Low serum neutralizing anti-SARS-CoV-2 S antibody levels in mildly affected COVID-19 convalescent patients revealed by two different detection methods

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INTRODUCTION
Within 6 months since its emergence, the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the cause of coronavirus disease 2019 (COVID-19), has spread rapidly worldwide. COVID-19 consists of a spectrum of clinical syndromes ranging from asymptomatic cases to mild, flu-like disease to severe illness requiring hospitalization mainly due to pulmonary complications.1–3 Although COVID-19 primarily targets the respiratory system, new data indicate that COVID-19 also affects the vascular system, causing thrombosis and inflammation in multiple organs, including the lungs.4–6 It is not surprising, therefore, that patients with pre-existing cardiovascular diseases, hypertension, and other comorbidities are at particular risk.3

SARS-CoV-2 utilizes angiotensin-converting enzyme 2 (ACE2) as a receptor for entry into target cells and employs TMPRSS2, a cellular serine protease, for activation of the viral spike (S) protein.8,9 Both ACE2 and TMPRSS2 are abundant in the upper respiratory tract,10 an early immune response against SARS-CoV-2 involves interleukin-6 and interferon signature gene expression in alveolar macrophages and infiltrating monocytes.11 Although this early immune response is accompanied by severe lymphopenia,12,13 increasing data indicate that successful recovery from COVID-19 relies on antibody and T-cell responses.12,14–17 Importantly, there appears to be a strong correlation between circulating SARS-CoV-2-specific CD4+ and CD8+ T cells and IgG antibodies against the nuclear (N) and/or the spike (S) protein of SARS-CoV-2.16,17

Current data indicate that anti-SARS-CoV-2 IgM antibodies appear within one week after infection and are present for a month before they gradually decrease.18,19 In contrast, anti-SARS-CoV-2 IgG antibodies appear within 10–21 days after infection and appear to remain more-or-less stable for up to 3 months.8,18 It is not surprising, therefore, that antibody responses against SARS-CoV-2 have received attention as a method for accurate assessment of infection prevalence.20,21 Particularly interesting

Neutralizing antibodies targeting the receptor-binding domain (RBD) of the SARS-CoV-2 spike (S) block severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) entry into cells via surface-expressed angiotensin-converting enzyme 2 (ACE2). We used a surrogate virus neutralization test (sVNT) and SARS-CoV-2 S protein-pseudotyped vesicular stomatitis virus (VSV) vector-based neutralization assay (pVNT) to assess the degree to which serum antibodies from coronavirus disease 2019 (COVID-19) convalescent patients interfere with the binding of SARS-CoV-2 S to ACE2. Both tests revealed neutralizing anti-SARS-CoV-2 S antibodies in the sera of ~90% of mildly and 100% of severely affected COVID-19 convalescent patients. Importantly, sVNT and pVNT results correlated strongly with each other and to the levels of anti-SARS-CoV-2 S1 IgG and IgA antibodies. Moreover, levels of neutralizing antibodies correlated with the duration and severity of clinical symptoms but not with patient age. Compared to pVNT, sVNT is less sophisticated and does not require any biosafety labs. Since this assay is also much faster and cheaper, sVNT will not only be important for evaluating the prevalence of neutralizing antibodies in a population but also for identifying promising plasma donors for successful passive antibody therapy.

Keywords: COVID-19; SARS-CoV-2; Neutralizing antibody; Serum; ELISA

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requirement of a biosafety-2 lab. As reported by others,30 we also

