Defining the features and duration of antibody responses to SARS-CoV-2 infection associated with disease severity and outcome

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SARS-CoV-2–specific antibodies, particularly those preventing viral spike receptor binding domain (RBD) interaction with host angiotensin-converting enzyme 2 (ACE2) receptor, can neutralize the virus. It is, however, unknown which features of the serological response may affect clinical outcomes of patients with COVID-19. We analyzed 983 longitudinal plasma samples from 79 hospitalized patients with COVID-19 and 175 SARS-CoV-2–infected outpatients and asymptomatic individuals. Within this cohort, 25 patients died of their illness. Higher ratios of IgG antibodies targeting S1 or RBD domains of spike compared with nucleocapsid antigen were seen in outpatients who had mild illness versus severely ill patients. Plasma antibody increases correlated with decreases in viral RNAemia, but antibody responses in acute illness were insufficient to predict inpatient outcomes. Pseudovirus neutralization assays and a scalable ELISA measuring antibodies blocking RBD-ACE2 interaction were well correlated with patient IgG titers to RBD. Outpatient and asymptomatic individuals’ SARS-CoV-2 antibodies, including IgG, progressively decreased during observation up to 5 months after infection.

INTRODUCTION

A novel coronavirus first described in Wuhan, China in December 2019 (1) has led to the coronavirus disease 2019 (COVID-19) pandemic and a global economic shutdown amid unprecedented social distancing measures. The clinical spectrum of COVID-19 ranges from asymptomatic infection and mild upper respiratory tract illness in most patients to severe viral pneumonia with respiratory failure, multiorgan failure, and death (2–4). Older adults and people with serious underlying health conditions are at greatest risk for severe illness and death (5–8). Host immune responses may be one of the most important determinants for disease progression and outcome, but this remains to be established.

The virus causing COVID-19 belongs to the subgenus Sarbecovirus (genus Betacoronavirus) together with the severe acute respiratory syndrome coronavirus (SARS-CoV) and has been designated SARS-CoV-2 (9). Coronaviruses contain four structural proteins, including spike, envelope, membrane, and nucleocapsid (N) proteins. The spike surface glycoprotein contains the receptor binding domain (RBD), which binds strongly to human angiotensin-converting enzyme 2 (ACE2) receptors (1, 10), and plays a major role in viral attachment, fusion of viral and host membranes, and entry of the virus into host cells (11). Most individuals infected with SARS-CoV-2 develop antibodies to the spike and N proteins, which are therefore used as antigens in clinical serology assays. The spike protein is an important target for neutralizing antibodies, because they can prevent viral entry into host cells (12, 13). Current information on the role of antibodies in viral clearance and modulation of disease severity as well as the durability of these responses after primary infection is limited or controversial. Improved understanding of humoral immunity to SARS-CoV-2 is needed to inform strategies for vaccination and the use of therapeutics in the form of neutralizing antibodies or convalescent plasma. Reports about the longevity of antibody titers to SARS-CoV-2 are not in full agreement, with some finding a rapid waning of virus-specific immunoglobulin G (IgG) antibodies by about 3 months after infection (14, 15) and others emphasizing stable titers detected over several weeks or several months (16–18). Virus-specific antibody responses appear to be elevated in COVID-19 patients with severe disease as opposed to asymptomatic or mildly ill individuals, raising concerns about the effectiveness of antibody responses to SARS-CoV-2. A suggestion that the quality rather than quantity of antibodies may predict the outcome of infection is provided by a recent report applying a panel of serological assays to patients with COVID-19 who convalesced or died (19). Nonhuman primates challenged with SARS-CoV-2 after vaccination with spike-based DNA vaccines...
were identified either after routine serology testing or occupational
week to month of documented infection.
progressive decreases in SARS-CoV-2–specific antibodies after the first
patients and asymptomatic individuals show substantial and pro-
comparable with N were strongly associated with clinically milder infec-
tion. Viral RNAemia decreased to undetectable levels rapidly once
compared with RBD. Higher ratios of IgG antibodies targeting S1 or RBD
were well correlated with IgG specific for RBD.
individuals were younger and had the lowest levels of obesity
compared with more severely ill patients. Levels of viral RNA mea-
sured by rRT-PCR of nasopharyngeal swabs at diagnosis showed a
progressive increase [lower rRT-PCR cycle threshold (Ct)] with dis-
eease severity; patients who died had the highest viral loads (fig. S2).
Demographic and clinical characteristics of patients stratified by
disease status are presented in Table 1. The percentage of males and
those with comorbidities of hypertension or diabetes mellitus in-
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levels for in-depth serological testing were available from all
patients and 86 of the 160 outpatient survivors. Demographic and
clinical characteristics of outpatients and asymptomatic individuals
with and without plasma availability are presented in table S1. A total
of 828 samples were analyzed with enzyme-linked immunosorbent

