measuring antibodies to (i) trimeric Spike (S), (ii) Spike Receptor Binding Domain (Spike-RBD), (iii) Nucleocapsid, and (ii) Nucleocapsid RNA Binding Domain (N-RBD). Antibody levels were measured using eight 4-fold serial serum dilutions starting at 1:50 and reaching a maximum of 1:819,200, showing the great dynamic range of the ELISA assays. The maximal titer of 819,200 was detected in only 1 of 148 samples for Spike-RBD and 2 of 148 samples for N-RBD at the screening. The sensitivity of the assay is 100% because it detected antibodies in all the PCR+ samples tested. Specificity was tested with 17–20 human samples obtained in years 2015–2018, prior to SARS-CoV-2. In addition, we retrospectively compared our assays to other assays including the FDA-approved Elecsys Anti-SARS-CoV-2 S assay (Roche Diagnostics GmbH, Mannheim, Germany). Comparison ($N = 57$ samples) to our in-house Spike-RBD assay showed excellent correlation (Spearman r of 0.8675, $p<0.0001$).

The cut-off values were determined using 17–23 healthy human plasma samples collected between 2015 and 2018 tested against the different antigens. The cut-off values were defined using mean plus 2 STD (N-RBD, 3x mean) measured from OD values obtained between the 1:200 to 1:819,200 dilutions. Endpoint titers were defined as the last dilution value being higher than the cut-off. A sample below the threshold at 1:200 dilution is defined as background of the assay (dilution 1:50) and is entered as 50. A modelfit approach was conducted in R to model the curve to more accurately define endpoint titers. Antibody levels were also expressed as area-under-the curve (AUC) values covering serial serum dilutions (log10 transformed) above the endpoint titer cut-off. If a sample has an endpoint titer of 1:50, it is considered below threshold of the assay and the value is entered as 0.1. Comparison of AUC and modelfit measurments show significant correlations ($p<0.0001$) between these analysis for all the assays with Spearman r values from 0.933 to 0.972 ($N = 249$ to 375 data points for the 4 assays).

We employed ELISA assays to measure antibodies binding to the trimeric Spike and Nucleocapsid as well as to Spike-RBD and N-RBD. The rationale of this evaluation was based on our goal to (i) monitor the durability of two key SARS-CoV-2 antibodies (Spike and Nucleocapsid) and (ii) to compare antibody responses to the complete protein to those recognizing key functional regions (Spike-RBD, N-RBD).

SARS-CoV-2 Pseudotype Neutralization Assay: Functional neutralization assay was performed using SARS-CoV-2 pseudotyped virus, as previously described [9], in a sub-cohort of 86 patients. Briefly, serial dilutions (4x fold serial dilutions starting at 1:10) of heat-inactivated sera were incubated with an equal volume of the pseudotyped virions ($pHIV_{NL}\Delta$Env-Nanoluc [26]) and the virion-Ab mixture was used to transduce HEK293T/ACE2wt cells (1:2 dilution virus-Ab and cell culture medium). The highest serum concentration analyzed was a 1:40 dilution. Two days later, the luciferase levels were measured in the cell extracts and the ID50 (50% Inhibitory Dose) was calculated using GraphPad Prism version 8.0 for MacOS X (GraphPad Software, Inc, La Jolla, CA) with nonlinear regression curve fit using inhibitor vs responses variable slope (four parameters).

Statistical analysis: At first, descriptive statistics were calculated. Due to the deviation from normality, demonstrated with Shapiro–Wilk test, antibody values were log10-transformed prior to analysis. At the univariate analysis, log10-transformed antibody values at 6 and 8 month follow-up points were compared versus screening, using Kruskal-Wallis test.

For the modelfit determination of the endpoint titers, the right side of the sigmoid dilution curve (all points after the largest drop in measured value or the highest four dilution points, which ever was longer) was fit to a self-starting asymptotic regression model (R functions SSasymp() and nls() from the "stats" R package) used to determine the nonlinear least-squares estimate of the model parameters (https://www.rdocumentation.org/packages/stats/versions/3.6.2/topics/nls) [27,28].

Piecewise random-effects, generalized least squares multivariate regression analysis examining the associations between antibody levels (dependent variable), time (inflection point at 6 months), gender, age (≥50 vs. <50 years) and hospitalization (yes vs. no) was performed. Half-time was estimated from the formula – ($\log_{10} 2$) / slope, separately for each phase in the decay, as appropriate [29]. The level of statistical significance was set at 0.05. Statistical analysis was performed with STATA/SE version 13 statistical software (Stata Corp., College Station, TX, USA). Values below the threshold of the ID50 calculation were

entered as 0.5. Values below detection of the assay are considered negative and were entered as 0.1.

3. Results

Patients: Plasma donors ($N = 148$) were tested over time starting with the initial screening at 2.1 months (range: 0.5–4.1 months) from the day of the initial symptoms (or from the first positive PCR assay (PCR+) for those with asymptomatic disease). The characteristics of the patients are summarized in Table 1. The follow-up measurement ($n = 135$) was performed at a median of 5.9 months (range 2.9–7.2 months) post symptom onset, and termed "6-month follow-up" in this report. All these patients had recovered from symptomatic COVID-19. A subset of patients ($n = 94$) was tested again at a median of 8.3 months (range 6.8–10.5 months) and termed "8-month follow-up" in this report. The long-term follow-up was performed on patients ($N = 81$) analyzed at all three timepoints (2, 6 and 8 months).

The median age was 50 years (range: 18–65). Ninety-one patients did not need hospitalization and 57 patients were hospitalized. More than half of the patients reported fatigue (59.1%) and fever (53.7%), whereas other commonly reported symptoms included headache (49.4%), anosmia (48.3%), cough (45.1%), and loss of taste (43.0%). All hospitalized patients had pneumonia; the vast majority of them 28/33 (84.8%) had bilateral lung infiltrates in the CT scan of thorax on their admission day.