tively low throughput and relies on infectious viruses, with

as well as for identifying suitable plasma donors for passive

screening and will be valuable for epidemiological studies

COVID-19 contained signi-

time. In contrast, sera from convalescent patients with severe

patients exhibiting clinical symptoms for a short period of

anti-SARS-CoV-2 S titers could be detected in convalescent

anti-SARS-CoV-2 S antibodies in 93% of convalescent patients

IgA levels measured using a commercial S1 protein ELISA as

data obtained by sVNT and pVNT to anti-SARS-CoV-2 S1 IgG and

throughput analysis. Furthermore, we correlated the functional

on broadly available ELISA techniques and allows high-

SARS-CoV-2 S protein RBD to ACE2 in vitro. This assay is based

in 96-well plates. The next day, complement in test sera was

later, the pseudotype particle-containing supernatant was separated

cells transfected with the VSV-G expression construct. Twenty hours

transduced cells with the viral particles for 2 h at 37 °C, the

VSV-G open reading frame has been replaced by combined GFP and

recombinant VSV in which the

pVNT, Vero76 cells were seeded at 1 × 10^4 cells per well

Δ

G-FLuc, a replication-deficient recombinant VSV in which the

VSV-G control virus or pCG1-SARS-2 ΔΔ182 for the SARS-CoV-

spike protein. Eighteen hours later, the cells were infected with

VSV^2ΔG-FLuc stock virus was propagated in BHK-21 G43 cells.34

After incubating the transduced cells with the viral particles for 2 h at 37 °C, the supernatant was removed, and the cells were washed twice with PBS. The cells were then supplied with medium containing an mAb targeting VSV-G (supernatant from mouse hybridoma CRL-2700; ATCC) to neutralize residual VSV-G, a step that was omitted for the cells transfected with the VSV-G expression construct. Twenty hours later, the pseudotype particle-containing supernatant was separated from the cells by centrifugation and used for neutralization assays.

For the pVNT, Vero76 cells were seeded at 1 × 10^6 cells per well in 96-well plates. The next day, complement in test sera was

are antibodies targeting the receptor-binding domain (RBD) of the

S protein, as they can block virus entry into cells and thus prevent

infection and spread. Furthermore, these neutralizing antibodies

may be used for passive antibody therapy,20,21 as approved by the

United States Food and Drug Administration on March 24th, 2020,
as an emergency investigational new treatment for severe or life-

threatening COVID-19.23

In addition to general safety measures for plasma donation,
a crucial parameter in convalescent plasma donor selection for

COVID-19 is an adequate neutralizing antibody titer.24

However, the field is rapidly evolving, and there is still no

consensus about the diagnostic value of divergent ELISA-based

antibody tests for SARS-CoV-2 seropositivity.25 Moreover, there is

uncertainty regarding the durability of anti-SARS-CoV-2 antibody

responses, especially as there are indications that antibody responses to other coronaviruses are variable and transient.26–28 It is also not clear whether all COVID-19 patients, especially those with mild disease, will produce sufficient amounts of neutralizing antibodies against SARS-CoV-2 to prevent early reinfection.

In the present study, we compared different experimental

approaches to qualitatively and quantitatively assess antibody

response to SARS-CoV-2 infection primarily in cohorts of

convalescent individuals with mild COVID-19 disease. We applied

a SARS-CoV-2 S protein-pseudotyped-vesicular stomatitis virus

(VSV) vector-based neutralization assay (pVNT)8 that has rela-

tively low throughput and relies on infectious viruses, with

requirement of a biosafety-2 lab. As reported by others,30 we also

MATERIAL AND METHODS

 Serum samples

 Serum samples were collected from convalescent COVID-19 individuals who volunteered to donate plasma at Hannover Medical School’s (HMS) Institute of Transfusion Medicine and Transplant Engineering. All donors had PCR-diagnosed SARS-CoV-2 infection and showed only mild clinical symptoms. Serum was also collected from inpatients with severe COVID-19 symptoms and from healthy controls without any COVID-19-related symptoms (Tables 1, 2, S1, and S2). The blood donors provided consent prior to blood donation and the inpatients at the time of hospital admission for their samples to be used for research purposes. Written informed consent was obtained from all participants. Studies investigating serum samples from healthy controls and COVID-19 patients were approved by the HMS institutional review board (#9001_BO_K2020, #8973_BO_K2020, and #7901_BO_K2018).