**RESULTS**

**Study design and patient demographics**

A total of 254 individuals with a positive SARS-CoV-2 real-time re-
verse transcription polymerase chain reaction (rRT-PCR) nasophary-
geal swab test were included in the study (fig. S1). Study subjects
were identified either after routine serology testing or occupational
health screening in the Stanford Health Care Clinical Laboratories
for anti–SARS-CoV-2 RBD IgM and IgG antibodies (136 asym-
tomatic individuals or outpatients) or after they reported to Stanford
This included 24 outpatients, 35 hospitalized patients who were not
admitted to the intensive care unit (ICU), and 20 ICU inpatients
who survived their illness. To evaluate serological responses associ-
ated with patient mortality, we also analyzed specimens from 25 pa-
tients who died of COVID-19 (1 outpatient, 7 admitted non-ICU
patients, and 17 admitted ICU patients). Of the patients who were
treated in the ICU, 26 of 37 (70%) required mechanical ventilation,
including 15 patients who died.

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clinical characteristics of outpatients and asymptomatic individuals
with and without plasma availability are presented in table S1. A total
of 828 samples were analyzed with enzyme-linked immunosorbent

Table 1. Patient demographic and clinical characteristics. IQR, interquartile range; NA, not available; DM, diabetes mellitus; SOB, shortness of breath; GI, gastrointestinal.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Outpatients and asymptomatic individuals (n = 160)</th>
<th>Admitted, non-ICU (n = 35)</th>
<th>Admitted, ICU (n = 20)</th>
<th>Deceased* (n = 25)</th>
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<tbody>
<tr>
<td>Age, median (IQR)</td>
<td>41 (32–56)</td>
<td>55 (40–71)</td>
<td>44 (37–63)</td>
<td>76 (64–85)</td>
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<tr>
<td>Sex (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>101 (63)</td>
<td>20 (57)</td>
<td>10 (50)</td>
<td>10 (40)</td>
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<tr>
<td>Male</td>
<td>59 (37)</td>
<td>15 (43)</td>
<td>10 (50)</td>
<td>15 (60)</td>
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<td>Diagnostic rRT-PCR Ct, median (IQR)</td>
<td>29.7 (21.8–37.3)</td>
<td>30.6 (21.1–35.4)</td>
<td>25.2 (22.5–29.9)</td>
<td>20.4 (16.5–28.8)</td>
</tr>
<tr>
<td>Comorbidities, number of individuals (% present)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Obesity</td>
<td>43 (27 NA)</td>
<td>15 (1 NA)</td>
<td>11 (55)</td>
<td>10 (40)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>30 (19)</td>
<td>8 (23)</td>
<td>7 (35)</td>
<td>19 (76)</td>
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<tr>
<td>DM</td>
<td>16 (10)</td>
<td>7 (20)</td>
<td>7 (35)</td>
<td>14 (56)</td>
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<tr>
<td>Cough</td>
<td>93 (58)</td>
<td>27 (77)</td>
<td>17 (85)</td>
<td>15 (60)</td>
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<tr>
<td>Fever</td>
<td>72 (45)</td>
<td>23 (66)</td>
<td>15 (75)</td>
<td>11 (44)</td>
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<td>SOB</td>
<td>30 (19)</td>
<td>25 (71)</td>
<td>15 (75)</td>
<td>18 (72)</td>
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<tr>
<td>Myalgia</td>
<td>60 (38)</td>
<td>15 (43)</td>
<td>11 (55)</td>
<td>3 (12)</td>
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<tr>
<td>GI</td>
<td>29 (18)</td>
<td>19 (54)</td>
<td>10 (50)</td>
<td>5 (20)</td>
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<tr>
<td>Fatigue</td>
<td>56 (35)</td>
<td>17 (49)</td>
<td>6 (30)</td>
<td>7 (28)</td>
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<tr>
<td>Chills</td>
<td>29 (18)</td>
<td>10 (29)</td>
<td>8 (40)</td>
<td>3 (12)</td>
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<tr>
<td>Headache</td>
<td>37 (23)</td>
<td>6 (17)</td>
<td>5 (25)</td>
<td>1 (4)</td>
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<tr>
<td>Mechanical ventilation (%)</td>
<td>0</td>
<td>0</td>
<td>11 (55)</td>
<td>15 (60)</td>
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<tr>
<td>Length of hospital stay, median days (IQR)</td>
<td>NA</td>
<td>5 (2–8)</td>
<td>17 (9–49)</td>
<td>15 (5–29)</td>
</tr>
<tr>
<td>Number of plasma specimens per patient, median (IQR)</td>
<td>1 (1–2)</td>
<td>5 (4–8)</td>
<td>13 (8–21)</td>
<td>8 (3–11)</td>
</tr>
</tbody>
</table>

