Qualitative Changes in the SARS-CoV-2 Antibody Response in the Post-Infection Phase Impact the estimates of infections in Population-Based Seroprevalence Studies

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Abstract

In the present study, we have determined SARS-CoV-2-specific antibody responses in a cohort of 96 individuals during the acute phase of infection and in a cohort of 578 individuals enrolled in a seroprevalence population study in Switzerland including three groups, i.e. subjects with previous RT-PCR confirmed SARS-CoV-2 infections (n=90), ‘positive patient contact’ (n=177) and ‘random selected subjects’ (n=311). Six serological assays detecting predominantly IgG antibodies targeting either the Spike (S) and/or the nucleocapsid (N) proteins were used including also a Luminex based assay using an S protein in its native trimeric form. Antibody responses against the S and/or the N proteins were equally sensitive in the acute infection phase although differences in sensitivity (range 83 to 97% 16-33 days post-initial symptoms) were observed between the different assays and the Luminex S protein trimer assay was the most sensitive. Interestingly, antibody responses against the N protein appear to wane in the post-infection phase of the infection while those against the S protein persist over time, as indicated by the drop in sensitivity of the assays targeting the N protein (sensitivity range 71-77%). Assays detecting anti-N IgG antibodies may substantially underestimate the proportion of SARS-CoV-2 infections in the groups of ‘patient positive contacts’, i.e. 10.9 to 32.2% reduction (P<0.05-<0.0001) and in the ‘random selected’ general population, i.e. up to 45% reduction (P<0.05). The overall reduction in seroprevalence for the total cohort ranged from 9.4 to 31% (P<0.0009-<0.0001). Of note, the assay using the S protein in its native trimeric form was more sensitive as compared to those using monomeric S proteins.

These results indicate that assays targeting the S protein, ideally the trimeric form, should be implemented as reference test to estimate SARS-CoV-2 infections in seroprevalence population studies.
Introduction

The SARS-CoV-2 is currently causing a devastating pandemic with more than 12.7 million documented infections and more than 566’000 deaths, according to the latest WHO situation report from July 13th, 2020. However, the true incidence of the infection is largely underestimated, since in most countries asymptomatic and paucisymptomatic people are tested only if they came in direct contact with sick patients or belong to at-risk subgroups. Therefore, it is a public health urgency to perform large-scale population-based studies in order to determine rates of seroprevalence during the first wave of the SARS-CoV-2 pandemic and to implement continued surveillance with the combined used of viral detection tests such as RT-PCR and serological testing. Seroprevalence studies are also instrumental to determine the proportion of individuals with potential protective immunity.

Most commercially available SARS-CoV-2 serology assays detect IgG and some also IgA, IgM and/or all three immunoglobulin (Ig) subtypes. Detecting both IgA and IgG may increase sensitivity, particularly for people experiencing paucisymptomatic or asymptomatic infection. However, IgM does not seem to be of great benefit to overall sensitivity, since IgM appearance coincides with IgG antibodies during the early phase of infection, i.e. less than 15 days after the onset of symptoms and may increase the likelihood of false positive results due to cross-reactivity.

One of two proteins serve as baits in the majority of serological assays: the nucleocapsid protein (N) and the Spike protein (S). It has been suggested that the serological assays targeting the S protein are more specific while those targeting at N may be more sensitive, particularly in the early phase of infection.

A comparison of 17 different serological assays using sera from hospitalized patients with moderate to severe COVID-19 disease confirmed the overall higher sensitivity of assays targeting the N protein investigating. In this study, the DiaSorin CLIA that targets the S protein only exhibited 92% sensitivity whereas the Epitope Diagnostics ELISA and the Snibe assay that both target the N protein exhibited a sensitivity of 96%. However, this increased
sensitivity of N-based assays might be at the expense of specificity, given the relatively high protein sequence similarity of the N protein of SARS-CoV-2 with nucleocapsid proteins of other Coronaviridae and other viruses. Moreover, during the SARS outbreak (2002-2004), Chia et al observed that anti-N antibodies waned earlier than anti-S antibodies\(^9\). Thus, assays targeting the S proteins might be more specific and circumvent a possible decrease of antibodies, previously observed with N protein of the SARS virus. Thus, assays targeting the S protein might be more specific and circumvent a possible decrease in antibody titers, as the one previously observed after SARS. Furthermore, it is still unknown the durability of SARS-CoV-2 antibody response. Previous studies have shown early disappearance of antibodies to SARS-associated coronavirus after recovery\(^10\) while other studies have shown longer durability of the antibody response\(^11-14\).

In a recent study, the only test scoring negative on 404 sera taken before the pandemics (in 2018 & 2019) was the DiaSorin test, which targets the S protein; however, this high specificity was at the expense of a lower sensitivity (92%). We hypothesized that the lower sensitivity of most S-based assays reported so far might be explained due to their use of a monomeric form of S moieties, including the S1 or RBD domains, and that using S in its native trimeric conformation might increase sensitivity while preserving specificity.

We thus developed an IgA and IgG serological assay using the trimeric form of the S protein and the Luminex technology. This new assay was first evaluated using 96 sera obtained during the first 33 days of the acute phase of infection from subjects with moderate to severe symptoms, and further validated on 578 sera obtained in the post-infection phase mostly paucisymptomatic participants enrolled in the population-based seroprevalence study of the Vaud Canton in Switzerland. All sera were tested in parallel with five commercial assays targeting either the N and/or the S proteins. The Luminex assay exhibited the best accuracy with very high sensitivity in sera from both the acute and post-infection settings. In contrast, the tests targeting the N protein were less sensitive in detection seropositive donors in the post-infection phase.
The newly developed Luminex assay had the highest sensitivity in the sera of the post-
infection phase thus providing more accurate estimates of SARS-CoV-2 infections in the
general population.