ELISA

Serum samples were analyzed in the Clinical Virology Laboratory and Clinic for Rheumatology und Immunology of HMS using the CE-certified versions of Euroimmun SARS-CoV-2 S1 IgG and IgA ELISA (Euroimmun, Lübeck, Germany) according to the manufacturer’s recommendations.

Pseuodtyped virus neutralization assay

A pVNT was performed at HMS’s Institute of Virology and the Primate Center in Göttingen as described earlier.35 In brief, pseudotyped VSV particles were produced by calcium-phosphate transfecting HEK293T cells carrying expression plasmids for the respective glycoproteins, either pCAGGS-VSV-G36 for expression of VSV-G of the control virus or pCG1-SARS-2 ΔΔ182 for the SARS-CoV-2 spike protein. Eighteen hours later, the cells were infected with VSV^2ΔG-FLuc, a replication-deficient recombinant VSV in which the VSV-G open reading frame has been replaced by combined GFP and firefly luciferase expression cassettes.33 This VSV^2ΔG-FLuc stock virus was propagated in BHK-21 G43 cells.34 After incubating the transduced cells with the viral particles for 2 h at 37 °C, the supernatant was removed, and the cells were washed twice with PBS. The cells were then supplied with medium containing an mAb targeting VSV-G (supernatant from mouse hybridoma CRL-2700; ATCC) to neutralize residual VSV-G, a step that was omitted for the cells transfected with the VSV-G expression construct. Twenty hours later, the pseudotype particle-containing supernatant was separated from the cells by centrifugation and used for neutralization assays.

For the pVNT, Vero76 cells were seeded at 1 × 10^6 cells per well in 96-well plates. The next day, complement in test sera was

Table 1. Main cohort characteristics

| Group        | n  | Sex (M/F/ND) | Average age [years (range)] | Average symptom duration [days (range)] | Average sampling post symptom onset [days (range)] |
|--------------|----|--------------|-----------------------------|----------------------------------------|--------------------------------------------------|
| Mild COVID   | 40 | 20/19/1      | 42 (19–68)                  | 10 (0–25)                              | 25 (10–61)                                       |
| Severe COVID | 10 | 9/1/0        | 54 (23–67)                  | 36 (19–71)                             | 26 (14–46)                                       |
| HC           | 12 | 3/9/0        | 39 (25–56)                  | NA                                     | NA                                               |

HC healthy controls, M male, F female, NA not applicable, ND not disclosed

Table 2. Characteristics of the confirmation COVID-19 cohort with mild disease

| Group        | n  | Sex (M/F/ND) | Average age [years (range)] | Average symptom duration [days (range)] | Average sampling post symptom onset [days (range)] |
|--------------|----|--------------|-----------------------------|----------------------------------------|--------------------------------------------------|
| Mild COVID   | 44 | 29/15/0      | 47 (23–64)                  | 13 (4–24)                              | 33 (14–64)                                       |

M male, F female, NA not applicable, ND not disclosed

**Table 1. Main cohort characteristics**

**Table 2. Characteristics of the confirmation COVID-19 cohort with mild disease**

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inactivated by heating the samples to 56 °C for 30 min. The sera were then serially diluted, mixed 1:2 with the pseudotyped VSV and incubated for 30 min at 37 °C. The medium was removed from the Vero76 cells and replaced in triplicate wells with the serum/pseudotype mixture particle mixture. Twenty hours postinfection, the supernatant was removed from the cells and replaced with 1× luciferase lysis buffer (2× Lysis juice, 102511, PJK). The cells were lysed for 30 min at room temperature. The lysates were transferred to white plates with luciferase substrate (Beetle juice, 102511, PJK), and luciferase activity was measured with a High Sensitivity plate luminometer (Hidex) or a GloMax Discover Microplate Reader (Promega). Data were plotted after background subtraction and normalization to the "no serum" controls. Pseudotyped virus neutralizing titers 50 and 90 (pVNT50/90) were defined as the last serum dilution that reduced the transduction efficiency of biological triplicates by at least 50% or 90%, respectively.

