Persisting Salivary IgG against SARS-CoV-2 at 9 Months After Mild COVID-19: A Complementary Approach to Population Surveys

Hassan Alkharaan¹#, Shaghayegh Bayati⁵#, Cecilia Hellström⁵, Soo Aleman²,⁴, Annika Olsson², Karin Lindahl²,⁴, Gordana Bogdanovic³, Katie Healy¹, Georgios Tsilingaridis¹, Patricia De Palma¹, Sophia Hober⁶, Anna Månberg⁶, Peter Nilsson⁶, Elisa Pin⁵#, Margaret Sällberg Chen¹#

¹Karolinska Institutet, Department of Dental Medicine, 14152, Stockholm, Sweden
²Karolinska University Hospital, Department of Infectious Diseases, 14186, Stockholm, Sweden
³Karolinska University Hospital, Department of Clinical Microbiology, 14186, Stockholm, Sweden
⁴Karolinska Institutet, Department of Medicine, 14186, Stockholm, Sweden
⁵Division of Affinity Proteomics, Department of Protein Science, School of Engineering Sciences in Chemistry, Biotechnology and Health (CBH), KTH Royal Institute of Technology, SciLifeLab, Stockholm, Sweden
⁶Division of Protein Technology, Department of Protein Science, KTH Royal Institute of Technology, Stockholm, Sweden

#These authors contributed equally
**Corresponding author:** Professor Margaret Sällberg Chen

**Footnote page**

**Conflict of interest statement:** NO

**Financial support statement:** This study was supported by grants from Region Stockholm, Knut and Alice Wallenberg foundation, Science for Life Laboratory (SciLifeLab), and the Erling-Persson family foundation (to SH).

**This study has not been presented at any conference or meeting**

**Corresponding author contact information** Professor Margaret Sällberg Chen, Karolinska Institutet, Sweden. Email: Margaret.Chen@ki.se

**Short summary (40 words)**

Mucosal defense plays an important role in COVID-19. Multiplex-based analysis of self-collected saliva demonstrates that broad-specific antibodies to Sars-Cov-2 persist up to nine months after recovery of mild COVID-19. Saliva is promising in monitoring and screening of Covid-19 immunity.
Abstract

**Background:** Declining humoral immunity in COVID-19 patients and the possibility of reinfection have raised concern. Mucosal immunity, particularly salivary antibodies, may be short-lived although long-term studies are lacking.

**Methods:** Using a multiplex bead-based array platform, we investigated antibodies specific to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) proteins in 256 saliva samples from convalescent patients 1-9 months after symptomatic COVID-19 (n=74, Cohort 1), undiagnosed individuals with self-reported questionnaires (n=147, Cohort 2), and individuals sampled pre-pandemic time (n=35, Cohort 3).

**Results:** Salivary IgG antibody responses in Cohort 1 (mainly mild COVID-19) were detectable up to nine months post-recovery, with high correlations between spike and nucleocapsid specificity. At nine months, IgG remained in both blood and saliva in majority of patients. Salivary IgA was rarely detected at this timepoint. In Cohort 2, salivary IgG and IgA responses were significantly associated with a recent history of COVID-19 like symptoms. Salivary IgG also tolerated temperature and detergent pre-treatments.

**Conclusions:** Unlike SARS-CoV-2 salivary IgA that appeared short-lived, the specific IgG in saliva appeared stable even after mild COVID-19 as noted for blood serology. This non-invasive saliva-based SARS-CoV-2 antibody test with home self-collection may therefore serve as a complementary alternative to conventional blood serology.

**Keywords:** COVID-19, saliva, antibody, serology, convalescence, immunoassay
**Introduction**

The novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) outbroke in an abrupt fashion after its initial identification in Wuhan, China, in late December 2019 (1), and obligated the World Health Organization to declare a global health emergency which escalated the concern to a pandemic situation in March 2020. As of March 2021, SARS-CoV-2 has caused over 114 million cases of coronavirus disease (COVID-19) and up to 2.5 million global deaths (2). The human adaptive immune system plays a key role in eliminating and memorizing pathogens by launching a cascade of activities that activate B and T lymphocytes. B lymphocytes produce antibodies that recognize and neutralize SARS-CoV-2 and protect against reinfection (3–5). IgG, IgA, and IgM antibodies are activated against SARS-CoV-2 and detected in the circulating blood of >90% of infected individuals from the 11-13 day post-symptom onset (PSO) (6–8). A recent study showed that circulating antibodies post-SARS-CoV-2 infection can persist for up to eight months (9), while other studies have shown that this immunological memory persists for a certain period followed by a slight decline, especially in asymptomatic infected individuals (10–14).