Taken together, these results indicate that S protein based assays should be used in
population-based seroprevalence studies and that the trimeric form is associated with greater
sensitivity than the monomeric form.
Results

Novel Serological Binding Assay using the S protein trimer

A stabilized trimer of the full-length S protein, encompassing both its S1 and S2 moieties, was coupled to beads for capturing antibodies in a new Luminex assay. We hypothesized that conformational epitopes would be preserved in the trimeric S protein, providing a greater sensitivity to detect IgG antibodies (Supplementary Figure 1A and B). First, the specificity for IgG antibody binding was established with sera from 256 pre-COVID-19 pandemic healthy adults from 18 to 81 years of age and an additional set of 108 patients (Figure 1A), which included: pregnant women, individuals infected with alphacoronaviruses (NL63 and 229E), betacoronaviruses (OC43 and HKU1), HIV, Rubella, HSV1, HSV2, RSV, CMV, EBV, influenza or varicella, as well as patients suffering from autoimmune diseases such as Lupus. The signal distribution for all SARS-CoV-2 negative sera was similar for the 256 pre-COVID-19 healthy adults and for the diverse panel of 108 subjects. A cut-off for positivity was set at 4-fold above a negative control standard, which is slightly more than four standard deviation above the mean of all negative control samples (mean MFI ratio 0.84 + 4×0.75 SD). Using this threshold, only one sera of the 256 pre-COVID-19 people and two patients with acute HIV or CMV viral infections gave a positive signal (Figure 1A). As such, the Luminex assay using the stable trimeric S protein gave a high overall specificity of 99.2% and no cross-reactive antibodies were detected in sera from people infected with pre-pandemic coronaviruses or from patients with autoimmune diseases that can produce polyreactive antibodies. Considering that two of the three false positives from the 364 SARS-CoV-2 negative donors had MFI signals less than 6 (Figure 1A), an additional criteria for positivity was established for large general population screens, including the post-infection cohort. Here, sera with signal intensities between 4 and 6 were defined as being at the limit of positivity, which increases the assay sensitivity to 99.7% with only one acute HIV infected subject having a 6.8 MFI signal.

The sensitivity of the assay was next evaluated using sera from 96 acutely infected SARS-CoV-2 PCR-positive patients with blood sampling at 0-5 days, 6-10 days, 11-15 days and 16-33 days post-onset of symptoms (POS). As anticipated, sera collected during the early stage of the infection (0-5 days POS) had low or undetectable levels of anti-S protein IgG antibodies, with a rate of positivity of 12.5% (1 in 8 subjects; Figure 1B). Seropositivity increased to 42.1%
(8/19) at 6-10 days POS and to 91.7% (33/36) at 11-15 days POS. Almost all patients with symptoms for 16-33 days (28/29; 96.6%) displayed high antibody titers for the S protein. A mean MFI signal ratio of 85 was obtained with the Luminex assay with sera collected from these patients 16-33 days POS, which was significantly higher than the signal obtained with sera from all other time categories ($P =0.05$ relative to 11-15 days and $P < 0.0001$ relative to 0-5 days and 6-10 days POS; Mann-Whitney test). Interestingly, the only subject that was negative in the S protein trimer assay at day 25 post-onset of symptoms became seropositive when re-tested seven days later. Since signal intensity often comes at the expense of specificity, it is all the more important that the Luminex assay using the S protein trimer provides high MFI signals, high detection of PCR-positive donors yet exceptional specificity with negative results with sera from pre-COVID-19 healthy donor and patients hospitalized prior to November 2019.

Quantitative Luminex assay for anti-Spike IgG antibodies

Apart from the identification of seropositive subjects, the quantitation of the anti-SARS-CoV-2 antibodies provides valuable insight into the extent of the humoral immune response and its persistence over time. Using the S protein trimer Luminex assays, we generated a standard binding curve with the commercially available CR3022 anti-S1 IgG1 antibody (Figure 1C). This clone was initially identified as a neutralizing antibody for SARS-CoV-1, but was subsequently shown to bind specifically to the SARS-CoV-2 Spike protein. Evaluation of serum dilutions from 1/300 down to 1/24,000 allowed to precisely determine the anti S protein IgG antibody concentrations that ranged from 54 µg/ml to 820 µg/ml in a panel of sera tested from patients with acute infection (Figure 1D). All the commercially available assays are semi-quantitative and thus the Luminex based S trimer assay is the only one providing an accurate quantification of the magnitude of the SARS-CoV-2 antibody response.

Specificity and sensitivity of IgA antibody binding in the Luminex S protein trimer assay

We next evaluated the Luminex S protein trimer assay for the detection of anti-SARS-CoV-2 IgA antibodies. We established assay specificity and a cut-off threshold for positivity by screening sera from pre-COVID-19 healthy adults. Using four standard deviations above the standard negative control, this assay provided a 98.5% specificity in the 256 sera tested. The sensitivity of the Luminex IgA assay was estimated on 81 out of 96 acute infected SARS-CoV-
211 patients’ sera with positive detections ranging from 33.3% of patients at 0-5 days POS with
212 seropositivity increasing to 68.8% in patients from the 6-10 days group. At 11-15 and 16-33
days POS, IgAs were detected in 94.4% and 90% of the cases, respectively (Figure 2).