Anti-SARS-CoV-2 antibody responses over time: At the initial screen, all donors showed positive antibody responses recognizing trimeric Spike, Spike-RBD, Nucleocapsid and N-RBD, except 1 of 148 donors, who did not have measurable antibodies to N-RBD (Table 2). At the 6-month follow-up, positive responses were found to Spike (100%), whereas 1/135 (0.74%), 1/82 (1.2%) and 8/135 (5.9%) of donors showed values below the threshold of the respective assays (Table 2). At the 8-month follow-up, all donors ($N = 92$) had positive responses to Spike (100%) and Spike-RBD (100%), whereas 2/81 (2.4%) and 2/92 (2.2%) had responses below the threshold of the assay to Nucleocapsid and N-RBD, respectively (Table 2). These data show better persistence of antibodies to Spike than to Nucleocapsid (see below).

Monitoring responses over time, we found a significant contraction of antibody levels to all four antigens (paired t-test, Table 3). A significant correlation of Spike antibody levels was found between the values at screening and the 6-month follow-up (Fig. 2A); this correlation became even stronger when comparing antibody levels from the 6-month and the 8-month follow-up (Fig. 2B). Similar observations were made for the S-RBD, Nucleocapsid and N-RBD measurements (Fig. 2C). The stronger correlation may be the result of rapid antibody changes during the first period (early after infection) compared to the later stages, that reflected the activity of long-lasting antibody producing cells.

We further analyzed the relation between antibodies to Spike and Spike-RBD and between antibodies to Nucleocapsid and N-RBD over time. We previously reported significant correlations of these measurements at screening using a smaller cohort [9]. Here, we report that this significant correlation was maintained for Spike and Spike-RBD at the 6-month (Fig. 2D) and at the 8-month (Fig. 2E) evaluation. Similar significant correlations were maintained for Nucleocapsid and N-RBD (Fig. 2F). Together, our data indicate better persistence of antibodies to Spike than to Nucleocapsid (see below) and that the Spike and Nucleocapsid antibody relations were maintained over time.

Anti-Spike antibody showed better durability: We next compared the persistence of the antibody responses to Spike and Nucleocapsid in more detail. We calculated the level of antibody persistence for the four different assays at the 8-month follow-up in relation to their respective screening measurements (Fig. 3). This analysis showed that Spike antibodies have better persistence than Nucleocapsid antibodies (Fig. 3A, $p < 0.0001$). In addition, antibodies recognizing only Spike-RBD persisted less than the mixture of antibodies recognizing the complete Spike protein (Fig. 3B, $p < 0.0001$). These data indicate that Spike-RBD antibodies are less durable than other specificities within the mixture of antibodies recognizing the complete Spike.

No difference was found between antibodies recognizing N-RBD and the complete Nucleocapsid protein in this comparison (Fig. 3C; $p = 0.0656$).

The antibody measurements were also used to determine their half-life over the entire period. We employed the piecewise, linear random-effects generalized least squares multivariate regression analysis to evaluate decay in two sequential time intervals. This analysis showed that time emerged as an independent factor inversely associated with antibody levels (Table 4). Up to 6 months post symptom onset, the

Table 1
Characteristics of the study cohort ($n = 148$).

Variables	
Gender	**n (%)**
Female	71 (48%)
Male	77 (52%)
Age (years)	**n (%);** Median [range]
<50	72 (49%); median 41 [range 20–49]
≥50	76 (51%); median 58 [range 50–78]
Hospitalization	**n (%)**
no	91 (61.5%)
Yes	57 (38.5%)
First measurement (screening)	Median [range]
Time since symptom onset (months) ($N = 148$)	2.1 [0.5–4.1]
Second measurement (6-month value)	Median [range]
Time since symptom onset (months) ($N = 135$)	5.9 [2.9–7.2]
Third measurement (8-month value)	Median [range]
Time since symptom onset (months) ($N = 94$)	8.3 [6.8–10.5]

Table 2
Percentage of patients with values below the threshold of detection, per time point.

	Screening	1st follow-up	2nd follow-up
Time post first symptom (median)	2.1 months	5.9 months	8.3 months
Spike ELISA	0/148 (0.0)	0/135 (0.0)	0/92 (0.0)
Spike-RBD ELISA	0/148 (0.0)	1/135 (0.74)	0/92 (0.0)
Nucleocapsid ELISA	0/86 (0.0)	1/82 (1.2)	2/81 (2.4)
N-RBD ELISA	1/148 (0.6)	8/135 (5.9)	2/92 (2.2)
NAb (pseudotype assay)	0/86 (0)	3/76 (3.9)[a]	3/29 (10.3)[b]

[a] An additional 8 donors (10.5%) have low NAb levels below the threshold of quantification.
[b] An additional 9 donors (31%) have low NAb levels below the threshold of quantification.

Table 3
Descriptive statistics of the measured antibody levels.

	Screening, median [IQR]	6-month follow-up, median [IQR]	8-month follow-up, median [IQR]
Spike[a]	4.27 [0.76]	3.90 [0.80]*	3.79 [0.69]*
Spike-RBD[a]	4.17 [0.71]	3.65 [0.79]*	3.51 [0.59]*
Nucleocapsid[a]	4.13 [0.77]	3.50 [0.78]*	3.06 [0.74]*
N-RBD[a]	3.87 [0.90]	3.14 [0.93]*	3.00 [0.63]*
NAb[b]	2.75 [1.73]	1.75 [1.48]*	1.20 [2.25]*

[a] Endpoint titer, log10.
[b] ID50, log10.
* $p<0.0001$ for all comparisons versus screening (non-parametric unpaired Kruskal-Wallis test).