*Of the 25 deceased patients, 1, 7, and 17 were categorized as outpatient, admitted non-ICU, and admitted ICU, respectively. The 14 individuals that were part of the validation cohort were not included in this table.
assays (ELISAs) measuring IgM, IgG, and IgA specific for SARS-CoV-2 RBD, S1 or N, and the RBD-ACE2 blocking assay (Fig. 1, A to C). Representative samples were also tested for antibody cross-reactivity to SARS-CoV RBD, viral RNA in the blood (RNAemia), and neutralization of spike-pseudotyped lentivirus (Fig. 1D). We also tested 45 plasma specimens from a validation cohort of 14 asymptomatic and outpatient SARS-CoV-2 rRT-PCR+ individuals with monthly prospective sample collection for up to 4 months after enrollment.

**Anti-RBD, S1, and N antibody responses and duration are associated with disease severity**

Lower antibody responses in patients with mild symptoms compared with those with more severe disease have been reported for other coronavirus infections, such as MERS-CoV (21–23) as well as for SARS-CoV-2 (15, 24). The detection rate of RBD-binding antibodies at 1-week intervals after inpatient symptom onset is shown in table S2; most individuals seroconverted by week 2 after onset of symptoms (Fig. 2, A and B). Positivity rates for RBD IgM, IgG, and IgA reached their maximum at weeks 4, 6, and 5, respectively, with most patients negative for IgM and IgA after 12 weeks, whereas RBD IgG levels showed a slower but progressive decline or a continued negative result in those who failed to generate IgG at earlier time points (Fig. 2A, fig. S3, and table S2). Outpatients had lower titers of RBD IgM, IgG, and IgA compared with inpatients and a more rapid decline of titers (Fig. 2B and fig. S4). Patients who required ICU care and those who died developed the highest levels of IgM, IgG, and IgA, as well as RBD-ACE2 blocking antibodies, throughout the time course (Fig. 2C).

S1-specific IgM, IgG, and IgA showed antibody kinetics that were very similar to those seen for RBD (figs. S3 to S5 and table S2). Whereas N-specific IgG responses showed high positivity rates with antibody kinetics similar to those for RBD and S1, IgM antibody responses to N were low in most patients (figs. S3 to S6 and table S2). There was no consistent difference in RBD, S1, and N antibody titers or RBD-ACE2 blocking antibodies between ICU patients who survived and patients who died (fig. S7).

We further evaluated the breadth of antibody responses in different disease severity categories by testing for SARS-CoV RBD binding. Most monoclonal antibodies targeting SARS-CoV RBD fail to bind SARS-CoV-2 RBD, indicating distinct antigenicity despite the sequence and structural similarity of the two proteins (25, 26). Nine of 13 ICU patients, 3 of 25 admitted non-ICU patients (fig. S8A), and 5 of 82 outpatients (fig. S8B) developed SARS-CoV RBD IgG titers during the course of their infection. The time course of anti–SARS-CoV RBD positivity in serial samples from individual patients did not always mirror anti–SARS-CoV-2 RBD IgG responses, suggesting limited clonal or oligoclonal B cell responses with this broader reactivity within the overall polyclonal anti–SARS-CoV-2 serological response (fig. S8, C and D).

**Neutralizing antibodies are increased in inpatients compared with outpatients and correlate with RBD-ACE2 blocking and RBD IgG titers**

Antibody neutralization of live SARS-CoV-2 or of pseudotyped viruses such as lentiviruses expressing the SARS-CoV-2 spike may represent the most physiologically relevant surrogates for humoral
immunity in vivo but are poorly scalable due to restrictive biosafety requirements for SARS-CoV-2 and less easily standardized assay components and protocols, compared with tests using purified proteins. We tested SARS-CoV-2 spike–pseudotyped lentivirus neutralization (27) in HeLa cells overexpressing ACE2 (28) by using inpatient and outpatient samples and evaluated correlations with the more scalable ELISAs for RBD and RBD-ACE2 blocking (Fig. 3, A and B). As with RBD-ACE2 blocking, neutralizing antibody activity was higher in inpatients compared with outpatients and began to decrease after about 1 month after symptom onset. Neutralization was well correlated with RBD IgG titers and RBD-ACE2 blocking (linear regression coefficient of determination $R^2$ of 0.6995 and 0.6824 for inpatients and 0.7338 and 0.6839 for outpatients). The RBD-ACE2 blocking assay was less sensitive than neutralization or RBD IgG ELISA. RBD IgM and IgA ELISA results were much more variable and did not correlate as well with neutralization or RBD-ACE2 blocking compared with RBD IgG.