Expression and purification of recombinant soluble ACE2-IgG1 protein
HEK293T cells were grown in DMEM/10% ultralow IgG FBS/PenStrep and transiently transfected with the plasmid pcDNA3-sACE2(WT)-Fc (a gift from Erik Procko; Addgene plasmid #14516339) by applying standard calcium-phosphate procedures. Supernatants were collected and separated using a protein A-Sepharose column (ThermoFisher). The bound recombinant protein was eluted with 0.1 M sodium citrate pH 3.5. The buffer was exchanged with PBSd, and integrity as well as purity was confirmed by analyzing 2 μg of protein on a 10% SDS polyacrylamide gel.

Surrogate virus neutralization assay
The surrogate virus neutralization assay was developed based on the hypothesis that virus neutralizing antibodies should also interfere with the binding of the RBD of SARS-CoV-2 (SARS-CoV-2 S RBD) to soluble, surface-immobilized ACE2, as described by others,24 with several modifications. Depending on the amount of neutralizing antibodies present in convalescent sera, the binding of SARS-CoV-2 S RBD to ACE2 should be blocked to various degrees that should correlate with the optical density of this enzyme-linked immune sorbent-based assay. In the assay reported herein, the hACE2 protein (Trenzyme or in-house produced) was coated with different concentrations in 100 mM carbonate-bicarbonate coating buffer (pH 9.6) on F96-Maxisorp Immuno plates (Thermo Scientific) at 4 °C overnight. After washing in phosphate-buffered saline (PBS) with 0.05% Tween 20 (PBST), the plates were blocked with 2% bovine serum albumin (BSA, Sigma) and 0.1% Tween 20 in 1× PBS for 1.5 h at 37 °C. Then, His-tag-conjugated SARS-CoV-2 S RBD (Trenzyme) was added to the carrier buffer (1% BSA and 0.05% Tween in 1× PBS) at different concentrations and incubated for 1 h at 37 °C. Unbound SARS-CoV-2 S RBD was removed by four PBST washes before an anti-His peroxidase-labeled monoclonal antibody (mAb; Clone 3D5, preincubated in house) in carrier buffer was added for 1 h at 37 °C. After the final wash, the colorimetric signal was developed by adding the chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (Sigma) and stopped by adding an equal volume of stop solution (0.2 M H2SO4). Finally, absorbance readings at 450 and 570 nm were acquired using the SpectraMax ID3 microplate reader ( Molecular Devices). The Kd values of the SARS-CoV-2 binding affinity to ACE2 were calculated from binding curves based on their global fit using one-site specific binding analysis (GraphPad Prism).

To test for the presence of neutralizing anti-SARS-CoV-2 S serum antibodies, 6 ng of SARS-CoV-2 S RBD was preincubated with test sera at final dilutions between 1:20 and 1:540, as indicated on the graphs, for 1 h at 37 °C before adding them to plates coated with 150 or 300 ng/well ACE2. For each reaction, the percent inhibition was calculated from optical density values after subtraction of background values as: Inhibition (%) = (1 – Sample OD value/

Average SARS-CoV-2 S RBD OD value) × 100. Neutralizing sVNT titers were determined as the dilution with binding reduction > mean ± 2SD of values from sera of healthy controls.

Statistical analysis
Linear data were analyzed using an unpaired t-test with Welch’s correction (for 2 groups) or Welch’s ANOVA followed by Dunnett’s T3 multiple comparisons test (for 3 groups) and were correlated using the Pearson r test. Categorical data were analyzed using the χ² test or Fisher’s exact test for unpaired proportions, as indicated beneath each figure. Correlation between linear and categorical data was assessed using ordinary one-way ANOVA followed by the test for linear trend. All statistical analyses were conducted using GraphPad Prism 8.4 (GraphPad Software, USA).