Table 4
Determination of antibody half-life.

		0–6 months interval post symptom onset: increment per month	6 months or more post symptom onset: increment per month
Spike[a]	Coefficient (95% CI)	−0.09 (−0.11 to −0.08)	−0.05 (−0.08 to −0.02)
	p[c]	**<0.001**	**<0.001**
	Estimated half-life[d]	97 days	169 days
Spike-RBD[a]	Coefficient (95% CI)	−0.14 (−0.16 to −0.13)	−0.04 (−0.08 to −0.01)
	p[c]	**<0.001**	**0.015**
	Estimated half-life[d]	62 days	212 days
Nucleocapsid[a]	Coefficient (95% CI)	−0.19 (−0.22 to −0.17)	−0.09 (−0.12 to −0.06)
	p[c]	**<0.001**	**<0.001**
	Estimated half-life[d]	47 days	100 days
N-RBD[a]	Coefficient (95% CI)	−0.19 (−0.21 to −0.17)	−0.05 (−0.09 to −0.01)
	p[c]	**<0.001**	**<0.001**
	Estimated half-life[d]	47 days	168 days
NAb[b]	Coefficient (95% CI)	−0.19 (−0.25 to −0.13)	−0.33 (−0.53 to −0.14)
	p[c]	**<0.001**	**<0.001**
	Estimated half-life[d]	47 days	27 days

[a] Endpoint titer, log10.
[b] ID50, log10.
[c] Bold lettering denotes statistically significant associations.
[d] Half-life was estimated in cases of decrease along with time with $p<0.05$.

estimated half-life for Spike, Spike-RBD, Nucleocapsid, and N-RBD was 97, 62, 47 and 47 days, respectively. These data support a significant difference between Spike and the Nucleocapsid antibody half-life, in agreement with data shown in Fig. 3A, pointing to the better durability of Spike antibody. After 6 months, the estimated half-life was considerably longer for both types of antibodies, corresponding to 169, 212, 100 and 168 days, respectively. Thus, our data show that the Spike antibodies and the Nucleocapsid antibodies have bi-phasic decay kinetics with a significantly longer half-life after the 6-month inflection point, as suggested in Fig. 1.

Evaluation of SARS-CoV-2 neutralizing antibody (NAb) responses: We employed a neutralization assay to interrogate the functional ability of Spike antibodies to neutralize pseudotyped SARS-CoV-2 virus using samples collected at screening, the 6- and the 8-month follow-up from a sub-cohort of donors. All donors (N = 86) had detectable NAb at screening time. At the 6-month and the 8-month follow-up timepoints, 76 and 29 patients, respectively, were analyzed, with a subset of 18 patients being measured at both time points. As expected from the Spike and Spike-RBD antibody evaluation shown above (Fig. 1,Table 4), the neutralization ability also contracted. Functional NAb persisted fairly well (Fig. 4A) with 73 of 76 donors (96%) and 26 of 29 donors (90%) showing positive responses at the follow-up time points, respectively (Table 2). Of note, at each of these time points some of the donors (8 of 76 at 6-month follow-up and 9 of 29 at 8-month follow-up) showed very low neutralization abilities below the threshold of quantification in the pseudotype-virus inhibition assay.

Using the piecewise, linear random-effects generalized least squares multivariate regression analysis further showed that the NAb half-life was also biphasic: 47 days during the first 6 months of infection declining to 27 days after 6 months (Table 4). Examination of the relation of the levels of NAb and Spike antibodies (Fig. 4B) and of Spike-RBD antibodies (Fig. 4C) at the screening showed strong correlations, as we previously reported for a smaller cohort [9]. Comparison of such relation at the follow-up 6- and 8-month time points (Fig. 4B, 4C, middle and lower panels) showed a stronger contraction of NAb over time reaching