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Comparison of Immunoassays targeting the S and N SARS-CoV-2 proteins during the acute
216 phase of infection
The new Luminex S protein trimer IgG assay was compared with five commercially available
218 SARS-CoV-2 immunoassays: i) two ELISAs from Euroimmun and Epitope Diagnostics detecting
219 IgG against the S1 and N proteins, respectively, ii) two CLIA from Diasorin and Snibe detecting
220 IgG against S and S+N proteins, respectively, and iii) a pan-Ig ECLIA from Roche targeting the
221 N protein. Since the commercial assays provide semi-quantitative estimates of the antibody
222 response, semi-quantitative estimates generated from the Luminex assay were used for the
223 comparison analyses. We performed the comparison on the same set of 96 sera from patients
224 with acute infection and stratified based on time between symptoms onset and sera
225 collection as shown in Figures 1B. Small differences in the number of sera tested across assays
226 is due to insufficient volume of some samples. We evaluated the specificity of the six tests on
227 a common panel of 65 pre-COVID-19 pandemic sera sampled before November 2019.
228 An increased in sensitivity was observed over time post-symptoms with all six assays (Figure
229 3 A-B, Table 1). After 10 days POS, all assays but the Diasorin SARS-CoV-2 IgG kit (57%)
230 reached a sensitivity above 70 %. Three assays, including the Luminex S protein trimer IgG
231 assay, reached a sensitivity greater than 90% after 15 days POS. The three other assays
232 exhibited a sensitivity slightly lower than 90% at 16-33 days POS, i.e. the Epitope diagnostic
233 ELISA IgG test (89%), the Euroimmun ELISA IgG test (88%), and the Diasorin SARS-CoV-2 IgG
234 CLIA kit (83%) (Figure 3 B and C; Table 1). All six tests had a specificity equal or above 97%
235 (Figure 3 C) and none displayed cross-reactivity with sera from patients positive for 229E,
236 OC43, HKU1, NL63 coronaviruses (Figure 1A).

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Taken together, these results indicate that the sensitivity of serological assays targeting the S
239 and/or the N proteins are similar on sera taken during the acute phase of infection.
240 Of note, we have also performed head-to-head comparison of trimeric versus monomeric S
241 proteins IgG responses within the Luminex assay. The responses observed in the Luminex
242 assay with the S monomeric proteins were similar in sensitivity to those detected in other
assays using monomeric proteins but inferior to those obtained with the trimeric S protein (Supplemental Figure 2A and B).

We also compared the Luminex S protein trimer IgG and IgA assay with the Euroimmun ELISA IgG and IgA test. We evaluated these four tests using 81 sera from the acute phase patients. We observed that the two IgA tests detected anti-SARS-CoV-2 antibodies earlier than the IgG assays. Between 0 and 5 days POS, the Luminex IgA and Euroimmun IgA tests showed a sensitivity of 33% and 22%, respectively, and the corresponding IgG tests had sensitivities of only 11% (Table 2). Between 6 and 10 days POS, the Luminex IgA and Euroimmun IgA tests showed a sensitivity of 69% and 63%, respectively, compared to the Luminex IgG test with a sensitivity of 44% and Euroimmun IgG of 31%. Comparing IgA and IgG positivity for 0 to 10 days POS showed a clear trend with anti-S protein IgA antibodies detected earlier than IgG during the acute phase of infection ($P=0.077$ for Luminex and Euroimmun tests independently that increases to $P=0.006$ with the artificial combining of results from the two assay; McNemar test). Although both IgA antibody tests show this same trend, it appeared that the Luminex S protein trimer IgA assay was systematically more sensitive (56%) than the Euroimmun ELISA IgA tests (48%) in all early-infected subjects up to 10 days POS (Table 2).

Comparison of Immunoassays targeting the S and N SARS-CoV-2 proteins during the post-infectious phase of infection and for seroprevalence studies

We next evaluated the performance of the six SARS-CoV-2 assays targeting the N and/or S proteins on 578 sera as part of a seroprevalence population study of the Vaud Canton in Switzerland, while being blind to the seropositivity status. These comparisons included 90 sera sampled from mildly to paucisymptomatic patients tested positive by RT-PCR, 177 sera sampled from positive contacts of RT-PCR positive subjects, and 311 sera sampled from undefined, randomly selected people from the general population aged 6 months and over. Results of the comparisons are shown in Figure 4, and Table 3. As expected, a good correlation of signal intensities was observed between the Luminex IgG assay using the S protein trimer and the other S protein targeting assays, while a poorer correlation was observed with anti-N assays (Figure 4).
In the 90 sera sampled from RT-PCR positive patients (Figure 4, blue dots), the Luminex IgG assay demonstrated the best sensitivity (96.7%), followed by the Roche pan-Ig ECLIA (95.6%). The two tests detecting anti-S antibodies, Diasorin and EuroImmun, both had significantly higher sensitivity (88.9%) than the Epitope Diagnostics ELISA anti-N (76.7%). Finally, the Snibe CLIA IgG test, which targets mainly the N protein and an undefined part of the S protein, showed a much lower sensitivity, i.e. 71.1% (Table 3).

In the sera of positive contact subjects (Figure 4, red dots), the Luminex IgG assay had the highest positivity rate (36.2%), again followed by the Roche pan-Ig ECLIA (32.2%). The EuroImmun and Diasorin gave similar positive rates of 29.9% and 28.2%, respectively while the Epitope Diagnostics and Snibe CLIA IgG tests exhibited the lowest positivity rate (26.0% and 24.3%, respectively) (Figure 4 and Table 3).

With regard to the sera from randomly selected subjects for the population based seroprevalence study (Figure 4, green dots; Table 3), we observed that tests detecting anti-S antibodies identified greater percentages of SARS-CoV-2 positive people (between 6.4 to 4.2%), than the tests targeting anti-N antibodies (4.5 to 3.5%). Importantly, the Roche pan-Ig assay using the N protein antigen was the second most sensitive assay in the acute infected patients and the PCR-positive and positive contact subjects in the post-infection cohort, but conversely, was one of the least sensitive assays (3.9%) in detecting seropositive people randomly selected from the general population. The significantly higher sensitivities of the Luminex S protein trimer assay in the post-infection setting compared to all five other serological assays is highlighted in Figure 5. Compared to the other tests, the Luminex IgG assay detected 10.9% to 32.8% more positive sera in the positive contact group, 30% to 45% more positive tests in the randomly selected population-based samples and 17.9% to 35.7% more positive tests in a combined analysis of the contact group and population-based samples. In the overall post-infection set of 578 sera, the Luminex IgG assay performed significantly better and detected between 9.4% and 31% more seropositive participants than the other five assays (Figure 5).