Validation of decreasing antibody responses in mildly ill and asymptomatic individuals

Most individuals who become infected with SARS-CoV-2 do not require hospitalization to recover from their illness, and a sizeable fraction (about 40 to 45%) (29) remain asymptomatic. We carried out further analysis of antibody responses in a larger set of samples from 136 outpatients and asymptomatic individuals who tested positive by rRT-PCR for SARS-CoV-2 RNA in nasopharyngeal swabs and had serological testing conducted in the Stanford Health Care Clinical Laboratory. We also tested an independent validation set of 45 plasma samples from an additional 14 asymptomatic or mildly ill individuals. As seen for outpatients in Fig. 2B, the asymptomatic and outpatient individuals showed relatively low titers and rapid decline of RBD IgM and IgG (Fig. 4A and table S3). The timing of infection in asymptomatic individuals is less certain than for symptomatic individuals, whose symptoms usually develop within 2 to 14 days after exposure to the virus (30). Plotting of serological responses of outpatients and asymptomatic individuals relative to the date of their first positive rRT-PCR test for infection nonetheless showed a similar time course to that seen for outpatients plotted as days after onset of symptoms (Fig. 2B). The amount of viral RNA detected in diagnostic nasopharyngeal swabs was correlated with the antibody titers measured in these individuals (Fig. 4B).

Plasma specimens from the independent validation cohort of asymptomatic or outpatient individuals were collected prospectively during monthly visits up to 4 months after recruitment (Fig. 4, C and D, and fig. S9). RBD IgM, IgG, and IgA and RBD-ACE2 blocking as well as neutralization assays all showed a progressive decrease during the months sampled. Neutralizing antibodies were best correlated with RBD IgG titers and somewhat less well with RBD-ACE2 blocking ELISA in these individuals ($R^2 = 0.751$ and 0.5221, respectively) (Fig. 4E).

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**Fig. 2. Development of anti–SARS-CoV-2 RBD antibody responses in patients with COVID-19.** A total of 828 longitudinal plasma samples collected from 80 COVID-19 inpatients and deceased individuals (714 samples) (A) and 86 outpatients (114 samples) (B) were tested by ELISA at a dilution of 1:100 for the presence of SARS-CoV-2 RBD–specific IgM, IgG, and IgA antibodies and for antibodies blocking the binding of ACE2 to RBD. ELISA data stratified by the 86 outpatients (Outpt), 35 hospitalized patients who did not require ICU care (Admit), and 20 ICU patients and 25 patients who died, from week 1 to ≥7 weeks after onset of symptoms (C). Boxes indicate the interquartile range, and whiskers show the minimum and maximum values for each group. Dotted lines denote the assay cutoff. Mean values for duplicate measurements are shown. Statistical testing comparisons are as follows: P1, Outpt versus Admit/ICU/Deceased; P2, Admit versus ICU/Deceased; by two-sided Wilcoxon rank sum test. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Data for 14 samples from two patients (one admitted non-ICU patient and one deceased patient) are not plotted because the time of symptom onset was unknown. Mean ELISA OD$_{450}$ values of duplicate measurements are shown for each sample.

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Outpatients and non-ICU inpatients show preferential antibody targeting of RBD and S1 compared with N

It is an open question whether antibody responses in the initial weeks of SARS-CoV-2 infection have a role in modulating disease severity. Having found that patients with milder illness or asymptomatic infection had lower levels of SARS-CoV-2 antibodies to RBD, S1, and N compared with severely ill patients, we hypothesized that the relative targeting of the antibody response between antigens might be associated with different disease severity. In a recent study, serological analysis of samples from patients who died of COVID-19 compared with individuals who convalesced suggested an enrichment of an aggregate measure of spike antibodies or antibody functional activities in the convalescent (19). We calculated the ratios of RBD to N (Fig. 5A) and S1 to N (Fig. 5B) ELISA results for IgM, IgG, and IgA for all specimens that had detectable antibodies. In weeks 1 and 2 after onset of symptoms, outpatient IgG RBD/N and S1/N ratios were higher than those of admitted non-ICU patients and patients who died of their illness; admitted and ICU patients also had higher RBD/N ratios compared with patients who died. In weeks 3 and 4 and after week 4 after onset of symptoms, the IgM ratios to these antigens were more variable and did not show a consistent relationship to disease severity.