Oral and nasal cavities are considered the main gate for SARS-CoV-2 virus entry, and saliva secretory antibodies may be the first immunity arm that combat the infection through virus recognition. Salivary antibodies to SARS-CoV-2 can be detected early after symptom onset and persist for at least three months post-infection (8,10,12). Hence, saliva sampling could be a sensible and non-invasive way to indicate SARS-CoV-2 exposure. Similar to the previous SARS-CoV and MERS-CoV viruses, the spike protein (S) of SARS-CoV-2 recognizes the angiotensin-converting enzyme 2 (ACE2) receptor and uses it to enter host cells (15–17). Although antibodies play an important role in virus clearance (11,18), differential features of anti-SARS-CoV-2 antibodies negatively impacting disease severity, especially those related to complement deposition and systemic inflammation is also described (19). Understanding the dynamics and durability of antibody memory to SARS-CoV-2 is an instrumental step to manage the pandemic and may be useful in deploying
vaccination strategies. As the mucosal immunity is known to be short-lived, the durability of SARS-CoV-2 specific antibodies in saliva could be limited. Whether they permit detection 3-4 months after infection (8,10) is of great interest.

In this study, we exploited a highly sensitive and specific multiplex SARS-CoV-2 serology platform previously validated for seroprevalence studies (20) to investigate SARS-CoV-2 antibodies in saliva. Samples from individuals 1) with a diagnosis of mild COVID-19 in the convalescent phase; 2) 1-9 months after diagnosis of COVID-19, and 3) with or without a history of COVID-19 symptoms (undiagnosed), were analysed and compared to pre-pandemic samples. Our data indicates that spike-specific IgG reactivity is detectable in saliva in the vast majority of patients at 1-9 months post-infection. This result was similar to those detected by blood serology performed in a clinical diagnostic laboratory. The IgA reactivity on the other hand was short-lived in saliva, detectable only during the first three months. Moreover, IgG and IgA reactivity to both spike and nucleocapsid significantly correlated with a history of COVID-19 like symptoms in undiagnosed individuals.

Materials and methods

Experimental design

We applied a bead-based serology assay to detect IgG and IgA to SARS-CoV-2 proteins in saliva samples to evaluate its performance. The assay method is originally developed for detection of SARS-CoV-2 specific IgG in serum and plasma (20) where it showed 99.7% sensitivity and 100% specificity, and no cross-reaction when testing samples positive for other Coronaviruses. Salivary antibody responses to three different SARS-CoV-2 antigens (two spike and one nucleocapsid proteins) were first tested. The antigens’ performance in classifying positive and negative samples was evaluated for the single antigens as well as for antigens combined in panels. Best performing representations of spike and nucleocapsid were chosen in subsequent assessments.
Cohort design

The study was approved by the human ethical authority (dnr 2020-01702, 2020-06381) and complied with the declaration of Helsinki. All participants were recruited after signing informed consent forms. Saliva samples (total n=256) were collected and arranged in the following groups. Cohort 1: convalescence COVID-19 samples (n=74) of 72 patients (2 participants donated twice at six months apart) diagnosed with COVID-19 during March-April 2020, were collected from June to December 2020; Cohort 2: samples from undiagnosed individuals donated during May-Nov 2020 (n=147); Cohort 3: anonymous saliva samples taken before the COVID-19 outbreak (2018; pre-pandemic, n=35).

All convalescent patients (Cohort 1) had COVID-19 diagnosis confirmed by SARS-CoV-2 RT-PCR, except one patient who had positive SARS-CoV-2 antibodies at four time points in the convalescence phase. Seroconversion was tested by clinical SARS-CoV-2 blood serology assays (described below). The patients were recruited from department of Infectious Diseases, Karolinska University Hospital (n=65), and University Dental Clinic of Karolinska Institutet (n=7). Clinical demographic data of convalescent patients was compiled from medical journal records or questionnaire. Among 72 patients, 95.8% had mild COVID-19, without hospitalization due to COVID-19 symptoms. Three were admitted to hospital for the purpose of isolation, and three were admitted due to COVID-19 symptoms. In the latter group, two were hospitalized without any required oxygen treatment and one received a maximum of 1.5 L oxygen treatment during hospitalization, indicating no severe disease outcome. The time-points of serum and saliva samples collection were grouped according to time post-symptom onset (PSO), i.e. (i) PSO less than 3 months; (ii) PSO of 3-8 months; (iii) PSO of 9 months. Cohort 2 constituted of anonymous participants visiting the premises of University Dental Clinic of Karolinska Institutet or Eastman Institute, Stockholm during the study time, such as patients, staff, or their relatives. A questionnaire was used to collect
COVID-19 related data of undiagnosed participants: (i) Symptomatic; (ii) Non-symptomatic, based on their health condition in the three months prior to sampling.