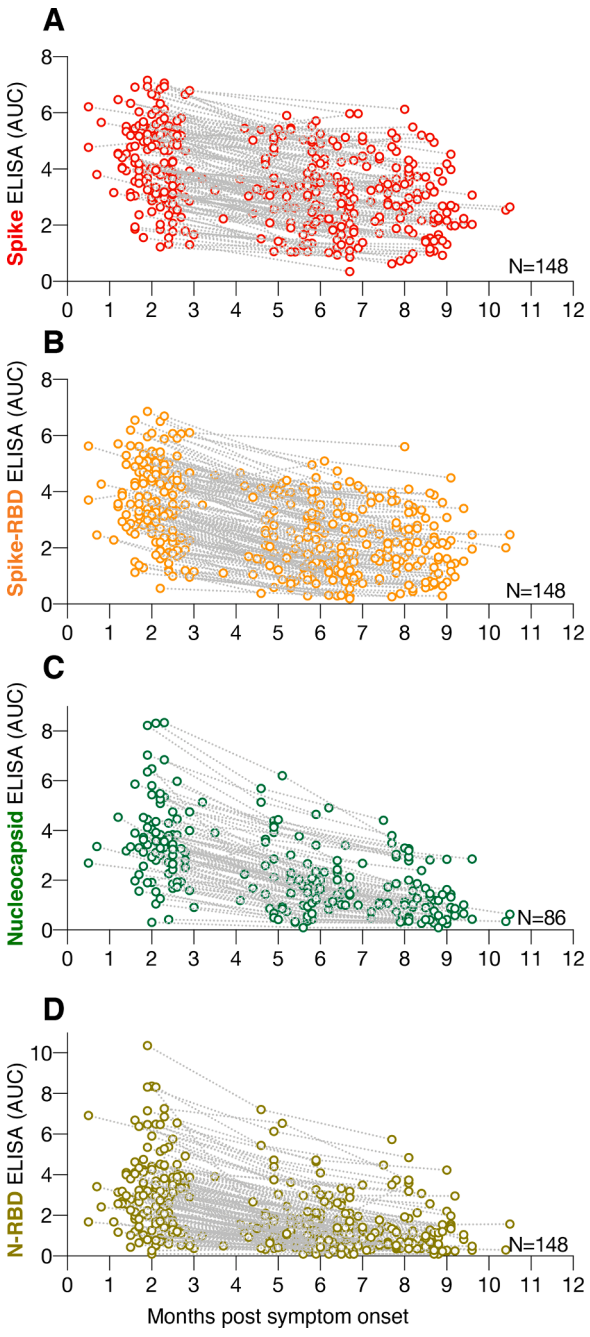
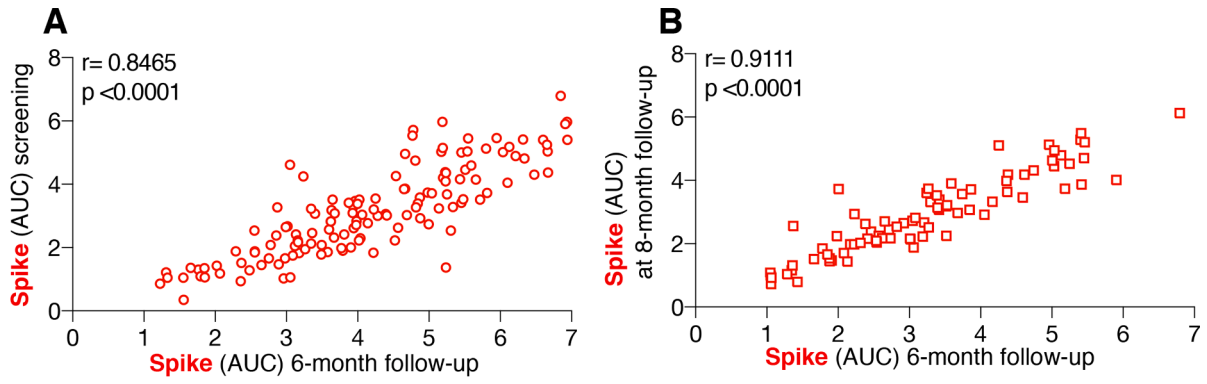


Fig. 1. Longevity of SARS-CoV-2 binding antibodies for up to 10 months of follow-up. Binding Ab levels were measured by ELISA using serial dilutions of serum samples and were expressed as area-under-the curve (AUC) values. ELISA assays measured antibodies recognizing (A) trimeric Spike, (B) Spike Receptor Binding Domain (Spike-RBD), (C) complete Nucleocapsid, or (D) Nucleocapsid RNA Binding Domain (N-RBD). The number of donors is given.

the threshold level of the assay despite detectable ELISA responses. These findings corroborated the measurements of the half-life of binding antibodies and NAb. The greater loss of NAb is in agreement with its predicted half-life (Table 4).

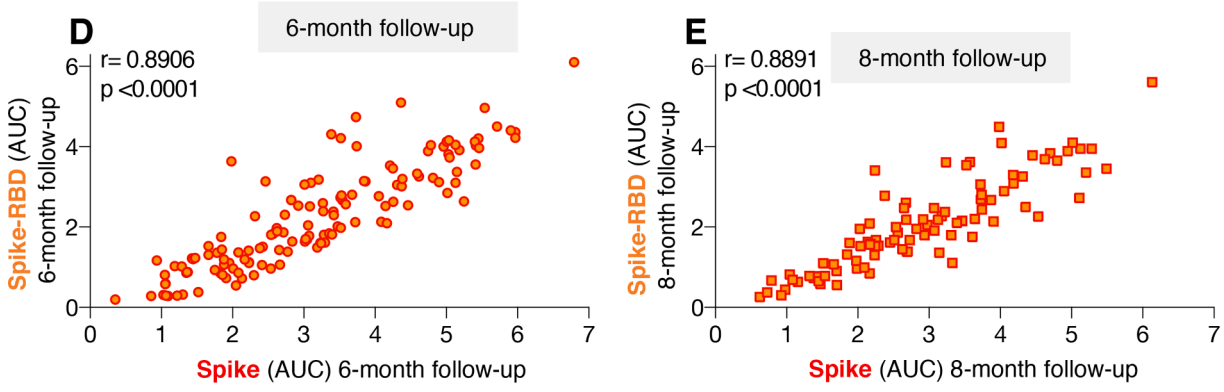
In summary, measuring the decline of CoV-2 Spike antibodies in these sequentially collected sera, we found that the 100% positivity at the screening was reduced to 76% at 6 to 8-month follow-up. Of note, 7% of the negative samples are below the detection threshold of the assay and 17% show very low positivity but below the level of quantification. These data show lack of long-term durability of NAb in CoV-2 infected persons despite the better persistence of Spike binding



C

		Comparison: screening to 6-month follow-up	Comparison: 6-month to 8-month follow-up
Spike	r[a] (N)[b]	0.8465 (N=135)*	0.9111 (N=81)*
Spike-RBD	r (N)	0.8440 (N=135)*	0.9228 (N=81)*
Nucleocapsid	r (N)	0.7629 (N=82)*	0.8936 (N=67)*
N-RBD	r (N)	0.8383 (N=135)*	0.8385 (N=81)*

[a] Spearman r
[b] number of samples
* p<0.0001 for all comparisons



F

Correlation		at screening	at 6-month follow-up	at 8-month follow-up
Spike Ab to S-RBD Ab	r[a] (N)[b]	0.9163 (N=148)*	0.8906 (N=135)*	0.8891 (N=94)*
Nucleocapsid Ab to N-RBD Ab	r (N)	0.8394 (N=86)*	0.7802 (N=82)*	0.7310 (N=83)*

[a] Spearman r
[b] number of samples
* p<0.0001 for all comparisons

Fig. 2. Correlation of CoV-2 antibody levels measured at screening and at the 6- and the 8-month follow-up periods. Binding Ab levels were measured by ELISA. Correlations of Spike antibody at (A) screening and the 6-month follow-up and (B) at 6-month and 8-month follow-up are shown. (C) Spearman r values of correlations shown in panels A and B for all ELISA measurements (Spike, Spike-RBD, Nucleocapsid and N-RBD antibodies). (D, E) Correlation of Spike and Spike-RBD antibody levels at (D) the 6-month and (E) 8-month time points. (F) Spearman r values of the comparisons of Spike and Spike-RBD and of Nucleocapsid and N-RBD antibody levels at 6-month and the 8-month follow-up are given.