Inpatient SARS-CoV-2 antibody time course patterns are associated with decreases in RNAemia but not disease outcome

We hypothesized that detailed examination of the time course of SARS-CoV-2 antibody responses could identify distinct patterns associated with admitted non-ICU patients (Fig. 6 and fig. S10), ICU patients who recovered from their illness (Fig. 7 and fig. S11A), or those who died (Fig. 8 and fig. S11B). We identified three main patterns of response: At the time point closest to their discharge from hospital or death, group 1 individuals had SARS-CoV-2 antibody responses with no RBD IgG or RBD-ACE2 blocking activity; group 2 individuals had up to 25% RBD-ACE2 blocking activity; and group 3 individuals developed high levels of antibodies and more than 25% RBD-ACE2 blocking activity. These patterns of response were not strongly associated with disease severity but instead were shared across disease categories. Group 1 patients not only included admitted patients who recovered from their illness rapidly and were discharged before they had developed detectable...
antibody responses but also included patients who recovered from their illness without antibody production after prolonged ICU stays and patients who died of COVID-19 before antibody development. Similarly, group 2 patterns with low levels of RBD-ACE2 blocking were shared across all three patient categories. In group 3 (high levels of antibody production and >25% RBD-ACE2 blocking activity), admitted non-ICU patients differed from ICU or deceased patients in that they developed their robust antibody responses during short hospital stays before discharge, whereas ICU or deceased patients typically had prolonged hospital courses. In patients tested for neutralizing antibodies (13 admitted non-ICU patients, 16 ICU surviving patients, and 16 deceased patients), the results were closely correlated with IgG titers to RBD or RBD-ACE2 blocking.

Viral RNAemia is detected in up to a third of patients with COVID-19, most often in patients with severe disease (3, 31). RNAemia was evaluated in a subset of patients and detected in 15 of 25 admitted patients, 13 of 15 ICU patients who recovered, and 2 of 2 patients who died of their disease. Reduction in RNAemia was strongly correlated with the appearance of plasma antibodies (Spearman’s correlation coefficients of −0.47 for IgM, −0.43 for IgG, and −0.44 for IgA, P < 0.001 for each) (Figs. 6 to 8 and figs. S10 and S11).

**DISCUSSION**

Key clinical questions in the ongoing COVID-19 pandemic are the role of antibodies in modulating disease severity during infection,
the duration of individuals’ serological responses, and the extent to which patient antibody responses may be protective against reinfec-
tion. As SARS-CoV-2 vaccine candidates progress through clinical
trials, comparison of vaccine-induced immune responses to those
stimulated by viral infection and those of individuals who become
reinfected will help clarify immunological correlates of protection.
More comprehensive understanding of the role of antibodies in
acute COVID-19 illness will guide the effective use of therapeu-
tic convalescent plasma products and recombinant antibodies targeting
SARS-CoV-2. Assuming that spike-targeting vaccines are shown to
be safe and effective and become widely used, monitoring N-specific
antibodies may be of utility in distinguishing vaccine-related anti-
bodies from those stimulated by infection.

Here, we have analyzed serological responses to SARS-CoV-2 in
254 individuals ranging from asymptomatic individuals to ICU pa-
tients, with detailed analysis of antibody responses to SARS-CoV-2
RBD, S1, and N antigens and functional assays testing antibody
blocking of RBD-ACE2 binding or spike-pseudotyped virus neu-
tralization. At early time points after onset of symptoms, we saw no
evidence of preexisting antibodies that recognize SARS-CoV-2,
suggesting that there is minimal cross-reactivity of SARS-CoV-2
antigens with community coronaviruses. These findings do not
preclude the possibility that memory B cells stimulated by prior
coronavirus exposure may be cross-reactive and form part of the
response to SARS-CoV-2.

IgM, IgG, and IgA antibodies specific for SARS-CoV-2 antigens
typically become detectable in patients’ blood at similar median
times of about 2 weeks after onset of symptoms [this study and
(32, 33)], although IgA timing is the most variable. As we have re-
cently reported, large polyclonal proliferations of recently class-
switched B cells expressing IgG and IgA subtypes with low antibody
somatic mutation frequencies appear in the circulation around the
time of seroconversion, likely including plasmablasts that contribute
to the observed serological responses (34).

Which features of patient antibody responses were associated
with disease severity? To our surprise, we found that outpatients
with the mildest illness showed higher ratios of spike RBD and S1
domains compared with N antigen, beginning in the first 2 weeks
after onset of symptoms. These findings could suggest that early hu-
moral immune responses focused on spike antigens help to con-
strain the viral infection, perhaps even at times when titers are not yet
high enough to be measured in the blood. Outpatients and admitted
non-ICU patients had the lowest viral loads in their nasopharyngeal
swabs, but whether this is due to their antibody responses cannot be