Saliva sample collection

Expectorated unstimulated whole saliva was used throughout this study. All samples were self-collected using standardized instructions and sample tubes provided by this study. Samples were processed and stored at -80°C within 24 h. Salivary stability tests were performed on sample subgroups to evaluate antibody reactivity following viral inactivation with either 1% Triton X-100 for 1 h at room temperature (RT), or heat-treatment at 56°C for 30 min (19). Eighteen antibody-positive from Cohort 1 and, then antibody-negative samples from Cohort 2 were included in the comparison. Incubation at RT for one to three days was also tested in five samples to simulate the standard circumstances of the mailed-in saliva self-collection procedure. Saliva samples from convalescent patients (Cohort 1) were collected on same day as venous blood during a COVID-19 follow-up examination at department of Infectious Diseases, Karolinska University Hospital.

Clinical serology tests

Paired serum samples of all convalescent patients were tested at the Karolinska University Hospital Clinical Microbiology Laboratory. Three automated and one in-house diagnostic method was used for the included convalescent blood samples: SARS-CoV2-IgG test iFlash 1800 YHLO (CLIA), LIAISON® SARS-CoV-2 S1/S2 IgG test DiaSorin (CLIA), and SARS CoV-2 IgG in-house ELISA for samples taken prior to June 2020 (mainly early convalescent samples; <9 months). The Elecsys® Anti-SARS-CoV-2 antibody test Roche (ECLIA) was used for all late convalescent samples (9 months). YHLO determines antibodies against the SARS-CoV-2 nucleocapsid and spike protein, DiaSorin against spike protein, whilst
Elecsys® and in-house ELISA against recombinant nucleocapsid protein. The tests use different techniques such as chemiluminescence immunoassay (CLIA), electrochemiluminescence immunoassay (ECLIA), and enzyme-linked immunoassay (ELISA).

**Antigen Production**

The proteins were produced as follows: 1) Spike glycoprotein (Spike-f) in a soluble trimeric form stabilized in its prefusion-conformation, was expressed in HEK293 cells and purified using a C-terminal Strep II tag; 2) Spike S1 domain was expressed in CHO cells and purified using a C-terminal HPC4-tag; 3) Nucleocapsid C-terminal (NC-C) chain, were both expressed in *E. coli* and purified using a C-terminal His-tag (21,22).

**SARS-CoV-2 antibody detection by a bead-based assay**

The analysis of salivary antibodies was performed as previously described (20) with some modifications. Briefly, each antigen was diluted to a final concentration of 80 µg/ml (100mM) with 2-(N-morpholino) ethanesulfonic acid buffer, pH 4.5 (SigmaAldrich) and immobilized on a uniquely color-coded bead type (bead ID) (MagPlex-C, Luminex corp.). The antigen-immobilized beads were then pooled to form the bead array. Anti-human IgG (309-005-082, Jackson Immunoresearch), anti-human IgA (800-338-9579, Bethyl), and the EBV EBNA1 protein (ab138345, Abcam) were also included as sample loading controls. Saliva samples were diluted 1:5 in assay buffer composed of 3% bovine serum albumin (w/v), 5% non-fat milk (w/v) in 1X PBS supplemented with 0.05% (v/v) Tween20 (VWR, 437082Q), and incubated with bead array for 1 h at RT and 650 RPM. Afterwards, antigen-antibody complexes were cross-linked by adding 0.2% paraformaldehyde (AlfaAesar, 30525-89-4) in PBS 0.05% Tween 20 (PBS-T) for 10 min at RT. Detection was performed by applying R-
phycoerythrine-conjugated anti-human IgG (H10104, Invitrogen) diluted to 0.4 µg/mL, or R-
phycoerythrine-conjugated anti-human IgA (800-338-9579, Bethyl) diluted to 0.2 µg/mL in
PBS-T for 30 min at RT. The read-out was performed by using a FlexMap3D system and
xPONENT software (Luminex Corp.).