RESULTS
Most individuals with mild COVID-19 disease develop anti-SARS-CoV-2 antibodies
Between March 23rd and May 11th, 2020, we enrolled as a first step 50 convalescent COVID-19 patients diagnosed with SARS-CoV-2 infection by RT-PCR and 12 healthy control subjects who were not exposed to SARS-CoV-2. The samples from the convalescent COVID-19 cases were split into two groups according to disease severity. Eighty percent of the individuals (n = 40) had a mild clinical course, with an average symptom duration of 10 days (range, 0–25 days), and did not require an inpatient hospital stay (Tables 1 and S1). Ten patients had severe COVID-19 and required hospital stays longer than 2 weeks and respiratory support. The patients with severe COVID-19 had an average disease duration of 37 days (range from 19–71 days).

To estimate overall antibody responses against SARS-CoV-2 in the serum of COVID-19 convalescent individuals, we analyzed the presence of anti-SARS-CoV-2 IgG and IgA antibodies targeting the S1 protein by ELISA. Anti-SARS-CoV-2 S1-specific IgG antibodies were detected in 35/37 (94.6%) of the mildly affected and in 10/10 severely affected COVID-19 patients tested. One individual with mild disease was considered to have borderline serum positivity, and one patient was negative according to the manufacturer’s classification (Fig. 1A). Similarly, anti-SARS-CoV-2 S IgA antibodies were present in 33/37 (89.2%) of the tested sera; two samples were diagnosed as borderline positive and two as negative...
The surrogate virus neutralization test (sVNT) detects neutralizing antibodies interfering with SARS-CoV-2 S RBD binding to human ACE2. Binding of SARS-CoV-2 S RBD to human ACE2 from commercial vendor (A) and produced in-house (B). Plates were coated with ACE2 as indicated. His-tagged SARS-CoV-2 S RBD was titrated as indicated and detected with an anti-His peroxidase-labeled mAb. Representative assays performed in duplicate are presented as the mean ± SD. C Inhibition of the interaction of SARS-CoV-2 S RBD with ACE2 by the addition of sera from convalescent patients with mild (blue lines) or severe (red lines) COVID-19 and healthy controls (HC; black lines). Assay performed in duplicate; mean percentages of neutralization ± SD. D–G Inhibition of the interaction of SARS-CoV-2 S RBD with ACE2 at the serum dilutions indicated. Individual values (dots) and means (line). Shaded areas represent the mean ± 2SD of values from sera of healthy controls. *P < 0.05; ***P < 0.001; Welch’s ANOVA followed by Dunnett’s T3 multiple comparisons test. H Relative distributions of SARS-CoV-2 neutralizing serum titers determined as the dilution retaining binding reduction > mean ± 2SD of HC. **P < 0.01; Fisher’s exact test (HC vs. mild or severe) or the Chi-squared test was used to assess the trend (mild vs. severe).
supporting the finding that patients with mild COVID-19 only produce low amounts of SARS-CoV-2 neutralizing antibodies. In contrast, the median pVNT 50 and pVTN90 in severely affected COVID-19 convalescents were 1:1600 and 1:400, respectively, further indicating that patients recovering from severe disease produce higher neutralizing anti-SARS-CoV-2 antibody titers than patients with mild disease.

Positive correlation between total anti-S1 protein and neutralizing antibody levels in sera of convalescent individuals with mild COVID-19

We then analyzed the correlation between total levels of anti-S1 IgG and IgA and the amount of neutralizing antibodies in our cohort of mild COVID-19 convalescent patients and healthy controls. As expected, an initial comparison showed a strong positive correlation between the levels of S protein-specific IgA and IgG antibody in sera (Fig. S2). More importantly, there was a robust positive correlation between the percent inhibition of SARS-CoV-2 S RBD binding to ACE2 at a 1:20 serum dilution (sVNT1:20) and pVNT90 as well as pVTN50 inhibitory titers (Fig. 4A, B). Furthermore, there was a strong positive correlation between sVNT and pVNT90 as well as pVTN90 inhibitory titers (Fig. 4C, D).