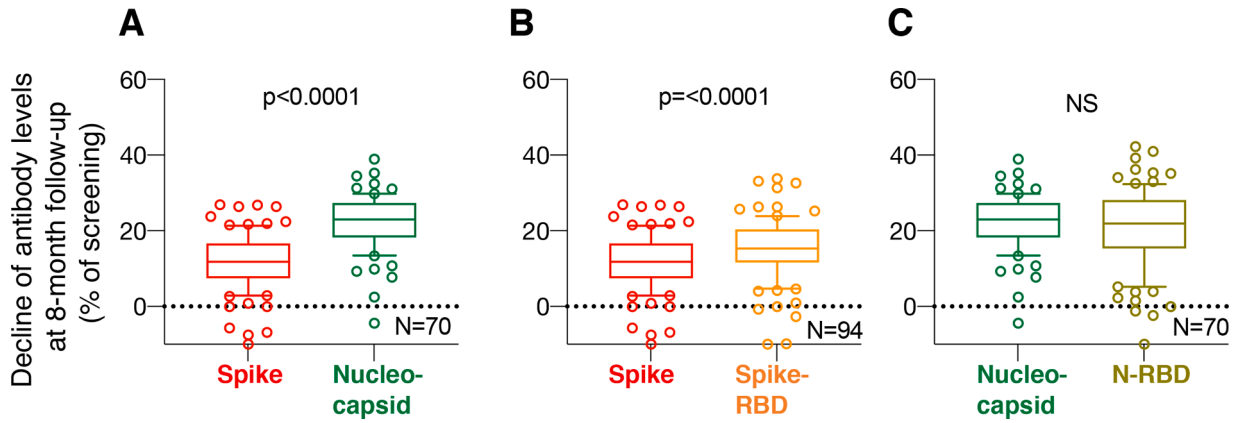


Fig. 3. Persistence of binding antibodies. (A-C) Antibody levels (expressed as Modelfit endpoint titers, log10) were determined at screen time and the 8-month follow-up. Persistence of antibody levels determined at the follow-up was calculated as percent of the screening measurements. Comparisons of% declines are shown for (A), Spike and Nucleocapsid, (B) Spike and Spike-RBD, and (C) Nucleocapsid and N-RBD. p values are from paired non-parametric t tests (Wilcoxon matched-pairs signed rank test). Plots show the median with box and whiskers at the 10–90 percentile.

antibodies.

3.1. Associations of anti-SARS-CoV-2 antibodies with time and clinical characteristics-multivariate analysis

The data from the sequentially analyzed antibodies (Fig. 1) were further examined according to clinical characteristics (Fig. 5) in relation to time post symptom onset including age (<50 years vs >50 years) (Fig. 5A), hospitalization (Fig. 5C) and gender (Fig. 5D). We previously reported [9] the presence of higher CoV-2 antibody levels at screening analyzing a smaller cohort of patients ($N = 60$). Here we expanded this study and examined the antibody levels in these subgroups of donors over 10 months of follow-up.

Cross-sectional analysis showed that donors with age >50 years showed higher antibodies levels for Spike, Spike-RBD, Nucleocapsid and N-RBD from the screening to 8-month follow-up (Fig. 5B). Similar data were obtained for Nucleocapsid and N-RBD antibodies (Table 5). Using the piecewise, multivariate regression analysis, we found that older age (≥50) was independently associated with higher log10-transformed antibody levels (Spike, $p = 0.023$; Spike-RBD, $p = 0.002$; Nucleocapsid, $p<0.001$; N-RBD, $p = 0.013$) (Table 5).

Comparative analysis of hospitalized versus not hospitalized donors showed significantly higher antibodies to all four antigens (Fig. 5D). Hospitalization correlated with higher antibody values (Spike, $p<0.001$; Spike-RBD, $p<0.001$; Nucleocapsid, $p = 0.003$; N-RBD, $p = 0.024$) (Table 5).

The univariate analysis showed further that there is a significant association between antibody levels and gender against Spike and Nucleocapsid at the screening time point (Fig. 5F). Anti-SARS-CoV-2 antibody levels were higher in male donors, however, this association did not persist over time in this cohort (Fig. 5F, Table 5). Gender differences in anti-Spike antibodies were also observed by others [30-32].

We also show the NAb data measured overtime, separated by age, hospitalization and gender (Fig. 5B, 5D, 5F, right panels). These data mirror the results found for the binding antibodies measured by ELISA showing higher levels in male, in donors <50 years of age and in donors with previous hospitalization.

In a sub-cohort of 86 patients, age ($p<0.001$) and hospitalization ($p = 0.003$) correlated with higher neutralizing antibody titers (Table 5).

Together, over the 10 months of follow-up, clinical characteristics of patients including age and prior hospitalization continue to be associated with higher CoV-2 antibody levels and NAb.