Statistical analysis

Statistics and visualization of the multiplex bead array generated data were performed using
R (version 3.6.1) with RStudio (version1.2.1335) and additional packages heatmap (1.0.10)
and reshape2 (1.4.3). In-house developed functions were used for instrument file import and
quality control. Bead array results were acquired as Median Fluorescent Intensity (MFI) per
sample and bead identity. A cutoff for positivity was calculated per antigen as the mean + 6X
SD of 12 negative pre-pandemic reference samples carefully selected based on their signal
intensity distribution. GraphPad Prism Version 9.0.0 (86) was used for nonparametric
comparisons: Mann-Whitney test and Spearman correlation analysis. Datasets also initially
underwent normality distribution testing. The N-1 chi-squared test was used for comparisons
of binomial datasets in MedCal software calculator. Two-sided p-values <0.05 were
considered significant.

Results

Salivary antibody reactivity to SARS-CoV-2 proteins

The assay performance was evaluated by comparing the ability of each of the three antigens
included in the assay to classify convalescent samples (Cohort 1, n=74) and pre-pandemic
samples (Cohort 3, n=35), of which 12 samples from Cohort 3 were used to set the assay
cut-offs. Among the three antigens, spike glycoprotein (Spike-f) and C-terminal fragment of
the nucleocapsid (NC-C) showed the best performance in classifying SARS-CoV-2
convalescent samples from the pre-pandemic samples. Spike-f showed 88% sensitivity and
100% specificity, with one negative control sample reaching intensity signal at the cut off level. NC-C showed 66% sensitivity and 100% specificity (Table 1). Here, we also evaluated the assay performance for all combined antigen panels of 2 and 3 antigens, considering a sample as positive when reactive to both antigens in a panel-of-two antigens and to at least two out of three antigens in a panel (Table 1). As noted, the best performance was reached by the Spike-f, S1, NC-C triple combination, showing 72% sensitivity and 100% specificity. On the other hand, the IgA reactivities were identified only in a minority of cases, with higher prevalence of reactivity to Spike-f (17%) in this cohort (Table S1). It should be noted that larger sample sets are needed in order to establish and validate these sensitivity and specificity levels.

**Serum and salivary antibody reactivity overtime post Covid-19**

As shown in Table 2, Cohort 1 mainly comprised patients who have had mild COVID-19 and were grouped according to duration after confirmed diagnosis. Some were hospitalized for isolation, but none received oxygen treatment or required ventilation-related treatment. All individuals were free from respiratory symptoms at the nine month follow-up but some residual symptoms were still noted in a minority of patients across all three groups (data not shown). As shown in Table 3, the vast majority of serum samples up to nine months post-infection tested positive in clinical SARS-CoV-2 serology, with high seroprevalence across the whole time span of collection. Interestingly, paired saliva samples from Cohort 1 tested with the multiplex bead-array showed that the anti-Spike-f IgG positivity rate in saliva remained remarkably high and in similar range (100%-87.5%) as was noted for serum antibodies (88.9%-96.9%) from early (<3 months) through to late convalescence (9 months) (Table 3, Figure 1a, and Figure S1a). However, the NC-C specific IgG in saliva dropped significantly after three months (from 88.9% to 69.7-56.2%). As stated earlier, specific IgA responses to these antigens were detected only in a minority of the saliva samples, and
were enriched in early convalescence (<3 months, 55.6% for Spike-f and 22.2% for NC-C), while dropping to minority in late convalescence (p<0.01).

Moreover, salivary IgG to Spike-f and NC-C were highly correlated in this cohort (r=0.88, p<0.0001, Spearman correlation test), with concordant serostatus in the majority of samples (Figure 1b). Significant, albeit moderate, correlations were also seen between IgA to Spike-f and NC-C (r=0.62, p<0.001), and between Spike-f specific IgA and IgG (r=0.45, p<0.001) (Figure 1b).

Salivary antibody reactivity to SARS-CoV-2 in healthy donors is associated with the recent history of COVID-19 like symptoms.

