Anti-Spike, anti-Nucleocapsid and neutralizing antibodies in SARS-CoV-2 hospitalized patients and asymptomatic carriers

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Abstract

Using longitudinal plasma samples from thirty COVID-19 patients, we observed that virus-specific antibodies are detectable in 100% of patients two weeks after symptom onset. We also show that these patients produced variable levels of neutralizing antibodies which reached a plateau two weeks after symptom onset and then declined in the majority of patients. Furthermore, we report that neutralizing antibodies were undetectable in 56% (14/25) of asymptomatic carriers.

Keywords

SARS-CoV-2; COVID-19, coronavirus, spike, nucleocapsid, RBD, neutralizing antibodies
The Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has recently emerged and caused a human pandemic of coronavirus disease 2019 (COVID-19). Among the four SARS-CoV-2 structural proteins, the Spike (S) and the Nucleocapsid (N) proteins are the main immunogens. The S protein consists of two subunits, S1 which contains the receptor-binding domain (RBD) and S2. The kinetics of antibody detection is essential for the selection of commercial serological assays and the interpretation of the results. Some manufacturers have decided to target the S1 and/or the S2 whereas others chose the RBD or the N protein. Furthermore, the neutralizing antibody (NAb) response against the SARS-CoV-2 remains poorly understood and it is still unknown whether cured patients are protected against new infection.

In order to accurately assess the time of seroconversion, we used 151 samples from 30 patients hospitalized at the Amiens University Hospital for a COVID-19 (see Supplementary Table 1) to monitor the kinetics of detection of anti-S1, anti-S2, anti-RBD and anti-N antibodies with in-house ELISAs. We observed that antibodies targeting the N protein and the RBD were the earliest to be detected (Fig. 1a). Thirteen days post-symptom onset, 100% of patients had detectable antibodies to both proteins. A similar profile was observed for anti-S2 antibodies but with a mean time lag of two days. Antibodies to the S1 subunit were the last to be detected and remained undetectable for two patients. High levels of anti-N and anti-RBD antibodies were detected in the large majority of samples obtained fourteen days post-symptom onset whereas very heterogeneous levels of anti-S1 antibodies were found in the same samples (Fig. 1b). The positive correlations between each ELISA are shown in Extended Data Fig. 1. Significant differences were observed between intensive care versus non-intensive care patients for anti-S1, anti-S2 and anti-N antibody levels, from eight days post-symptom onset (Extended Data Fig. 2a). A slight difference was observed for anti-N antibody levels according to the sex, from fourteen days post-symptom onset (Extended Data Fig. 2b). In contrast, no significant difference was observed according to age, from fourteen days post-symptom onset (Extended Data Fig. 2c).
We also monitored the presence of NAbs in all plasma samples using retroviral particles pseudotyped with the S glycoprotein of the SARS-CoV-2 (SARS-CoV-2pp). The results obtained for each patient are presented in Extended Data Fig. 3. One sample of each patient was also used to perform dose-response curves with particles pseudotyped with the G glycoprotein of the Vesicular Stomatitis Virus and no inhibition was observed, demonstrating that the neutralization observed with the COVID-19 patient plasmas was specific to the SARS-CoV-2 (Extended Data Fig. 4a).

Furthermore, plasmas from twelve patients that had previously been infected with other coronaviruses (OC43 (n=5), 229E (n=4), NL63 (n=2) or HKU1 (n=1)) did not have any effect on SARS-CoV-2 pseudotype infectivity (Extended Data Fig. 4b and Supplementary Table 2). As expected, our results demonstrate that the production of NAbs correlates with the production of antibodies targeting the S1, S2 and RBD domains and we detected NAbs in all COVID-19 patients fifteen days post-symptom onset (Fig. 1a). The NAb titers increase from one week post-symptom onset and reaches a plateau one week after (Fig. 2a, 2b and Extended Data Fig. 3). However, the NAb titers reached were variable between patients, 17% generated low levels of NAbs (40 ≤ titers < 160), 73% intermediate levels (160 ≤ titers < 1280) and 10% high levels (1280 ≤ titers) (Fig. 2a). Positive correlations between NAb titers and anti-S1, anti-S2, anti-N or anti-RBD antibody levels, as well as white blood cells and lymphocytes counts are shown in Extended Data Fig. 5. Significantly higher NAb titers were observed in patients with severe forms (p=0.04) and in women (p=0.03) from 14 days post-symptoms onset (Extended Data Fig. 6). In contrast, no significant difference was observed according to the age. We also had the opportunity to monitor the presence of NAbs in late samples of twelve patients (≥40 days post-symptom onset) and we observed that the NAb titer dropped to low or undetectable level in most of these samples (Fig. 2b and Extended data Fig. 3).