4. Discussion

Elucidating the characteristics of human immune response against SARS-CoV-2 is among the top research priorities on COVID-19 and it is highly relevant in terms of formulating new vaccination strategies. Herein, we show that the B-cell mediated immunity against SARS-CoV-2 persists at 8 months post initial COVID-19-related symptom onset or positive PCR test. A two-phase reduction in all measured antibody specificities (Spike, Spike-RBD, Nucleocapsid, Nucleocapsid-RBD, Neutralizing antibodies) was documented.

In our cohort, analyzing paired samples from patients monitored up to 10.5 months post symptom onset, we found good persistence of Spike and Nucleocapsid antibodies. In line with our results, a decay of anti-SARS-CoV-2 anti-Spike RBD antibodies after 3 months from the onset of symptoms of COVID-19 has been reported [18-21,26,33-35] with few reports extending the observations beyond 6 months [36-38]. Differences in detection methods and disease severity in different cohorts may be responsible for variations reported in the literature regarding the timing of antibody decay [22,39]. In agreement with others [29], we noted an initial phase of stronger contraction of the antibody responses. In our study, we present data from a sequential analysis over 10 months post symptom onset of 148 CoV-2 infected persons, addressing both the presence of binding and neutralizing antibodies. This analysis showed the expected contraction of antibody responses over time after infection. We report a biphasic reduction of antibody responses with a shorter half-life during the contraction period (months 2–6) and longer half-life thereafter which is also in agreement with the observation of presence of long-the lived memory cells [29]. Although more time in necessary to evaluate the SARS-CoV-2 antibody responses over time in this new infection in humans, previous studies of SARS-CoV showed remarkable antibody durability [40,41].

We found that Spike antibodies showed better durability than Nucleocapsid antibodies. During the first six months, spike antibodies also showed better durability (half-life 97 days) than the subset of Spike-RBD antibodies (half-life 62 days), whereas both Nucleocapsid and N-RBD antibodies showed similar persistence (half-life 47 days). Similar conclusions were drawn in a study by Fenwick et al. [42] who reported differential waning of SARS-CoV-2 humoral responses, with antibodies recognizing the trimeric Spike being more persistent compared to antibodies recognizing nucleocapsid. The analysis of our cohort revealed a bi-phasic decline of antibodies with an inflection point at ~6 months post symptom onset.

Regarding neutralizing antibodies, 76% of the convalescent donors showed positive NAb at 6–8 months post symptom onset. Durability calculation also showed a bi-phasic decline with a half-life of 47 days