Taken together, these results indicate that assays detecting anti-N IgG antibodies may substantially (i.e. up to 45%) underestimate the proportion of SARS-CoV-2 exposed individuals.
compared to those detecting anti-S IgG antibodies in population-based seroprevalence studies.

**Discussion**

Population-based seroprevalence studies are important to monitor the dynamics of the pandemic, to have a better appreciation of the number of infections and to determine the proportion of the population that has developed specific SARS-CoV-2 immunity. Seroprevalence studies performed in Switzerland, Spain and in New York City indicate that a minor percentage of the population, ranging from 10 to 20% of individuals, has been infected with SARS-CoV-2. The estimates of SARS-CoV-2 infected individuals from seroprevalence studies may be substantially influenced by the sensitivity of the assays used to determine the antibody responses.

A large number of assays have been developed to detect IgG, IgA and IgM SARS-CoV-2 specific antibodies. These assays target either the S protein or the N protein. The validation of the assays with regard to the sensitivity and specificity has been mostly performed on sera from patients during the acute phase of infection. Despite some differences in sensitivity between the various assays, assays targeting the N and the S proteins were considered as equally sensitive, with a generally higher sensitivity for those targeting N. Either assays, targeting either N or S, were therefore considered as equally suitable for population-based seroprevalence studies. However, limited information is yet available on the evolution of the antibody response during progression from the acute to the post-infection phase and, in particular, on the antibody responses against the two targets, S and N proteins. For these reasons, we evaluated antibody responses using six different assays targeting the N and/or the S proteins.

Our results indicate a substantial drop in sensitivity of the three assays targeting the N protein thus strongly suggesting a waning of the anti-N antibody responses in the post-infection phase. Importantly, the estimated seroprevalences are mostly impacted in the ‘positive patient contacts’ group and ‘randomly selected’ population-based samples with an underestimation ranging from 11 to 33% for the former and 30 to 45% for the latter group. Of note, the new Luminex-based assay using the trimeric S protein showed a greater sensitivity than two assays using monomeric S1 or RBD. The underestimation of SARS-CoV-2
seropositive individuals was in the range of 18-22% in the ‘positive patient contacts’ and 30-35% in the population-based samples. The greater sensitivity of the Luminex assay using the trimeric S protein likely results from the conservation of conformational epitopes. This increased sensitivity was not obtained at the expense of cross-reactivity, since the specificity of the novel Luminex assay was 99%, in spite of including sera typically prone to cross-reactivity (i.e. from individuals with documented infection with other viruses, including coronaviruses or with lupus). Overall, the underestimation of SARS-CoV-2 seropositive individuals was less important in the ‘Positive RT-PCR patients’ group ranging from 1 to 26%.

A recent study has shown that 40% of asymptomatic individuals became seronegative over time. However, an assays detecting anti-N antibodies was used in this study. Based on our results demonstrating a waning of anti-N antibody response in the post-infection phase, it is likely that the loss of antibody response observed is due to the use of the anti-N assay rather than to a disappearance of the SARS-CoV-2 antibody response.

Additionally, the Luminex S protein trimer assay provides quantitative levels of anti-S protein IgG antibodies in donor sera, which may correlate with an effective neutralizing activity and the duration of a protective humoral response. Given that N protein antibodies are not neutralizing, this same correlation with N protein assays would be indirect at best and potentially misleading given our demonstration that antibody responses to N wane in post-infection subjects.

In addition to seroprevalence population studies, the use of a highly sensitive test is also important for patient care and diagnostic tests should exhibit high performance also during the post-infection period. In this regard, a number of late complications have been reported following SARS-CoV-2 infection, including Guillain-Barré syndrome, Kawasaki syndrome, hair losses, thrombo-embolic events and all these late events are recognized indications for serological testing. Highly sensitive tests are also warranted for immunocompromised subjects suffering from hematological malignancies (lymphoma or leukemia) who may fail to develop any antibody response up to 38 days post-infection despite severe disease requiring prolonged intubation and artificial ventilation in the intensive-care unit.

Furthermore, the availability of highly sensitive serological assay quantifying anti-S protein IgG antibodies together with assays measuring neutralizing antibodies are critical for the development of effective vaccines against SARS-CoV-2.
In conclusion, these results provide new insights in the evolution of the SARS-CoV-2 antibody response from the acute to the post-infection phase and indicate that the detection of antibody responses against the S protein and the use of trimeric S protein should be implemented as reference test to avoid large underestimation of SARS-CoV-2 infections in population-based seroprevalence studies. Therefore, the detection of antibody responses against the S protein and the use of trimeric S protein as antigenic target should be implemented as reference test to provide the best estimates of SARS-CoV-2 infections in seroprevalence population studies.
Material and Methods

Study populations

Patients with acute infections

Comparison of tests for acute/sub-acute phase of the infection was performed on 161 sera, including i) 96 sera, expected to be positive, sampled from hospitalized patients with severe to mild symptoms 0 to 45 days post onset of the symptoms and documented with a positive SARS-CoV-2 RT-PCR; ii) 65 sera, expected to be negative, sampled before November 2020, presented as pre-COVID-19 sera, and including 18 samples from patient documented positive for a Human coronavirus (E229, OC43, HKU1, NL63) RT-PCR. Date of the symptoms onset were extracted from the electronic record of the 96 SARS-CoV-2 RT-PCR positive patients.