Next, we applied this assay platform to evaluate a second independent cohort: Cohort 2. Participants here were self-reporting symptom-free individuals visiting the University Dental Clinic's premises of Karolinska Institutet and the Eastman Institute in Stockholm. A total of 147 individuals from May to November 2020 participated and donated saliva samples. Samples were collected and tested using the same standard operating protocol as for Cohort 1. Shown in Figure 2a and based on antigen-specific cutoffs calculated on 12 negative controls, antibody reactivities to Spike-f and NC-C in this cohort were as follows: IgG was detected in 14% to Spike-f and 15% to NC-C, while 11% had detectable IgG to both antigens; for IgA, 14% and 6% of the samples showed reactivity to Spike-f and NC-C respectively, while only 6% showed reactivity to both. Salivary positivity was particularly enriched among participants with a self-reported recent history of COVID-19-like symptoms (14 days to three months prior to sampling time). Significant reactivities of IgG (p=0.004, and p=0.01) and IgA (p<0.0001, and p=0.044) to either Spike-f or NC-C was found to associate with a recent history of symptoms compared to pre-pandemic controls (Figure 2a). Adding risk factors with symptoms further enriched the salivary IgG positivity. This includes recent
Covid-19 contact, travelling abroad, or clinical duties which increased IgG positivity to 23%, 15% or 13% respectively to Spike-f; and 23%, 19% or 17% to NC-C (Figure S1b).

A correlation analysis (Figure 2b) gave a similar result as was observed for Cohort 1, with the highest reported correlation between salivary IgG to Spike-f and NC-C (r=0.81, p<0.0001, Spearman correlation test). Significant, albeit moderate, correlations were also seen between IgA to Spike-f and NC-C (r=0.73, p<0.001), and IgG and IgA to Spike-f (r=0.49, p<0.001), and Spike-f IgA to NC-C IgG (r=0.53, p<0.001).

**Saliva antibody stability – the influence of inactivation pre-treatment and room temperature**

Next, the effects of virus inactivation by heat treatment (HT) at 56°C for 1 h, 1% Triton X-100 (Triton) as well as room temperature (RT) (identical aliquots left out for indicated time) on the antibody results were determined (Figure 3). Both HT and Triton showed little change in the cut-off (based on the ten included negative controls). A good correlation between treated and non-treated samples was noted (Figure 3 and S2), with a few exceptions of single samples that showed a drop in IgG reactivity. Simulation of RT storage (22°C) showed a slow decay in IgG signal intensity in positive samples (blue) over time, while the signal in negative samples remained low and stable (grey). Based on these data, inactivation by HT or Triton seems to have little effect on saliva samples. However, antibody decay variations showed slight IgG signal reduction by each day of RT storage.

**Discussion**

Comprehensive antibody testing and the subsequent interventions they generate are essential to monitoring and controlling SARS-CoV-2 transmission. The present study demonstrates that salivary SARS-CoV-2-specific IgG after mild COVID-19 can serve as a complementary measure of exposure or immunity to SARS-CoV-2, particularly due to their frequent concurrence with serum IgG responses. Key findings included: 1) SARS-CoV-2-
specific mucosal salivary antibodies co-existed with circulating blood antibodies for up to nine months post-natural infection in the majority of participants (88% in saliva vs. 97% in blood); 2) Natural infection induced salivary antibodies to recognize both viral spike and nucleocapsid proteins; 3) The response correlated significantly to recent COVID-19-like symptom history in undiagnosed individuals; 4) Salivary IgG is relatively stable, tolerating detergent and heat-based inactivation treatments. Taken together, saliva sampling represents a non-invasive approach suitable for population-based immunity surveys. Ideally, if the latter is sampled at home and mailed to the lab, it can help protect vulnerable persons at risk for severe COVID-19 by sparing the need to visit the laboratory units for blood samplings. It is therefore appealing, particularly during a pandemic and can serve as a complementary test for conventional blood IgG assays. Our data also showed that sample inactivation by heat treatment or Triton X-100 were both viable options for biosafety handling procedures and caused minimal variation on assay performance. Options to combine with other point-of-care tests, such as lateral flow-based tests validated for blood could be an interesting way forward (23)