Finally, we monitored the presence of NAbs in plasma samples from 25 asymptomatic carriers. It is important to note that we could not establish when these patients had been infected since they were asymptomatic but it probably occurred more than one week before sampling since they were confirmed seropositive using commercial serological assays and our in-house ELISAs (Supplementary...
Table 3). As shown in Fig. 2c and Extended Data Fig. 7, NAbs were below the detection limit of our assay in the majority of these plasma samples (56%, 14/25). Low NAb levels (40 ≤ titers < 160) were found in 28% of these patients (7/25). Three patients had intermediate NAb levels (160 ≤ titers < 1280) and only one showed a high NAb titer (≥1280).

Commercial serological assays that are complementary to direct viral detection of the SARS-CoV-2 by RT-PCR have recently become available but they need to be finely evaluated. With our four in-house ELISAs, we showed that the detection of the RBD and the N protein may be more suitable since it was highly or slightly more sensitive than the detection of S1 or S2, respectively. We only tested IgG detection since recent data showed that anti-SARS-CoV-2 IgG levels increase at the same time or earlier than IgM levels⁴. We also report that COVID-19 patients generate variable levels of NAbs that are likely to drop a few weeks after infection and that most of asymptomatic carriers do not generate NAbs. Thus, our results raise questions concerning the role played by NAbs in COVID-19 cure and protection against secondary infection. They also imply that anti-SARS-CoV-2 NAbs should be titrated to optimize convalescent plasma therapy. Finally, they suggest that induction of NAbs is not the only strategy to adopt for the development of a vaccine.
Methods

Study population and samples. Thirty patients diagnosed SARS-CoV-2 positive by RT-PCR on a nasopharyngeal swab sample, between 25 February and 23 March 2020, were enrolled in the study. The general information was extracted from electronic medical records and the clinical characteristics of the 30 patients are described in Supplementary Table 1. Samples from patients diagnosed positive for the human coronaviruses NL63, 229E, HKU1 and OC43 were also tested (Supplementary Table 2). Finally, we also used samples from 25 asymptomatic carriers (Supplementary Table 3) that were diagnosed positive using commercial serological tests (LIAISON® SARS-CoV-2 IgG from DiaSorin and/or ELISA SARS-CoV-2 (IgG) from EUROIMMUN). This study was approved by the institutional review board of Amiens University Hospital (number PI2020_843_0046, 21 April 2020).

In-house ELISAs. All plasmas were decomplemented at 56°C for 30 min. MaxiSorp Nunc-immuno 96-well plate were coated with a 1 µg/mL solution of SARS-CoV-2 S1, S2, RBD or N antigen (The Native Antigen Company, United Kingdom), overnight at 4°C. Wells were blocked with 1% fetal bovine serum for 1 hour at 37°C. Then 100 µL of diluted plasmas (1:100 for S1, S2, RBD and 1:200 for N) were added and incubated for 1 h at 37°C. After washing 4 times, plates were incubated with peroxidase conjugated mouse anti-human IgG (Southern Biotech, 1/6000). After 4 washes, 100 µL of o-phenylenediamine peroxidase substrate was added at room temperature in the dark. After 15 minutes, the reaction was stopped with H₂SO₄ solution. The optical density was measured at 490 nm. All samples were run in triplicate. To establish the specificity of each assay, 40 pre-pandemic sera from 2019 were tested. Each cut-off values were defined as the means plus 3 standard deviations obtained with these samples.

Neutralization assay. SARS-CoV-2pp were produced as described previously³ with a plasmid encoding a human codon-optimized sequence of the SARS-CoV-2 spike glycoprotein (accession
number: MN908947). Supernatants containing the pseudotyped particles were harvested at 48, 72
and 96 h after transfection, pooled and filtered through 0.45-μm pore-sized membranes. All plasmas
were decomplemented at 56°C for 30 min. Neutralization assays were performed by preincubating
SARS-CoV-2pp and diluted plasma for 1 h at room temperature before contact with Vero cells
(ATCC® CCL-81™) that were transiently transfected with the plasmids pcDNA3.1-hACE2 and
pcDNA3.1-TMPRSS2 48 h before inoculation. Luciferase activities were measured 72 h post-infection,
as indicated by the manufacturer (Promega). The NAbs titers were defined as the highest dilution of
plasma which resulted in a 90% decrease of the infectivity. Retroviral particles pseudotyped with the G
glycoprotein of the Vesicular Stomatitis Virus (VSVpp) were used to control the specificity of the
neutralization.