Post-infection cohort

A second comparison of tests was performed on sera from the seroprevalence study of the Vaud Canton in Switzerland (SerocoviD) performed by the Centre for Primary Care and Public Health, University of Lausanne (Unisanté). Out of the 1,942 participants who provided a blood sample between May 4 and June 27, 2020, a subset of 578 subjects were included in the present analysis, of which: i) 90 subjects were expected to be positive—sampled from mostly mildly to paucisymptomatic patients (only 21% had been hospitalized) documented with a positive SARS-CoV-2 RT-PCR; ii) 177 were sampled from contacts of RT-PCR positive subjects, and iii) 311 were randomly selected subjects in the general population. There were 304 women (52.6%), and the mean age was 39.2 years (SD 24.2, range: 6 months to 90 years).

Pre-COVID-19 pandemic donors

Negative control serum samples from 256 adult healthy donors with ages ranging for 18 to 81 years of age were collected prior to November 2019 as part of the Swiss Immune Setpoint study sponsored by Swiss Vaccine Research Institute. Specificity tests for the Luminex S-protein assay with a diverse set of 108 patient sera included the 65 sera collected prior to November 2019 and used in the blinded tested performed with all six assays and an additional 43 patient samples. This diverse set of samples consisted of sera from pregnant women (n=14), pre-pandemic coronavirus infected donors (OC43, E229, NL63 and HKU1; n=19),
patients with infectious diseases (HIV, Rubella, HSV1, HSV2, CMV, EBV, influenza and varicella; n=57) and patients with autoimmune diseases including Lupus (n=18). Study design and use of subject sera samples were approved by the Institutional Review Board of the Lausanne University Hospital and the ‘Commission d’éthique du Canton de Vaud’ (CER-VD) stated that authorization was not required.

**Preparation of Luminex beads**

Luminex beads used for the serological binding assays were prepared by covalent coupling of SARS-CoV-2 proteins with MagPlex beads using the manufacture’s protocol with a Bio-Plex Amine Coupling Kit (Bio-Rad, France). Briefly, 1 ml of MagPlex-C Microspheres (Luminex) were washed with wash buffer and then resuspended in activation buffer containing a freshly prepared solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysulfosuccinimide (S-NHS), (ThermoFischer, USA). Activated beads were washed in PBS followed by the addition of 50 μg of protein antigen. The coupling reaction was performed at 4 °C overnight with bead agitation using a Hula-Mixer (ThermoFischer). Beads were then washed with PBS, resuspended in blocking buffer then incubated for 30 minutes with agitation at room temperature. Following a final PBS washing step, beads were resuspended in 1.5 ml of storage buffer and kept protected from light in an opaque tube at 4 °C. Each of the SARS-CoV-2 proteins was coupled with different coloured MagPlex beads so that tests could be performed with a single protein bead per well or in a multiplexed Luminex serological binding assay.

**SARS-CoV-2 proteins evaluated in Luminex assay**

The S protein trimer was designed to mimic the native trimeric conformation of the protein in vivo and the expression vector was kindly provided by Prof. Jason McLellan, University of Texas, Austin; 25. It encoded the prefusion ectodomain of the SARS-CoV-2 Spike with a C-terminal T4 foldon fusion domain to stabilize the trimer complex along with C-terminal 8x His and 2x Strep tags for affinity purification. The trimeric Spike protein was transiently expressed in suspension-adapted ExpiCHO cells (Thermo Fisher) in ProCHO5 medium (Lonza) at 5 x10⁶ cells/mL using PEI MAX (Polysciences) for DNA delivery. At 1 h post-transfection, dimethyl sulfoxide (DMSO; AppliChem) was added to 2% (v/v). Following a 7-day incubation with agitation at 31 °C and 4.5% CO2, the cell culture medium was harvested and clarified using a
0.22 µm filter. The conditioned medium was loaded onto Streptactin (IBA) and StrepTrap HP (Cytiva) columns in tandem, washed with PBS, and eluted with 10 mM desthiobiotin in PBS. The purity of S protein trimer was determined to be > 99% pure by SDS-PAGE analysis.

Receptor binding domain (RBD) and S1 SARS-CoV-2 proteins were prepared as previously described. In initial characterization of the assays, serum dilutions of 1/50 down to 1/ 2'700 were evaluated for SARS-CoV-2 PCR-positive subjects and healthy donors. A 1/300 dilution of serum was selected for screening patient samples since it showed a high MFI signal for all donors and a low background staining with serum samples from pre-COVID-19 pandemic healthy donors. In addition to the high positive signal and low background, <1 µl of serum was needed to evaluate anti-SARS-CoV-2 antibody binding in the Luminex assay binding assays.

**Luminex anti-SARS-CoV-2 antibody binding assay**

Luminex beads coupled with the Spike, RBD or S1 proteins were diluted 1/100 in PBS with 50 µl added to each well of a Bio-Plex Pro 96-well Flat Bottom Plates (Bio-Rad). Following bead washing with PBS on a magnetic plate washer (MAG2x program), 50 µl of individual serum samples diluted at 1/300 in PBS, were added to the plate wells. Along with samples, three replicates of a 1/300 negative control pool of pre-COVID-19 pandemic healthy human sera (BioWest human serum AB males; VWR) were evaluated on each 96-well plate. Plates were sealed with adhesive film, protected from light with a dark cover and agitated at 500 rpm for 60 minutes on a plate shaker. Beads were then washed on the magnetic plate washer and anti-human IgG-PE secondary antibody (OneLambda ThermoFisher) was added at a 1/100 dilution with 50µl per well. Plates were agitated for 45 minutes, and then washed on the magnetic plate washer. Beads resuspended in 80 µl of reading buffer were agitated 5 minutes at 700 rpm on the plate shaker then read directly on a Luminex FLEXMAP 3D plate reader (ThermoFisher). MFI signal for each test serum samples was divided by the mean signal for the negative control samples to yield an MFI ratio that normalized values between plates and between different Luminex instruments tested.