Similarly, a strong positive correlation between sVNT1:20 and levels of SARS-CoV-2 S1-specific IgG and antibody levels in convalescents and healthy controls was observed, though levels of anti-S1 IgA showed a weaker correlation (Fig. 4E, F). These data demonstrate that sVNT reliably detects neutralizing serum antibodies against SARS-CoV-2. Furthermore, a strong correlation with $r^2$ values between 0.64 and 0.75 was revealed when comparing SARS-CoV-2 S1-specific IgG and IgA antibody levels with pVNT90 and pVTN50 (Fig. S3).

Total and neutralizing anti-SARS-CoV-2 S antibody levels in sera correlate positively with symptom duration but not with the timing of sampling or patient age

We next examined whether the level of the protective humoral response to SARS-CoV-2 in COVID-19 patients correlated with disease duration, as defined by the number of days patients showed symptoms (mild cases) or until they were discharged from the hospital (severe cases). Not surprisingly, severely affected patients had a 3.5 times longer disease duration, averaging 36 days, than the 10 days of the mildly affected patients (Fig. 5A). As severely ill patients produced higher neutralizing and total antibody titers, these data indicate that disease duration might directly influence antibody titers. This hypothesis is further supported by a positive correlation between the duration of symptoms and total anti-SARS-CoV-2 IgG, but not IgA, antibodies in convalescent patients with mild disease (Fig. 5A, B). Although weaker, there was also a positive correlation between symptom duration and levels of neutralizing antibodies, as determined by sVNT1:20, pVNT90, and pVTN50 (Fig. 5D, E). These data are in agreement with data reported by Robbiani et al.37 and with a recent publication indicating that asymptomatic SARS-CoV-2 infection induces lower antibody levels than symptomatic infection.38 Altogether, our results and publicly available data suggest that a certain threshold of disease severity and/or duration might be required for successful mounting of neutralizing anti-SARS-CoV-2 humoral responses.

Conversely, in our cohort of convalescent COVID-19 cases, we did not observe any correlation between levels of total and neutralizing anti-SARS-CoV-2 S antibodies with the timing of sampling after the occurrence of first symptoms (Fig. S4). Similarly, the levels of neutralizing anti-SARS-CoV-2 S IgG and IgA antibodies

Fig. 3 Frequency of neutralizing antibodies against SARS-CoV-2 measured by a pseudotyped virus neutralization test (pVNT) based on SARS-CoV-2 S protein-pseudotyped VSV. A Example of pVNT results. Sera from COVID-19 convalescent patients with mild or severe disease—but not from healthy controls (HC)—suppress entry of replication-defective VSV particles carrying the SARS-CoV-2 S protein into host cells (filled bars); neither sera suppressed the entry of control particles carrying the G-protein of VSV entry (open bars). Red lines indicate levels of 50 or 90% suppression of virus entry as indicated. Relative distributions of SARS-CoV-2 neutralizing serum titers that result in (B) 90% (pVNT90) or (C) 50% (pVTN50) reduction of luciferase production, as described in (A). ***P < 0.001; Fisher’s exact test (HC vs. mild or severe) or the Chi-squared test was used to assess the trend (mild vs. severe).
blood and therefore included only three elderly patients (aged 60 or over). Thus, there is not sufficient power to detect levels of total and neutralizing anti-SARS-CoV-2 S IgG and IgA antibodies in this age group. Similarly, most of the severely affected patients investigated in this study were males, but the relatively small cohort size did not allow analysis of sex associations using our data.