![Fig. 5. RBD/N and S1/N antibody response ratios in patients with different disease severity.](https://www.sciencemag.orgience.com/content/sciimmunol/6/50/eabe0240/F5.html)
determined from this observational study. Associations of mortality in patients with COVID-19 with SARS-CoV-2 viral load assessed by rRT-PCR applied to nasopharyngeal swab specimens have also been found in other studies (35, 36). Our data are consistent with results reported from a panel of antibody assays applied to single–time point samples from patients with COVID-19 who recovered or died of their disease, which found higher values of spike-targeting responses in the convalescents (19). Patients with more severe illness in our study eventually raised higher antibody titers than those with milder disease, consistent with prior publications (32, 37) and reports of other coronavirus infections (21–23). Patients with more severe disease also had somewhat higher viral loads than patients with milder illness, suggesting that larger initial amounts of viral antigen may contribute to their greater serological responses. Functional blocking of RBD-ACE2 interaction and spike-pseudotyped virus neutralization by patient antibodies appeared with a similar time course to IgM, IgG, and IgA but were most closely correlated in magnitude with IgG titers. Neutralization, RBD-ACE2 blocking, and RBD-specific IgG were all highly correlated in patients with high antibody levels, but RBD-ACE2 blocking was less sensitive than the neutralization assay, potentially because of antibodies that can neutralize by binding to non-RBD regions of the spike or lower-affinity antibodies that can neutralize in the cell culture assay but do not compete as well with binding of ACE2 under the blocking assay conditions. It is currently unclear, however, which of these assays will be the best predictor of in vivo immunological protection from SARS-CoV-2 infection or reinfection in vaccinated or previously infected individuals.

In the detailed serological time courses of the hospitalized patients in this study, it was evident that the patterns of antibody responses could not fully explain patient outcomes, including death. Substantial numbers of patients recovered from their illness and were discharged from hospital before they had formed detectable antibody responses, but minimal serological responses were also seen in patients who died of COVID-19 at early times after onset of symptoms. Similarly, individuals with moderate antibody production were seen across the full spectrum of inpatient disease severity, and many patients who died of their disease generated high levels of...
antibodies, RBD-ACE2 blocking activity, and neutralizing titers. Differences between individuals in other aspects of the immune response or disease course—such as production of inflammatory mediators, T cell responses, host cell and tissue vulnerability to the damage during viral infection, coagulopathy, and secondary infections—are all likely to contribute to patient outcomes.

There is an urgent need to understand how long antibody titers against the virus persist after infection, now that the pandemic has been underway for more than half a year in many countries, and initial case reports of proven reinfection by SARS-CoV-2 have begun to appear (38, 39). Studies differing in their patient populations, disease severity, and serological assays have disagreed on the duration of SARS-CoV-2 antibody responses (14, 16, 32). Our data derived from inpatients, outpatients, and asymptomatic individuals with an additional asymptomatic validation cohort show that not only IgM and IgA but also IgG titers to RBD, S1, and N antigens; RBD-ACE2 blocking activity; and spike-pseudotyped viral neutralization titers all begin to decrease in patients after about the first month after onset of symptoms. The decline in antibody titers is most evident in individuals who had asymptomatic infection or mild illness, who produce lower levels of antibodies at the peak of their responses. We note that reported results failing to find a decrease in SARS-CoV-2 antibodies after several months after infection have relied on “pan-Ig” assays that cannot evaluate each isotype separately (16). Our data do not permit us to predict what fraction of the population will be susceptible to reinfection at a given time after their initial illness or whether individuals will maintain sustained plateaus of lower antibody levels after an initial decrease; additional time and follow-up will be required to obtain this information. A limitation of our study is that, apart from the prospective validation cohort, the sampling time points for each patient were determined by their length of hospital stay and subsequent health care visits, enabling more detailed analysis of serological responses in patients with more severe illness. It is important to note that decreasing antibody levels do not necessarily indicate that all immunity will be lost. It is possible that local mucosal antibody production in the airways could help prevent or impede SARS-CoV-2 infection upon reexposure (40). Even if serum antibodies wane to undetectable levels, memory B and T cells stimulated by infection could provide a faster or more effective response after future exposure. Initial reinfection reports offer some hope that SARS-CoV-2 may behave similarly to other community coronaviruses, with reinfection generally producing milder illness than the initial infection (41, 42).

One implication of our finding of waning antibody levels is that seroprevalence studies may, over time, underestimate the proportion of the investigated population that has been previously infected with SARS-CoV-2. The decrease in antibodies after infection also raises the question of how long antibodies elicited by vaccination will last and whether frequent boosting will be needed to maintain protection, assuming that safe and effective vaccines are identified. The current vaccination strategies undergoing clinical trials differ from natural infection in a variety of ways, including the method for generating or introducing viral antigens into the body, the site of exposure, and the presence of adjuvants (43–46). It is possible that some of the vaccine approaches may generate more potent and long-lasting antibodies than natural infection, in which the virus may have currently unknown
mechanisms for subverting humoral immune responses. Further detailed study of the generation of memory B cell populations, short- or long-lived plasma cells, and T cell memory to SARS-CoV-2 as well as other coronaviruses should begin to clarify some of these key mechanistic points.