**Immunoassays**

ELISA and CLIA were performed according to the manufacturers’ instructions. EuroImmun and Epitope Diagnostic IgG ELISA were done manually for the essential of the protocol at the
exclusion of washing steps performed with a microplate washer (PW40, Bio-Rad, France).
Lecture of the Optical densities (OD) was done with a microplate reader (800 TSI, BioTek, USA). Each sample was measured in duplicates. The LIAISON® SARS-CoV-2 IgG kit was performed on a Liaison® XL (Diasorin, Italy), and the MAGLUMI™ 2019-nCoV IgG and IgM kits were performed on a MAGLUMI™ 800 (Snibe, China). The Elecsys anti-SARS-CoV-2 was performed on a COBAS 6000 (Roche, Switzerland).

**Statistical analyses**

The sensitivity of the different tests was calculated according to day post-symptoms on expected positive sera taken from patients with a positive RT-PCR. The RT-PCR was previously performed according to Corman et al. on our automated molecular diagnostic platform.

Sensitivity and specificity of the tests with 95% CI (Wilson/Brown method of GraphPad Prism 8.3.0) were calculated with Excel and GraphPad prism. For comparisons between the Luminex assay and the five other serological assays, $R^2$ values were calculated using the Pearson test and the McNemar’s test was used to determine the p-value significant differences for sensitivities in detecting seropositive subjects in the patient subsets within the post-infection cohort. All statistics were done with GraphPad prism.
Figure 1: Specificity and sensitivity of IgG antibody binding to the SARS-CoV-2 S protein trimer in a Luminex binding assay. Luminex beads covalently coupled with SARS-CoV-2 S protein trimer were used to monitor IgG binding in assays performed with pre-COVID-19 pandemic negative control sera and sera from SARS-CoV-2 PCR positive donors. MFI signals for serum antibody binding were expressed as a ratio compared to a negative control pool of pre-COVID-19 pandemic human serum tested in parallel. **A)** Assay specificity was evaluated using the sera from pre-COVID-19 pandemic healthy adults (n=256; ages ranging between 18 to 81 years of age), pregnant woman, pre-pandemic coronavirus infected donors (OC43, E229, NL63 and HKU1), patients with infectious diseases (HIV, Rubella, HSV1, CMV, EBV, influenza and varicella) and patients with autoimmune diseases including Lupus. **B)** The sensitivity of the S protein trimer Luminex assays was evaluated with sera from acute infected SARS-CoV-2 PCR-positive donors at 0-5 days, 6-10 days, 11-15 days and 16-33 days post-onset of symptoms. The red dashed line in A and B corresponds to the 4.0 cut-off for positivity in the IgG Luminex assay that was established by using mean value + 4×SD of all pre-COVID-19 pandemic serum samples shown in A. **C)** Quantitation of anti-S protein IgG antibody levels was performed by generating a standard curve for binding using the CR3022 IgG1 antibody that binds SARS-CoV-2 S protein. **D)** Using multiple dilutions of sera from COVID-19 patients, MFI signals between 100 and 10’000 in the linear part of the standard curve were used to calculate the serum concentration of anti-S protein IgG antibodies.
Figure 2: Specificity and sensitivity of IgA antibody binding to the SARS-CoV-2 S protein trimer in a Luminex binding assay. The Luminex S protein trimer assay was used to monitor IgA binding of antibodies in sera from pre-COVID-19 pandemic negative control donors and sera from acute SARS-CoV-2 PCR positive donors. The Luminex assay exhibited high specificity of 98.5% against a cohort of negative control donors and was effective at detecting IgA antibodies specific for S protein in most subjects in both the early stage (0 to 10 days) and later stage (11 to 33 days) after onset of symptoms in acute PCR-positive patients. The red dashed line corresponds to 6.5-fold MFI signal over the internal negative control and was established by using the mean value + 4×SD of the 256 pre-COVID-19 pandemic adult serum samples.
Figure 3: Comparative analysis of six different SARS-CoV-2 serological assays in the profiling of sera from acutely infected patients. Sensitivity in detecting anti-SARS-CoV-2 specific IgG antibodies is compared for our new Luminex S protein trimer assay and five other commercial assay. A-B) Serum samples were grouped by the number of days after initial onset of symptoms with sensitivity increasing in all assays with increased days post-symptom onset. C) In the 16 to 33 days post-symptom group, the Luminex assay is among the most sensitive and specific assays.
Figure 4: Comparative analysis of Luminex S protein trimer assay versus five commercial serological assays. Signal intensities for the different subject sera in the post-infection cohort were compared between the Luminex and the five other serological assays. Collected sera were from patients with a documented positive SARS-CoV-2 RT-PCR (90 sera; blue dots), contacts with a SARS-CoV-2 RT-PCR positive patient (177 sera; red dots) and participants randomly selected from the general population (311 sera; green dots). Pearson correlation $R^2$ values are given for all 578 participants (black text) or for 183 Luminex positive sera (blue text).
Figure 5: The Luminex S protein trimer assay has significantly increased sensitivity relative to five other commercial assays in detecting seropositive subjects in the post-infection settings. Analysis shows the percentage seropositive subjects relative to the Luminex assay (top) and the percentage of reduced sensitivity relative to the Luminex assay (bottom) which had the best performance in all subsets of participants. Assays with blue bars used the S protein as their bait for binding serum antibodies while the red bars used the N protein. Statistical analysis was performed using the McNemar test for matched participant samples where $P<0.045$ (*); $P<0.0022$ (**); $P<0.0009$ (***)$; $P\leq0.0001$ (****).
### Table 1: Performance of the serological tests on sera collected during the acute infectious stage from hospitalized patients with moderate to severe symptoms