sVNT allows rapid screening of sera of blood donors for the presence of neutralizing antibodies. To validate our findings, we recruited a second cohort of 44 convalescent patients with mild COVID-19 and analyzed their sera by ELISA and sVNT (Tables 2 and S2). In this group of patients, ELISA detected 51 protein-specific IgA and IgG antibodies in 38 of 44 analyzed serum samples (borderline counted as negative; Fig. 6A). As described above for the first group of mildly affected convalescent patients, levels of S protein-specific IgA and IgG antibodies showed a strong correlation (Fig. S6). Similarly, applying the sVNT confirmed that mildly affected COVID-19 convalescent patients possess relatively low titers of neutralizing anti-S RBD antibodies (median 1:180). This assay revealed neutralizing antibodies in the sera of 40 of 44 (90.9%) individuals with mild COVID-19 (Fig. 6B). Along the same line, a strong positive correlation between the sVNT1:20 and total levels of SARS-CoV-2 S-specific IgG and IgA antibody levels was also identified (Fig. 6C, D). Moreover, in this group of samples, we observed a weak positive correlation between symptom duration and sVNT1:20 or log-transformed SARS-CoV-2 S-specific IgG but not IgA levels (Fig. S7A–C). As expected, we did not detect any correlation between specific IgG, IgA, or sVNT1:20 and patient age or date of sampling (Fig. S7D–F and data not shown). As a final validation, we correlated the sVNT1:20 and total levels of SARS-CoV-2 S-specific IgG and IgA antibody levels as well as a weak positive correlation between symptom duration and sVNT1:20 and log-transformed SARS-CoV-2 S-specific IgG but not IgA levels. Altogether, the results from the validation cohort confirmed our initial results and further emphasize the usefulness of the sVNT for rapid screening of a larger number of samples for the presence of neutralizing anti-SARS-CoV-2 S RBD antibodies.

**DISCUSSION**

A detailed understanding of immune responses following SARS-CoV-2 infection will enable better treatment and diagnostic procedures, as well as the development of successful vaccines that will help to control the global COVID-19 pandemic. In this regard, it is important to gain a better understanding of the presence of neutralizing anti-SARS-CoV-2 serum antibodies in the population, as they potentially prevent (re)infection and might be a treatment option. 39,40 As reported by others, 39,40 we established an sVNT that is based on ELISA technology and thus can be adapted to allow for high-throughput analysis of samples. We validated this assay by comparing data from the sVNT with those derived from a classical pVNT and found a strong correlation between the results obtained with these two tests, which is in line with the results of Tan et al. 30,41 Our experience confirms that sVNT is technically less complicated, cheaper, and much faster than pVNT, making it more suitable for the rapid screening of a large number of samples. Of note, Tan et al. also showed that sVNT, apart from a small degree of cross-neutralization with anti-SARS-CoV antibo-

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**Fig. 4** sVNT positively correlates with pVNT and anti-SARS-CoV-2 S1 IgG and IgA antibodies. Correlation between sVNT1:20 and antibody titers resulting in 90% (A) or 50% (B) reduction of luciferase production in pVNT90 and pVNT50. The horizontal shaded area indicates the mean ± 2SD range of inhibition of sera from HC. Correlation between log-transformed sVNT titers (determined as in Fig. 2H) and log-transformed pVNT90 (C) and pVNT50 (D). To alleviate overplotting, the titer values were jittered by the addition of random values in the interval [−0.5, 0.5]. Correlation between sVNT1:20 and log-transformed SARS-CoV-2 S1-specific IgG (E) and IgA (F) levels measured by ELISA. The vertical shaded areas indicate the respective cutoff values recommended by the manufacturer to determine positive (right to), borderline (within) and negative (left to) shaded areas. The horizontal shaded area indicates the mean ± 2SD range of inhibition of sera from healthy controls. A–F Dots, samples of HC (black), mildly (blue), or severely (red) affected COVID-19 convalescent cases. C–F Linear correlation (solid line) and 95% confidence intervals (dotted lines). Correlation, one-way ANOVA followed by a test for the trend (A, B) or Pearson r (C–F).
also be applied to detect neutralizing antibodies in any animal species used for preclinical testing of SARS-CoV-2 vaccines. SVNT might also be adapted for the detection of immunoglobulin classes that most efficiently neutralize SARS-CoV-2 S or used for rapid screening of neutralizing capacities of monoclonal SARS-CoV-2 S RBD-specific antibodies. On the other hand, a disadvantage of SVNT, as compared to PVNT, is its intrinsic inability to detect neutralizing antibodies other than those binding to the SARS-CoV-2 S RBD. Nevertheless, these non-RBD-targeting antibodies appear to have only a minor role in SARS-CoV-2 neutralization, which is supported by the robust correlation between SVNT and PVNT reported in the present study and by others.30