Severe COVID-19 symptoms have been shown to induce strong antibody responses in 99% of convalescent individuals, but published data also shows that these antibody responses tend to decline slower than in mild symptomatic cases (6, 9, 16, 19). This may be attributed to the fact that tests developed earlier in the pandemic were based on detection of samples from severe COVID-19 cases resulting in sub-optimal sensitivity to mild infections (24). Furthermore, many of the initial test kits used the nucleocapsid as a target antigen and antibodies against it have been shown to decline more rapidly (25), as also demonstrated here. In this study, we deliberately recruited convalescent samples from mild COVID-19 patients, in order to evaluate the multiplex antibody platform was capable to detect SARS-CoV-2-specific antibodies in saliva in such patients. In the present study, saliva reactivities were compared against blood serology using certified diagnostics (including anti-N pan-Ig
ECLIA), which show high performance in detecting late convalescent blood samples. In fact, our result is in line with a recent South Korean group reporting this diagnostic antibody assay is, among several others, effective in detecting SARS-CoV-2 antibodies in blood (90%) up to eight months after either asymptomatic infection or mild symptomatic cases (26). Here, the persistence of salivary IgG to structural viral proteins in the saliva samples nine months post-recovery from mild COVID-19 is intriguing, and possibly explained by a secondary exposure or spill-over from blood circulating responses. More studies are therefore warranted to clarify the mechanism underlying the magnitude of salivary responses with better matched study participants. It has been shown that the mucosal antibody response is triggered slightly earlier than the systemic response upon infection (10). Information is still limited about the duration and kinetics of mucosal antibodies secreted into the mouth and nose, particularly in this patient group. A sensitive salivary antibody detection assay with the capability to identify infections with various severities would contribute to improving the current understanding of mucosal antibodies to SARS-CoV-2. For instance, such studies may compare low versus high avidity antibodies and their relation to neutralization or disease enhancement (10,27,28). The advantage of multiplexed assays for antibody detection is that they allow to minimize the sample consumption and increase the throughput by maintaining high sensitivity and specificity. One examples is the recent large-scale screenings of cross-reactivities to multiple pandemic or endemic coronaviruses (29), and capacity to contribute at-home testing for COVID-19 telemedicine diagnosis and monitoring as proposed recently by Torrente-Rodríguez et al. (30).

The hypothesis that antibodies towards previously known coronaviruses may block SARS-CoV-2 has raised questions about their functionality. However, such antibodies are known to be protective for only around six months after infection, and would therefore have disappeared by the time of emergence of SARS-CoV-2 (31,32). Clearly, further assessment of neutralizing capacity against Sars-Cov-2 virus and related coronavirus in human saliva is
necessary. Other important applications for saliva immunoassays include evaluation of vaccine-induced mucosal immunity, which is in fact ongoing in our laboratories for monitoring of local antibody recognition of virus mutations or vaccine-escape mutants. Since mouth and nose are the first ports of entry for SARS-CoV-2, sensitive and accurate methods for quantitative measurements of local immunity will lead to better means to combat COVID-19.

One limitation of our study was the relatively small sample size and the predominantly male population. Another weakness was that blood samples were not analyzed in the same way as saliva and as several diagnostic assays were used, only binary data was provided. Also, because of the cross-sectional design, we could not obtain baseline or longitudinal saliva samples. Moreover, we could not assess individual possibilities of re-exposure or reinfection. However, it is unlikely that humoral immunity was boosted because in Stockholm, where the study took place, the period June-Nov 2020 (second-wave) showed an increase in the daily incidence rate of COVID-19 from 30 to 400 cases/100,000 population (33). In conclusion, despite waning immunity concerns, the present study shows that our multiplex bead-based immunoassays can detect antibodies against SARS-CoV-2 in late convalescence saliva up to nine months after mild COVID-19.
Authors contributions: E.P., A.M., P.N., and M.S.C. conceived and designed the study. H.A., S.B., A.M., and A.O. collected the material and performed the experiments. H.A., S.B., C.H., K.H., A.M., E.P., and M.S.C. analyzed the data. E.P., AM, P.N., and M.S.C. supervised the work. K.L., S.A. G.B. S.H. contributed with material and data interpretations. H.A., S.B., E.P., and M.S.C. wrote the manuscript. K.H. and M.S.C proof-read the revised submission. All authors reviewed and revised the manuscript critically.

Acknowledgments

All study participants who took interest in this study.
References:

1. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A Novel Coronavirus from Patients with Pneumonia in China, 2019. N Engl J Med. 2020;382(8):727–33.

2. University of Johns Hopkins. Coronavirus Resource Center [Internet]. Available from: https://coronavirus.jhu.edu/map.html

3. Krammer F. SARS-CoV-2 vaccines in development. Nature [Internet]. 2020 Oct 22;586(7830):516–27. Available from: http://dx.doi.org/10.1038/s41586-020-2798-3

4. Wang H, Zhang Y, Huang B, Deng W, Quan Y, Wang W, et al. Development of an Inactivated Vaccine Candidate, BBIBP-CorV, with Potent Protection against SARS-CoV-2. Cell. 2020;182(3):713–721.e9.