| Test             | Days post-symptoms | N° sera from patients RT-PCR pos | IgG⁺ Se | IgG⁺ Se 95% CI | N° sera pre-COVID | IgG⁺ Sp | IgG⁺ Sp 95% CI |
|------------------|--------------------|----------------------------------|--------|---------------|-------------------|--------|---------------|
|                  | Total              | Positive in serology             |        |               | Total             | Positive in serology |     |               |
| Luminex          |                    |                                  |        |               | 65                | 0      | 1             | 0.94 to 1.0 |
|                  | 0-1                | 94                               | 71     | 0.76          | 65                | 0      | 1             | 0.94 to 1.0 |
|                  | 6-10               | 19                               | 8      | 0.42          | 0.23 to 0.64      | 65    | 0.98          | 0.92 to 1.3  |
|                  | 11-15              | 36                               | 33     | 0.92          | 0.78 to 0.97      | 65    | 0.98          | 0.92 to 1.3  |
|                  | 16-33              | 29                               | 28     | 0.97          | 0.83 to 1.0       | 65    | 0.98          | 0.92 to 1.3  |
| ELISA Epitope Dx |                    |                                  |        |               | 65                | 0      | 1             | 0.94 to 1.0 |
|                  | 0-1                | 93                               | 68     | 0.73          | 0.63 to 0.81      | 65    | 0.98          | 0.92 to 1.3  |
|                  | 6-10               | 18                               | 8      | 0.44          | 0.25 to 0.66      | 65    | 0.98          | 0.92 to 1.3  |
|                  | 11-15              | 36                               | 32     | 0.89          | 0.75 to 0.96      | 65    | 0.98          | 0.92 to 1.3  |
|                  | 16-33              | 29                               | 26     | 0.89          | 0.74 to 0.96      | 65    | 0.98          | 0.92 to 1.3  |
| ELISA Euroimmun  |                    |                                  |        |               | 65                | 0      | 1             | 0.95 to 1.3  |
|                  | 0-1                | 91                               | 58     | 0.64          | 0.53 to 0.73      | 65    | 0.98          | 0.95 to 0.99 |
|                  | 6-10               | 18                               | 5      | 0.38          | 0.12 to 0.51      | 65    | 0.98          | 0.95 to 0.99 |
|                  | 11-15              | 34                               | 27     | 0.79          | 0.63 to 0.90      | 65    | 0.98          | 0.95 to 0.99 |
|                  | 16-33              | 29                               | 25     | 0.86          | 0.69 to 0.95      | 65    | 0.98          | 0.95 to 0.99 |
| CLIA Disorex     |                    |                                  |        |               | 65                | 0      | 1             | 0.95 to 1.3  |
|                  | 0-1                | 96                               | 49     | 0.51          | 0.41 to 0.61      | 65    | 0.98          | 0.95 to 1.3  |
|                  | 6-10               | 19                               | 4      | 0.21          | 0.085 to 0.43     | 65    | 0.98          | 0.95 to 1.3  |
|                  | 11-15              | 37                               | 27     | 0.73          | 0.57 to 0.85      | 65    | 0.98          | 0.95 to 1.3  |
|                  | 16-33              | 29                               | 24     | 0.83          | 0.65 to 0.92      | 65    | 0.98          | 0.95 to 1.3  |
| CLIA Sube        |                    |                                  |        |               | 65                | 0      | 1             | 0.95 to 1.3  |
|                  | 0-1                | 96                               | 60     | 0.63          | 0.53 to 0.72      | 65    | 0.98          | 0.95 to 1.3  |
|                  | 6-10               | 19                               | 6      | 0.32          | 0.12 to 0.54      | 65    | 0.98          | 0.95 to 1.3  |
|                  | 11-15              | 37                               | 27     | 0.73          | 0.57 to 0.85      | 65    | 0.98          | 0.95 to 1.3  |
|                  | 16-33              | 29                               | 24     | 0.90          | 0.74 to 0.96      | 65    | 0.98          | 0.95 to 1.3  |
| ECLIA Roche      |                    |                                  |        |               | 65                | 0      | 1             | 0.95 to 1.3  |
|                  | 0-1                | 96                               | 60     | 0.75          | 0.62 to 0.80      | 65    | 0.98          | 0.95 to 1.3  |
|                  | 6-10               | 19                               | 9      | 0.47          | 0.27 to 0.68      | 65    | 0.98          | 0.95 to 1.3  |
|                  | 11-15              | 37                               | 32     | 0.86          | 0.72 to 0.94      | 65    | 0.98          | 0.95 to 1.3  |
|                  | 16-33              | 29                               | 27     | 0.93          | 0.78 to 0.99      | 65    | 0.98          | 0.95 to 1.3  |