MATERIALS AND METHODS

Study design and participants

The objective of this study was to investigate correlations between humoral immune responses to SARS-CoV-2, including antibodies blocking the binding of RBD to the human ACE2 receptor or neutralizing spike pseudovirus, and viral RNA loads in the nasopharynx and blood in different COVID-19 patient groups and individual patients. On 4 March 2020, the Stanford Health Care Clinical Virology Laboratory began rRT-PCR testing on nasopharyngeal specimens from suspected patients with COVID-19 using a laboratory-developed SARS-CoV-2 rRT-PCR assay (47, 48). For this study, we included specimens from patients with rRT-PCR–confirmed SARS-CoV-2 infection who reported with symptoms of COVID-19 to Stanford Healthcare–associated clinical sites between March and August 2020 and specimens from rRT-PCR–positive outpatients and asymptomatic individuals identified between April and May 2020 through occupational health screening including rRT-PCR and serology testing for RBD IgM/G at Stanford Clinical Laboratories. The screening program that detected asymptomatic SARS-CoV-2–infected individuals was offered to all Stanford Healthcare employees on a voluntary basis and screened employees with nasopharyngeal swab rRT-PCR testing and serology. In addition, we included a validation cohort of 14 asymptomatic and mildly ill individuals with prospective sample collection for up to 4 months after enrollment. This study was approved by the Stanford University Institutional Review Board (protocols IRB-48973 and IRB-55689).

Sample and data collection

Venipuncture blood samples collected in sodium heparin- or K$_2$EDTA-coated vacutainers were used for serology testing and rRT-PCR detection of RNAemia, respectively. After centrifugation for collection of plasma, samples were stored at –80°C.
Retrospective chart review was performed on all study participants. Collected data included age, gender, date of symptom onset, length of hospital stay, length of time admitted in the ICU, date and Ct value for the diagnostic nasopharyngeal swab rRT-PCR test result, the presence of underlying comorbidities, clinical symptoms, and mortality.

**Production of SARS-CoV and SARS-CoV-2 proteins and ACE2-mFc**
The SARS-CoV and SARS-CoV-2 RBD proteins were expressed in Exp293F cells and purified using nickel-NTA resin and size exclusion chromatography. The SARS-CoV construct (RBD-His_pTT5, GenBank accession number AAP13441.1) was synthesized commercially by Twist Bioscience (San Francisco, CA); the SARS-CoV-2 construct (RBD-His_pCAGGS, GenBank accession number MN908947.3) was provided by F. Krammer (49). SARS-CoV-2 S1 (spike residues 1 to 682) and ACE2-mFc, expressed in human embryonic kidney (HEK) 293 cells, and the N protein, expressed in Escherichia coli, were produced by the ATUM contract research organization. Soluble human ACE2 fused to a mouse Fc tag was constructed by synthesizing a gene encoding ACE2 (residues 1 to 615) joined by a (G4S)x2 linker to mouse IgG2a Fc and placed under control of a cytomegalovirus promoter by cloning into a mammalian expression plasmid.

**ELISA to detect anti–SARS-CoV-2 and anti-SARS-CoV antibodies in plasma samples**
The ELISA procedure in this study was modified from a protocol published by Stadlbauer et al. (49). Corning Costar 96-well high-binding plates (catalog no. 9018, Thermo Fisher Scientific) were coated with SARS-CoV RBD and SARS-CoV-2 RBD, S1, or N protein in phosphate-buffered saline (PBS) at a concentration of 0.1 μg per well (0.025 μg per well for the nucleocapsid IgG assay) overnight at 4°C. On the next day, wells were washed 3× with PBS and 0.1% Tween 20 (PBS-T) and blocked with PBS-T containing 3% nonfat milk powder for 1 hour at room temperature (RT). Wells were then incubated with plasma samples from patients with COVID-19 at a dilution of 1:100 in PBS-T containing 1% nonfat milk for 1 hour at 37°C. Two negative and two positive plasma pool wells and two blank wells incubated with PBS-T containing 1% nonfat milk powder were included on each plate. After washing 3× with PBS-T, horseradish peroxidase–conjugated goat anti-mouse IgG (1:6000 dilution, μ-chain specific; catalog no. A6907, Sigma-Aldrich), or IgM (1:6000 dilution, μ-chain specific; catalog no. A6907, Sigma-Aldrich), or IgA (1:5000 dilution, α-chain specific; catalog no. P0216, Agilent) in PBS-T containing 1% nonfat milk was added and incubated for 1 hour at RT. Wells were washed 3× with PBS-T and dried by vigorous tapping of plates on paper towels. TMB (3',5'-tetramethylbenzidine) substrate solution was added, and the reaction was stopped after 12 min by addition of 0.16 M sulfuric acid. The optical density at 450 nm (∆D450) was measured with an EMax Plus microplate reader (Molecular Devices, San Jose, CA). OD values were converted to percentage of blocking using the following formula: 100*(1 − (sample OD − 0.2)/(QC1 OD − 0.2)), taking into account the background noise of the assay of 0.2 as determined after testing preんどcontrol plasma samples. Additional details for competition ELISA assay setup and optimization are provided in figs. S14 and S15.