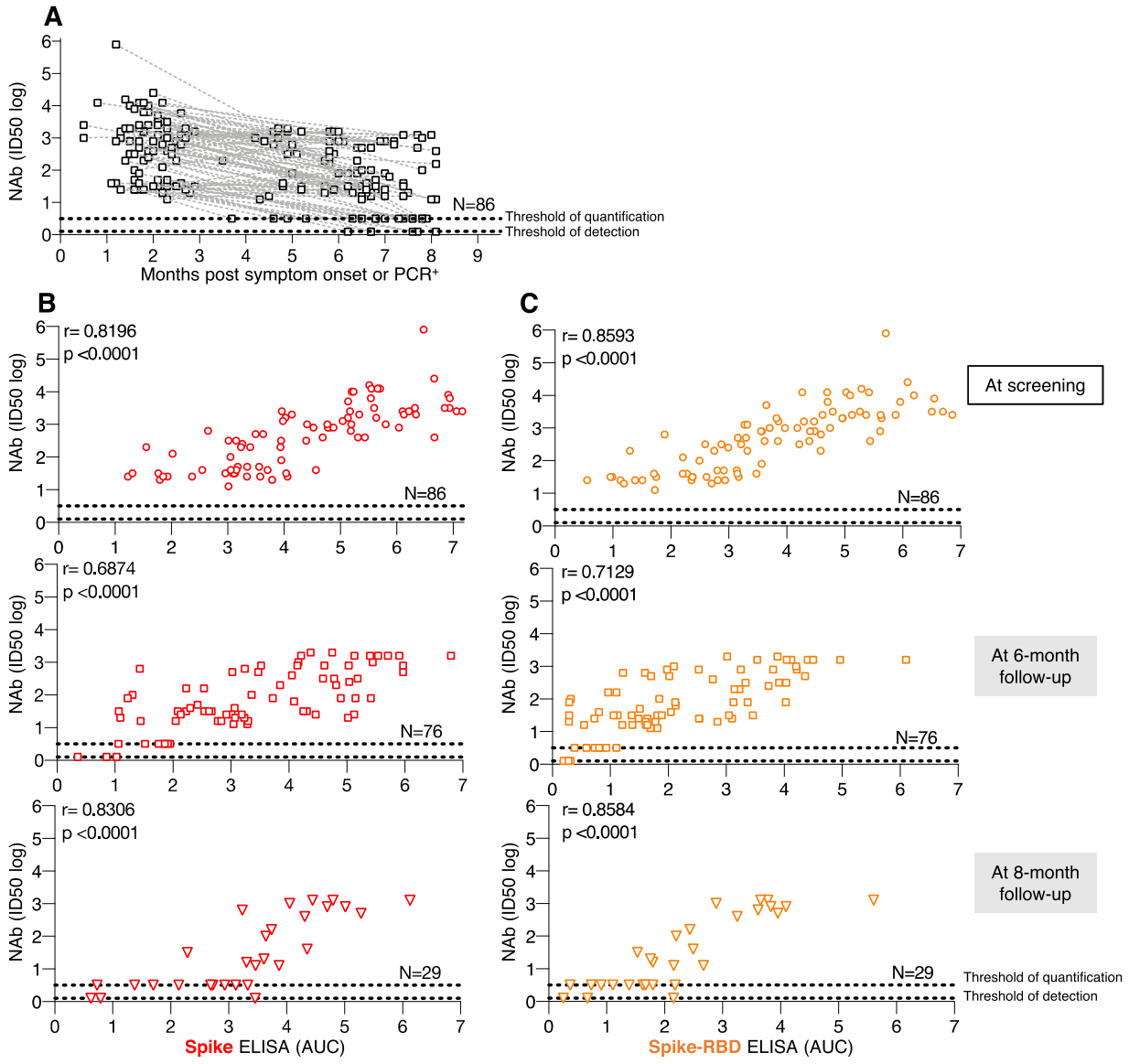


Fig. 4. Persistence of Neutralizing Antibodies (NAb) responses. (A) Neutralizing antibodies were measured using the pseudotype SARS-CoV-2 virus inhibition assay in a sub-cohort of patients ($N = 86$). The log serum dilution inhibiting virus by 50% (ID50) values are plotted over time. (B, C) Correlations of NAb and (B) Spike and (C) Spike-RBD ELISA antibody values (AUC) measured at the matching time points at screen time (top panels), at the 6-month follow-up (middle panels), and at the 8-month follow-up (bottom panels). The NAb ID50 threshold of quantification (0.5 log) and threshold of detection (0.1 log) in this assay are indicated.

during the first 6 months, followed by a shorter half-life of 27 days thereafter; nevertheless, this estimation should be deemed explorative, as it was based on a subcohort of 86 patients, for 29 of whom data were available regarding the 8-month follow-up. Additional measurements over longer periods of time post infection are necessary to fully determine the half-life of NAb in our convalescent patient cohort for longer periods of time. A decline in the titers of NAb over time has been also reported by Wajnberg et al. with more than 90% of the seroconverters maintaining their neutralizing capacity at 5 months after the onset of COVID-19 [43]. Crawford et al. [44] reported a 4-fold decline of NAb between month 1 and 4 post symptom onset. Mueksch et al. [33] reported a 45% decline per month in NAb titers. Gaebler et al. [37] reported a 5-fold decline between 1.3 and 6.3 months post symptom onset. The observed differences in the percentages might be due to different methodology in the neutralizing assays that have been applied in each study and/or the different cohorts. Overall, all the studies including ours find a parallel waning of Spike antibodies and NAb levels over time. Importantly, we show in this report that Spike antibodies can be readily detected even at 8 months post infection and the antibodies have maintained neutralization capacity, although on the average reduced.

Taking into consideration that the plasma half-life of IgG antibodies is approximately 21 days, it has been supported that long-lived plasma cells in the bone marrow produce SARS-CoV-2-specific sustainable antibody responses [43]. Clearly, the role of both B and T cell memory responses in the overall immunity to SARS-CoV-2 is of great importance and induction of long-lived memory cells is critical for inducing persistent virus-induced immunity [43,45,46]. Regarding other coronaviruses, antibodies against SARS-CoV-1 and MERS-CoV were detectable up to 2 and approximately 3 years, respectively [47,48]. Interestingly, although the titers of NAb strongly correlated with the values of Spike and Spike RBD antibodies at baseline, this correlation weakened at the 6- to 8-month analysis. This finding may have implications in the anticipated protection against re-infection over time. On the other hand, Dan et al. [29] and Hartley et al. [49] reported the presence of memory B cells in convalescent patients at 6 months post symptom onset. In addition, in a preprint, Kremsner et al. [50] reported that vaccination of previously infected individuals with a single dose of CVnCoV mRNA/LNP vaccine induced rapid recall responses, supporting