5. Lumley SF, O’Donnell D, Stoeesser NE, Matthews PC, Howarth A, Hatch SB, et al. Antibody Status and Incidence of SARS-CoV-2 Infection in Health Care Workers. N Engl J Med [Internet]. 2021 Feb 11;384(6):533–40. Available from: http://www.nejm.org/doi/10.1056/NEJMoa2034545

6. Amanat F, Stadlbauer D, Strohmeier S, Nguyen THO, Chromikova V, McMahon M, et al. A serological assay to detect SARS-CoV-2 seroconversion in humans. Nat Med [Internet]. 2020;26(7):1033–6. Available from: http://dx.doi.org/10.1038/s41591-020-0913-5

7. Premkumar L, Segovia-Chumbez B, Jadi R, Martinez DR, Raut R, Markmann AJ, et al. The receptor-binding domain of the viral spike protein is an immunodominant and highly specific target of antibodies in SARS-CoV-2 patients. Sci Immunol. 2020;5(48):1–10.

8. Long QX, Liu BZ, Deng HJ, Wu GC, Deng K, Chen YK, et al. Antibody responses to SARS-CoV-2 in patients with COVID-19. Nat Med. 2020;26(6):845–8.
9. Dan JM, Mateus J, Kato Y, Hastie KM, Yu ED, Faliti CE, et al. Immunological memory to SARS-CoV-2 assessed for up to 8 months after infection. Science (80-) [Internet]. 2021 Feb 5;371(6529):eabf4063. Available from: https://www.sciencemag.org/lookup/doi/10.1126/science.abf4063

10. Isho B, Abe KT, Zuo M, Jamal AJ, Rathod B, Wang JH, et al. Persistence of serum and saliva antibody responses to SARS-CoV-2 spike antigens in COVID-19 patients. Sci Immunol. 2020;5(52):1–21.

11. Baumgarth N, Nikolich-Žugich J, Lee FE-H, Bhattacharya D. Antibody Responses to SARS-CoV-2: Let’s Stick to Known Knowns. J Immunol [Internet]. 2020 Nov 1;205(9):2342–50. Available from: http://www.jimmunol.org/lookup/doi/10.4049/jimmunol.2000839

12. Pisanic N, Randad PR, Kruczynski K, Manabe YC, Thomas DL, Pekosz A, et al. COVID-19 Serology at Population Scale: SARS-CoV-2-Specific Antibody Responses in Saliva. Loeffelholz MJ, editor. J Clin Microbiol [Internet]. 2020 Dec 17;59(1):1–13. Available from: https://jcm.asm.org/content/59/1/e02204-20

13. Ripperger TJ, Uhrlaub JL, Watanabe M, Wong R, Castaneda Y, Pizzato HA, et al. Orthogonal SARS-CoV-2 Serological Assays Enable Surveillance of Low-Prevalence Communities and Reveal Durable Humoral Immunity. Immunity [Internet]. 2020 Nov;53(5):925–933.e4. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1074761320304453

14. Long Q-X, Tang X-J, Shi Q-L, Li Q, Deng H-J, Yuan J, et al. Clinical and immunological assessment of asymptomatic SARS-CoV-2 infections. Nat Med [Internet]. 2020 Aug 18;26(8):1200–4. Available from: http://www.nature.com/articles/s41591-020-0965-6

15. Gui M, Song W, Zhou H, Xu J, Chen S, Xiang Y, et al. Cryo-electron microscopy
structures of the SARS-CoV spike glycoprotein reveal a prerequisite conformational state for receptor binding. Cell Res [Internet]. 2017 Jan 23;27(1):119–29. Available from: http://www.nature.com/articles/cr2016152

16. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, et al. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. Cell [Internet]. 2020 Apr;181(2):271–280.e8. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0092867420302294

17. Li W, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, et al. Angiotsin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature [Internet]. 2003 Nov;426(6965):450–4. Available from: https://doi.org/10.1038/nature02145

18. Rydyznski Moderbacher C, Ramirez SI, Dan JM, Grifoni A, Hastie KM, Weiskopf D, et al. Antigen-Specific Adaptive Immunity to SARS-CoV-2 in Acute COVID-19 and Associations with Age and Disease Severity. Cell [Internet]. 2020;183(4):996–1012.e19. Available from: https://doi.org/10.1016/j.cell.2020.09.038