**Pseudotyped lentiviral neutralization assay**
SARS-CoV-2–pseudotyped lentivirus assays were performed as described previously. Briefly, spike-pseudotyped lentivirus was produced in HEK293T cells as described in (50) using a five-plasmid system described in (27). For viral neutralization assays, ACE2/HeLa cells (28) were plated in 96-well tissue culture plates 1 day before infection. Before neutralization assays, patient plasma samples were heat-inactivated for 1 hour at 56°C. Plasma samples and virus were incubated with the cells at 37°C for ~48 hours. After incubation, cells were lysed with BriteLite assay readout solution (PerkinElmer), and luminescence values were obtained with a BioTek plate reader. Single-dilution point neutralization assays were performed in technical duplicate in two different experimental replicates. Percent neutralization was determined by normalizing raw luciferase values to 0% infectivity (average from cell-only wells) and 100% infectivity (average from virus-only wells) in GraphPad Prism 8.4.1. Plasma dilution series shown in fig. S9 were performed in technical duplicate; normalized percentage infectivity values were fit with a three-parameter nonlinear regression inhibitor curve in GraphPad Prism 8.4.1, and fits were constrained to have a value of 0% at the bottom of the fit.

**Real-time PCR to detect SARS-CoV-2 RNA in plasma**
A volume of 400 μl of EDTA-anticoagulated plasma was extracted by using the Qiagen EZ1 Virus Mini Kit v2.0 (QIAGEN, Germantown, MD). Molecular testing for the presence of SARS-CoV-2 RNA in plasma was performed with a modification of a published rRT-PCR assay targeting the envelope (E) gene (47, 48). The standard Ct values of positive tests with this assay range from Ct < 20 to

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45 cycles. Testing of plasma samples with a Ct value of 40 or greater was repeated to ensure reproducibility of the positive result. Because viral culture was not performed as part of this study, the presence of SARS-CoV-2 in tested plasma was defined as RNAemia.

**Statistics**

GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA) software was used to visualize ELISA data, analyze for differences in antibody responses between disease categories by Wilcoxon rank sum testing, and carry out linear regression of pseudotyped virus neutralization, RBD-ACE2 blocking, and antibody ELISA results. The goodness of fit for linear regression analyses is reported as the coefficient of determination, \( R^2 \). Locally estimated scatterplot smoothing for the development and decrease of antibody responses over time was performed using the loess method in the R statistical package version 3.6.1 (51). Correlation between antibody OD 450 values, RNAemia, and ACE2-RBD blocking assay OD 450 values was calculated as Spearman correlations with the R cor function.

**SUPPLEMENTARY MATERIALS**

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Figs. S1 to S15

Supplementary methods, figures, and source data.

contributions throughout the study. K.R., B.N., and R.T. performed statistical analyses. K.R., A.E.P., O.F.W., B.A.P., C.A.H., S.G., R.T., and S.D.B. analyzed the data. K.R. and S.D.B. wrote the manuscript. All authors edited and approved the manuscript. Competing interests: M.H. is an employee of ATUM. S.D.B. has consulted for Regeneron, Sanofi, and Novartis on topics unrelated to this study. S.D.B., K.R., P.S.K., and A.E.P. have filed provisional patent applications related to serological tests for SARS-CoV-2 antibodies. K.C.N. reports grants from the National Institute of Allergy and Infectious Diseases (NIAID); Food Allergy Research & Education (FARE); End Allergies Together (EAT); National Heart, Lung, and Blood Institute (NHLBI); and National Institute of Environmental Health Sciences (NIEHS). K.C.N. is the director of the FARE and World Allergy Organization (WAO) Center of Excellence at Stanford University; advisor at Cour Pharmaceuticals; cofounder of Before Brands, Alladapt, Latitude, and IgGenix; National Scientific Committee member for the Immune Tolerance Network (ITN) of NIAID; recipient of a research sponsorship from Nestle; consultant and advisory board member at Before Brands, Alladapt, IgGenix, NHLBI, and ProBio; and Data and Safety Monitoring Board member at NHLBI. C.A.B. is on the board of Catamaran Bio. The other authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials.

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