Statistical analysis. Quantitative variables were expressed as the median and compared using
Student's t-test. The Pearson correlation coefficient was used to measure the strength of a linear
association between two quantitative variables. Statistical analyses were performed using GraphPad
Prism 5. A two-sided P-value < 0.05 was considered statistically significant.
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Author Contributions

EB, GD, CF, SC and FH designed the research and analyzed the data; JLS collected clinical samples; BD extracted clinical information from electronic medical records; AT, SB and JD provided key reagents; EB and FH performed the experiments; EB and FH wrote the manuscript.

Competing Interests statement

The authors declare no competing interests.
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### Supplementary Table 1. Characteristics of the 30 hospitalized patients included in the study

| Patient characteristics | Mild Disease (n=12) | Severe disease (n=18) |
|-------------------------|---------------------|----------------------|
| Female                  | 6 (50%)             | 6 (33%)              |
| Male                    | 6 (50%)             | 12 (66%)             |
| Median Age (Years)      | 77 (59-87)          | 63 (38-78)           |
| Chronic comorbidities   |                     |                      |
| Hypertension            | 7                   | 9                    |
| Chronic heart disease   | 3                   | 3                    |
| Chronic lung disease    | 3                   | 0                    |
| Chronic kidney disease  | 2                   | 0                    |
| Diabetes                | 3                   | 4                    |
| Hyperlipidemia          | 2                   | 1                    |

### Supplementary Table 2. Patients previously infected with endemic coronaviruses used as control in the study

| Patient | Coronavirus strain | Days post-diagnostic | Detection of IgG in our in house ELISA assays | SARS-CoV-2 NAb titer |
|---------|--------------------|----------------------|-----------------------------------------------|---------------------|
| C1      | OC43               | 174                  | -     -    +/   +/    -     -    <40 |
| C2      | 229E               | 39                   | -     -     -     -     -     -    <40 |
| C3      | NL63               | 561                  | -     -     -     -     -     -    <40 |
| C4      | OC43               | 170                  | -     -     -     -     -     -    <40 |
| C5      | OC43               | 176                  | -     -     -     -     -     -    <40 |
| C6      | NL63               | 16                   | -     -     -     -     -     -    <40 |
| C7      | 229E               | 14                   | -     -     +    +/-    +/-    +/   <40 |
| C8      | HKU1               | 24                   | -     -     -     -     -     -    <40 |
| C9      | OC43               | 301                  | +/-   -     -     -     -     -    <40 |
| C10     | 229E               | 56                   | -     -     -     -     -     -    <40 |
| C11     | 229E               | 761                  | -     -     -     -     -     -    <40 |
| C12     | OC43               | 86                   | -     -     -     -     -     -    <40 |

+, positive; -, negative; +/-, equivocal
### Supplementary Table 3. SARS-CoV-2 asymptomatic carriers included in the study

| Patient | Detection of IgG in in house ELISAs | Diasorin assay | Euroimmun assay | SARS-CoV-2 NAb titer |
|---------|-----------------------------------|----------------|-----------------|---------------------|
| AC1     | +                                 | +              | +/-             | 40                  |
| AC2     | +                                 | +/-            | +               | <40                 |
| AC3     | +                                 | +              | +               | 40                  |
| AC4     | +/-                               | +              | -               | <40                 |
| AC5     | -                                 | +              | -               | <40                 |
| AC6     | +/-                               | +              | +               | <40                 |
| AC7     | +                                 | +              | +               | 160                 |
| AC8     | +                                 | +              | +               | 640                 |
| AC9     | +                                 | +              | +               | <40                 |
| AC10    | +                                 | +              | +               | 80                  |
| AC11    | +                                 | +              | -               | <40                 |
| AC12    | -                                 | +              | +               | <40                 |
| AC13    | +                                 | +/-            | +               | <40                 |
| AC14    | +                                 | +              | +               | 480                 |
| AC15    | +                                 | +              | +/-             | <40                 |
| AC16    | +                                 | +              | +               | 120                 |
| AC17    | +                                 | +              | +               | 80                  |
| AC18    | +                                 | +              | +               | 5120                |
| AC19    | +                                 | +              | +               | <40                 |
| AC20    | +                                 | +              | +               | <40                 |
| AC21    | +                                 | +              | +               | 60                  |
| AC22    | +                                 | +              | +               | 60                  |
| AC23    | +                                 | +              | +               | <40                 |
| AC24    | +                                 | +              | +               | <40                 |
| AC25    | +                                 | +              | +               | <40                 |

+; positive; -; negative; +/-; equivocal
**Figure Legends**

**Fig. 1 | Antibody response in COVID-19 patients.**  
**a,** Kinetics of anti-S1, anti-S2, anti-RBD, anti-N and NAb detection in 30 COVID-19 patients after symptom onset.  
**b,** Evolution of the anti-S1, anti-S2, anti-RBD and anti-N antibody levels during the first month post-symptom onset.