Se : sensitivity ; Sp : specificity ; * serum sampled before 1st November 2019 considered as “negative”; ** IgG, except for the ECLIA Roche, which corresponds to a pan-Ig assay.
Table 2. Performance of the IgA serological tests on 81 sera collected during the acute infective stage from hospitalized patients with moderate to severe symptoms

| Test              | Days post-symptoms | No sera from patients | Ig Se | Ig5 Se 95% CI |
|-------------------|--------------------|-----------------------|-------|---------------|
|                   |                    | RT-PCR pos            |       |               |
|                   |                    | Total | Positive in serology |       |               |
| Laminex IgG       | All                | 81     | 61 | 0.75 | 0.65 to 0.83 |
|                   | 0-5                | 9      | 1 | 0.11 | 0.006 to 0.44 |
|                   | 6-10               | 16     | 7 | 0.44 | 0.23 to 0.67 |
|                   | 11-15              | 36     | 33 | 0.92 | 0.78 to 0.97 |
|                   | 16-33              | 20     | 20 | 1 | 0.839 to 1 |
| Laminex IgA       | All                | 81     | 66 | 0.81 | 0.72 to 0.88 |
|                   | 0-5                | 9      | 3 | 0.33 | 0.12 to 0.65 |
|                   | 6-10               | 16     | 11 | 0.69 | 0.444 to 0.858 |
|                   | 11-15              | 36     | 34 | 0.94 | 0.819 to 0.990 |
|                   | 16-33              | 20     | 18 | 0.9 | 0.699 to 0.982 |
| ELISA EuOImmun IgG| All                | 81     | 49 | 0.6 | 0.5 to 0.7 |
|                   | 0-5                | 9      | 1 | 0.11 | 0.006 to 0.44 |
|                   | 6-10               | 16     | 5 | 0.31 | 0.14 to 0.56 |
|                   | 11-15              | 36     | 25 | 0.69 | 0.53 to 0.82 |
|                   | 16-33              | 20     | 18 | 0.9 | 0.7 to 0.98 |
| ELISA EuOImmun IgA| All                | 81     | 61 | 0.75 | 0.65 to 0.83 |
|                   | 0-5                | 9      | 2 | 0.22 | 0.039 to 0.55 |
|                   | 6-10               | 16     | 10 | 0.63 | 0.39 to 0.82 |
|                   | 11-15              | 36     | 31 | 0.86 | 0.71 to 0.94 |
|                   | 16-33              | 20     | 18 | 0.9 | 0.7 to 0.98 |

Se: sensitivity; Sp: specificity
Table 3: Percentage of positive/negative/limit results of the different tests on sera collected from patients with a documented positive SARS-CoV-2 RT-PCR (90 sera), contacts with a SARS-CoV-2 RT-PCR positive patient (177 sera) and subjects randomly selected from the general population (311 sera).

|                   | Luminex (anti-S trimer) | Diasorin (anti-S) | Enolmun (anti-S) |
|-------------------|-------------------------|-------------------|------------------|
|                   | Positive RT-PCR | Patient positive contacts | Subjects randomly selected | Positive RT-PCR | Patient positive contacts | Subjects randomly selected | Positive RT-PCR | Patient positive contacts | Subjects randomly selected |
|                   | %   | N   | %   | N   | %   | N   | %   | N   | %   | N   | %   | N   |
| Positive          | 96.7 | 87  | 36.2 | 64  | 6.4  | 20 |
| Negative          | 2.2  | 2   | 62.7 | 111 | 90.7 | 282 |
| Limit             | 1.1  | 1   | 1.1  | 2   | 2.9  | 9   |

|                   | Roche (anti-N) | Epitope Dx (anti-N) | Symbi (anti-S + N) |
|-------------------|----------------|---------------------|--------------------|
|                   | Positive RT-PCR | Patient positive contacts | Subjects randomly selected | Positive RT-PCR | Patient positive contacts | Subjects randomly selected | Positive RT-PCR | Patient positive contacts | Subjects randomly selected |
|                   | %   | N   | %   | N   | %   | N   | %   | N   | %   | N   | %   | N   | %   | N   | %   | N   | %   | N   |
| Positive          | 95.6 | 86  | 32.2 | 57  | 3.9  | 12  |
| Negative          | 4.4  | 4   | 67.8 | 120 | 96.1 | 299 |
| Limit             | 0.0  | 0   | 0.0  | 0   | 0.0  | 0   | 5.6  | 5   | 3.4  | 6   | 1.9  | 6   |

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Supplementary Figure 1: Structural representation of the SARS-CoV-2 S protein Trimer.  A) Space filled representation of the S protein with trimer subunits shown in blue, red and green. The labeled S2, S1 and RBD portions of each subunit are in light, mid and dark colors, respectively. Compared to the monomeric S1 protein, this image demonstrates that the native S protein trimer has significantly greater conformational for antibody binding with epitopes that are only present in the higher order structure. B) S protein trimer with the blue subunit represented as a ribbon structural. The 6VSB PDB structure was used to generate these images.
Supplementary Figure 2: Specificity and sensitivity of IgG antibody binding to the SARS-CoV-2 RBD and S1 domains in a Luminex binding assay. A) Specificity of the Luminex RBD and S1 protein monomer binding assays was assessed using the cohort of negative control sera described in Figure 1A. B) Sensitivity of the Luminex RBD and S1 protein monomer binding assays was determined with the 94 acute infected serum samples described in Figure 1B. The cut-off for positivity used in the RBD and S1 Luminex IgG assays are 3.2- and 2.8-fold over the negative control, respectively and were established by using mean value + 4×SD of each for the 364 pre-COVID-19 pandemic serum samples in A.

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Author contributions
A.C and A.T.C. organized the testing of serum samples in the five commercial serological assays and contributed to data analysis and manuscript preparation; C.A. and C.P. organized the testing of sera with the Luminex assay and solidified the testing protocol into a robust diagnostics assay; A.F. prepared all batches protein coupled Luminex beads and performed validation studies along with J.C.; D.T., F.P., D.H. and K.L. produced and purified the S protein timer; B.-J.B. provided the S1 and RBD monomeric proteins; V.D’A., S.G.N. and M.B. were in charge of the Vaud post-infection cohort and performed the initial unblinded analysis; C.F. conceived of and designed the Luminex assays, the quantitative analysis strategies and contributed to data analysis and manuscript writing. D.T., G.G. and G.P. conceived the study design for testing of sera from acute and post-infection subjects and wrote the manuscript.
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