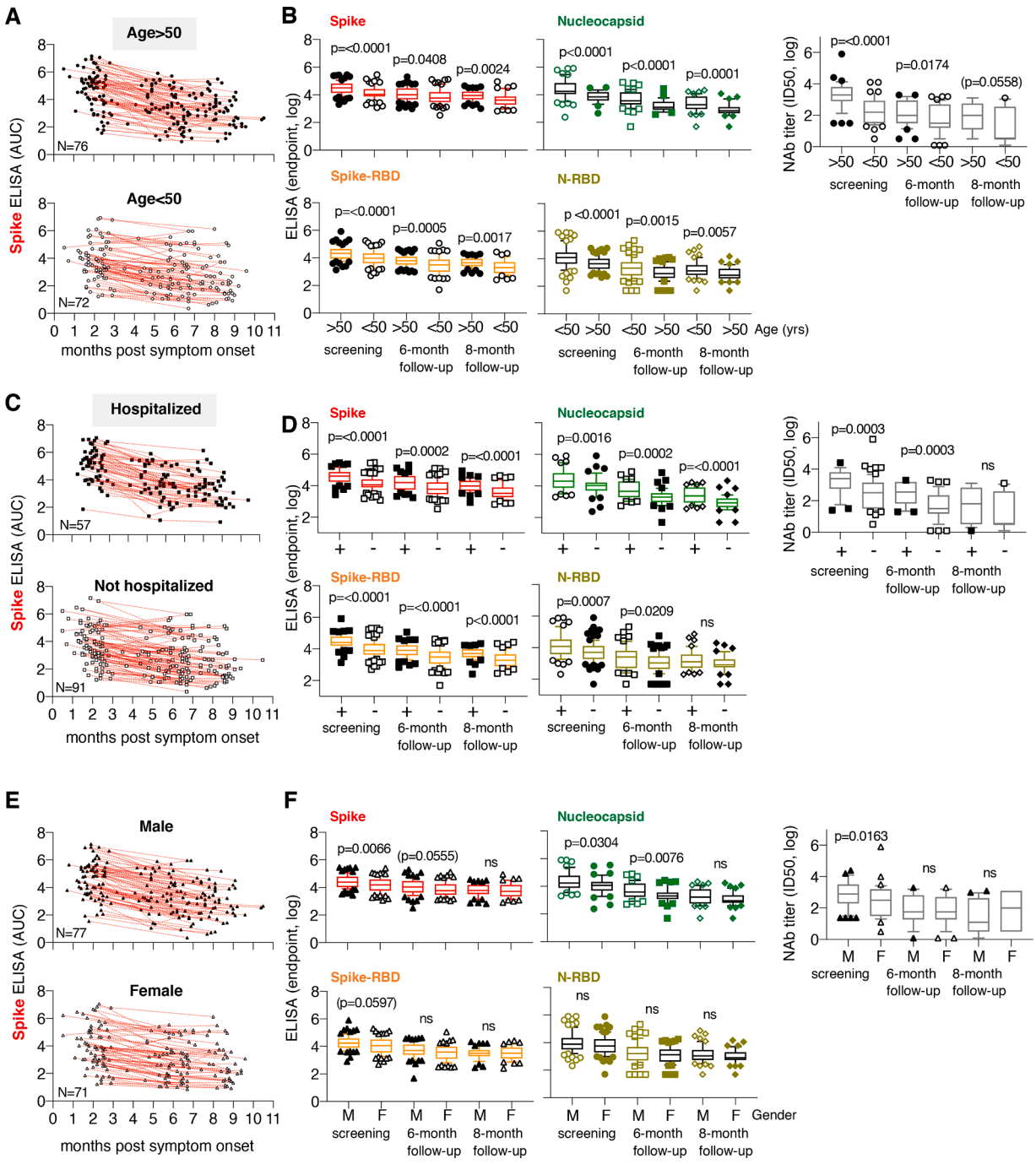


Fig. 5. Associations of antibodies levels with time and clinical characteristics. Spike antibody measurements as shown in Fig. 1 are analyzed for different clinical characteristics and plotted over time post symptom onset. Donors were grouped by (A, B) by age with ≥50 and <50 years; (C, D) hospitalization and not hospitalization; (E, F) by gender (male versus female). (B, D, F) Cross-sectional comparisons of endpoint antibody levels to Spike, Spike-RBD, Nucleocapsid and N-RBD and of NAb are shown. p values (Mann Whitney t-test) are given and the 3 time points of analysis are shown. p values in bracket denote trend.

the presence of robust memory responses. Therefore, it is important to evaluate both anti-Spike and neutralizing antibodies in subsequent studies assessing long-term humoral immunity against SARS-CoV-2.

Furthermore, it is noteworthy that different factors may predict an enhanced initial antibody response against SARS-CoV-2 and the persistence of antibodies over time. Inter-individual variability in antibody responses may be attributed to age, gender and genetic features [26,51]. Gender differences in anti-Spike antibodies were also observed by others [30-32]. Interestingly, gender differences detected in our data set lost statistical significance by six months post symptom onset. Age above 50 years was associated with increased antibody titers, whereas the need for hospitalization was associated with superior antibody response against Spike, Spike-RBD, Nucleocapsid, Nucleocapsid-RBD and neutralizing antibodies. Older age and more severe COVID-19 has been well described in the literature as predisposing factors for higher antibody production against SARS-CoV-2 [9,21,31,52-54]. In a recent preprint, Markmann et al. also reported a positive association between male sex, increased age, symptom severity and increased titers of convalescent antibodies [55]. The underlying pathophysiology for gender differences in antibody responses remains rather elusive despite several theoretical concepts. Additional analyses are required to determine any association with a distinct Th2 type response. Increased viral load in

Table 5
Association of CoV-2 antibody levels and clinical characteristics.

	Variables: Category:	Gender Male vs. female	Age (years) ≥*50 vs.* <*50*	Hospitalization Yes vs. No
Spike[a]	Coefficient (95% CI)	+0.04 (−0.11 to +0.18)	+0.17 (+0.02 to +0.32)	+0.32 (+0.17 to +0.48)
	p[c]	0.632	**0.023**	**<0.001**
Spike-RBD[a]	Coefficient (95% CI)	−0.01 (−0.16 to +0.13)	+0.24 (+0.09 to+0.39)	+0.35 (+0.19 to +0.50)
	p[c]	0.847	**0.002**	**<0.001**
Nucleocapsid[a]	Coefficient (95% CI)	+0.09 (−0.11 to +0.28)	+0.39 (+0.19 to +0.59)	+0.30 (+0.10 to +0.50)
	p[c]	0.385	**<0.001**	**0.003**
N-RBD[a]	Coefficient (95% CI)	+ 0.07 (−0.13 to +0.27)	+0.26 (+0.06 to +0.47)	+0.24 (+0.03 to +0.45)
	p[c]	0.486	**0.013**	**0.024**
NAb[b]	Coefficient (95% CI)	−0.11 (−0.43 to +0.20)	+0.62 (+0.30 to +0.94)	+0.49 (+0.16 to +0.83)
	p[c]	0.481	**<0.001**	**0.003**

[a] Endpoint titer, log10.
[b] ID50, log10.
[c] Bold lettering denotes statistically significant associations.

more severe cases, as well as a suboptimal T-cell response and an uncontrolled inflammatory status among older individuals, may ultimately result in a more potent antibody production against SARS-CoV-2 [52].

In conclusion, we showed that there is general persistence of anti-SARS-CoV-2 Spike antibodies as well as loss of neutralizing antibodies in 24% of convalescent donors at 6–8 months from initial symptoms of COVID-19. A prolonged follow-up of the donors is necessary to further characterize the kinetics of anti-SARS-CoV-2 B-cell mediated immunity over time and to establish a link between the presence of antibodies and the level of protection against re-infection. Such data should help to optimize vaccination strategies and public health decisions.

Declaration of Competing Interest

The authors declare they have no conflict of interest.

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Data sharing statement

Deidentified participant data available upon request from the authors.

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