Combining PVNT and SVNT, we found that ~90% of recovered patients with mild COVID-19 possessed neutralizing serum antibodies. These findings are in line with an early report suggesting that recovered COVID-19 patients have neutralizing SARS-CoV-2 S RBD antibodies in serum after discharge from the hospital.16 Other preliminary data indicate that neutralizing SARS-CoV-2 S RBD antibodies are undetectable in one-third of convalescent COVID-19 patients.37,42 Additional studies are therefore required to provide more detailed insight into the levels of neutralizing SARS-CoV-2 S antibodies in convalescent COVID-19 individuals from different countries. Those studies should also exclude false-positive PCR or COVID-19 misdiagnosis as the possible reason for the lack of antibodies in certain suspected COVID-19 patients. This would be particularly important, as convalescent COVID-19 individuals without neutralizing antibodies might still be susceptible to reinfection and would not be able to provide plasma for the prevention and treatment of COVID-19.

Our data also corroborate other studies indicating that levels of neutralizing antibodies in convalescent COVID-19 individuals are generally low.37,42 Interestingly, Robbiani et al. reported recently that individual neutralizing antibodies against SARS-CoV-2 S RBD have a half-maximal inhibitory concentration against authentic SARS-CoV-2 ranging from 3 to 709 ng/ml.37 Together, these data suggest that a considerable proportion of COVID-19 patients with mild disease can produce intermediate- to high-affinity IgG antibodies. Since such antibodies are not present in a certain proportion (5−30%) of COVID-19 cases, these findings indicate that

Fig. 5 The duration of symptoms correlates with total and neutralizing SARS-CoV-2 S-specific antibody levels. A Symptom duration of mild and severely affected COVID-19 patients. Dots, individuals; bars, mean, ***P < 0.001, Welch’s t test. Weak positive correlation between duration of symptoms and levels of log-transformed SARS-CoV-2 S1-specific IgG (B) and IgA (C) antibodies, SVNT1:20 (D), PVNT90 (E), or PVNT50 (F) neutralizing antibody titers. Dots, convalescent individuals with mild COVID-19, outliers are marked with x, horizontal lines, means. B, C, Shaded areas indicate vendor-defined cutoff values to determine positive (above), borderline (within), and negative (below gray area) samples. D The shaded area indicates the mean ± 2SD range of inhibition of sera from HC. Correlation, Pearson r (B−D) or one-way ANOVA followed by a test for the trend (E, F). An outlier was defined as a value with absolute residual value > 2SD of all residual values (D) or as a value > mean ± 2SD of values with the same titer

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In conclusion, this study reports a high-throughput sVNT for SARS-CoV-2. The results obtained with this assay correlate highly with data obtained by classical but laborious and time-consuming pVNT. Both assays revealed the presence of neutralizing anti-SARS-CoV-2 S antibodies, albeit at low titers, in the sera of many but not all convalescent COVID-19 patients with mild disease. Although these findings have implications for the selection of convalescent donors for passive immunization by plasma therapy, additional studies are required to understand why neutralizing anti-SARS-CoV-2 S antibodies do not develop in all patients and how long neutralizing antibodies are present in patients who have recovered from COVID-19.

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AUTHOR CONTRIBUTIONS
RF conceived and guided the study. R.F. and B.B. developed the sVNT. B.B., S.C.S., A.K.C., G.B., I.R., W.P., A.C., S.W., and C.R. performed the laboratory work, including the assay setup and data analysis. S.P., M.H., and H.X.-W. initially developed and performed and T.F.S. coordinated the conventional neutralization assays at HMS. R.B., N.G., M.Y., I.P., and J.M. set up the Hanover convalescent donor registry, coordinated the donor testing and provided the samples. C.R.S.-F. analyzed the clinical patient data and provided the healthy control samples. G.M.N.B., I.P., M.M.H., and T.F.S. also provided samples. B.B. and RF wrote the paper with input from all authors.

ADDITIONAL INFORMATION
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