**Fig. 2 | NAb response in COVID-19 patients and SARS-CoV-2 asymptomatic carriers.**  
**a,** Evolution of the NAb titer in 30 COVID-19 patients during the first month post-symptom onset.  
**b,** Evolution of the NAb titer in 12 COVID-19 patients after more than 40 days post-symptom onset.  
The dashed line indicates the cut-off of the assay.  
**c,** Determination of the NAb titer in plasma samples from 25 asymptomatic carriers.
Extended Data Fig. 1 | Correlations between anti-S1, anti-S2, anti-RBD and anti-N levels in in-house ELISAs.  

- a, anti-S1 versus anti-S2.  
- b, anti-S1 versus anti-RBD.  
- c, anti-S1 versus anti-N.  
- d, anti-S2 versus anti-RBD.  
- e, anti-S2 versus anti-N.  
- f, anti-RBD versus anti-N.  

Dashed lines indicate assay cut-offs for positivity. OD, optical density.

Extended Data Fig. 2 | Temporal profiles of anti-S1, anti-S2, anti-RBD and anti-N antibody levels.  

Patients samples were divided into three periods groups (day 0-7, day 8-14 and day >14).  

- a, The temporal profiles are presented according to the severity of the disease (SD, severe disease requiring intensive care ; MD, mild disease).  
- b, The temporal profiles are presented according to the sex (M, male ; F, female).  
- c, The temporal profiles are presented according to the age (< or > 60 years old).  

Dashed lines indicate assays cut-offs for positivity and lines indicate the median for each assay. OD, optical density. NS, not significant ; *, p<0.05 ; **, p<0.01.

Extended Data Fig. 3 | NAb response to SARS-CoV-2 in COVID-19 patients.  

SARS-CoV-2pp were preincubated with serially diluted plasma obtained from 30 COVID-19 patients (#1 to #30) at different days post-symptom onset (d2 to d58). Dose response curves represent the means of normalized infectivity (%) from two independent experiments performed in duplicate. Error bars have been omitted for clarity.

Extended Data Fig. 4 | Specificity of the neutralization assay.  

- a VSVpp were preincubated with serially diluted plasma obtained from 30 COVID-19 patients (#1 to #30). Dose response curves represent the means of normalized infectivity (%) from two independent experiments performed in duplicate.  
- b SARS-CoV-2pp were preincubated with serially diluted plasma obtained from 12 patients infected with 229E, NL63, HKU1 or OC43 coronaviruses (C1 to C12). Dose response curves represent the means of normalized infectivity (%) from two independent experiments performed in duplicate. Error bars have been omitted for clarity.
Extended Data Fig. 5 | Correlations between NAb titers and anti-S1, anti-S2, anti-RBD and anti-N antibody levels as well as white blood cells counts or lymphocyte counts. a, NAb titer versus anti-S1. b, NAb titer versus anti-S2. c, NAb titer versus anti-RBD. d, NAb titer versus anti-N. e, NAb titer versus white blood cells counts. f, NAb titer versus lymphocyte counts. Dashed lines indicate assay cut-offs for positivity. OD, optical density.

Extended Data Fig. 6 | Temporal profiles of NAb titers. Patients samples were divided into three periods groups (day 0-7, day 8-14 and day >14). a, The temporal profiles are presented according to the severity of the disease (SD, severe disease requiring intensive care; MD, mild disease). b, The temporal profiles are presented according to the sex (M, male; F, female). c, The temporal profiles are presented according to the age (< or > 60 years old). Dashed lines indicate assays cut-offs for positivity and lines indicate the median for each assay. NS, not significant; *, p<0.05; **, p<0.01.

Extended Data Fig. 7 | SARS-CoV-2 NAbs in asymptomatic carrier samples. SARS-CoV-2pp were preincubated with serially diluted plasma obtained from 25 asymptomatic carriers that were confirmed with commercial serological assays (AC1 to AC25). Dose response curves represent the means of normalized infectivity (%) from two independent experiments performed in duplicate. Error bars have been omitted for clarity.
Fig. 1
Brochot et al.

Fig. 2

a) NAb titer in hospitalized patients (n=30)

b) NAb titer in hospitalized patients

C) NAb titer in asymptomatic carriers (n=25)
Extended Data Fig. 1
Extended Data Fig. 2a
Extended Data Fig. 2b
Extended Data Fig. 2c
Extended Data Fig. 3
Extended Data Fig. 4
Extended Data Fig. 5
Extended Data Fig. 6
Extended Data Fig. 7