19. Opeyemi S. Adeniji1, Leila B. Giron1, Netanel F Zilberstein,2 Maliha W. Shaikh2, Robert A Balk2, James N Moy2, Christopher B. Forsyth2, Ali Keshavarzian2, Alan 10 Landay2 MA-M. COVID-19 Severity Is Associated with Differential Antibody Fc-mediated Innate Immune Functions. bioRxiv Prepr [Internet]. 2021; Available from: https://www.biorxiv.org/content/10.1101/2021.01.11.426209v1?s=09

20. Rudberg A-S, Havervall S, Månberg A, Jernbom Falk A, Aguilera K, Ng H, et al. SARS-CoV-2 exposure, symptoms and seroprevalence in healthcare workers in Sweden. Nat Commun [Internet]. 2020 Dec 8;11(1):5064. Available from: http://www.nature.com/articles/s41467-020-18848-0

21. Tegel H, Steen J, Konrad A, Nikdin H, Pettersson K, Stenvall M, et al. High-throughput protein production - Lessons from scaling up from 10 to 288 recombinant proteins per
week. Biotechnol J [Internet]. 2009 Jan;4(1):51–7. Available from: http://doi.wiley.com/10.1002/biot.200800183

22. Kanje S, Enstedt H, Dannemeyer M, Uhlén M, Hober S, Tegel H. Improvements of a high-throughput protein purification process using a calcium-dependent setup. Protein Expr Purif [Internet]. 2020 Nov;175(June):105698. Available from: https://doi.org/10.1016/j.pep.2020.105698

23. Mulchandani R, Jones HE, Taylor-Phillips S, Shute J, Perry K, Jamarani S, et al. Accuracy of UK Rapid Test Consortium (UK-RTC) “abC-19 Rapid Test” for detection of previous SARS-CoV-2 infection in key workers: Test accuracy study. BMJ. 2020;371(September):1–12.

24. Takahashi S, Greenhouse B, Rodríguez-Barraquer I. Are Seroprevalence Estimates for Severe Acute Respiratory Syndrome Coronavirus 2 Biased? J Infect Dis [Internet]. 2020 Nov 9;222(11):1772–5. Available from: https://academic.oup.com/jid/article/222/11/1772/5898481

25. Havervall S, Jernbom Falk A, Klingström J, Ng H, Greilert-Norin N, Gabrielsson L, et al. SARS-CoV-2 induces a durable and antigen specific humoral immunity after asymptomatic to mild COVID-19 infection. medRxiv [Internet]. 2021;2021.01.03.21249162. Available from: https://doi.org/10.1101/2021.01.03.21249162

26. Choe PG, Kim K-H, Kang CK, Suh HJ, Kang E, Lee SY, et al. Antibody Responses 8 Months after Asymptomatic or Mild SARS-CoV-2 Infection. Emerg Infect Dis [Internet]. 2021 Mar;27(3):928–31. Available from: https://wwwnc.cdc.gov/eid/article/27/3/20-4543_article.htm

27. Wen J, Cheng Y, Ling R, Dai Y, Huang B, Huang W, et al. Antibody-dependent enhancement of coronavirus. Int J Infect Dis [Internet]. 2020 Nov;100:483–9. Available
28. Lee WS, Wheatley AK, Kent SJ, DeKosky BJ. Antibody-dependent enhancement and SARS-CoV-2 vaccines and therapies. Nat Microbiol [Internet]. 2020 Oct 9;5(10):1185–91. Available from: http://dx.doi.org/10.1038/s41564-020-00789-5

29. Becker M, Strengert M, Junker D, Kaiser PD, Kerrinnes T, Traenkle B, et al. Exploring beyond clinical routine SARS-CoV-2 serology using MultiCoV-Ab to evaluate endemic coronavirus cross-reactivity. Nat Commun [Internet]. 2021;12(1):1–12. Available from: http://dx.doi.org/10.1038/s41467-021-20973-3

30. Torrente-Rodríguez RM, Lukas H, Tu J, Min J, Yang Y, Xu C, et al. SARS-CoV-2 RapidPlex: A Graphene-Based Multiplexed Telemedicine Platform for Rapid and Low-Cost COVID-19 Diagnosis and Monitoring. Matter. 2020;3(6):1981–98.

31. Pinto D, Park YJ, Beltramello M, Walls AC, Tortorici MA, Bianchi S, et al. Cross-neutralization of SARS-CoV-2 by a human monoclonal SARS-CoV antibody. Nature [Internet]. 2020;583(7815):290–5. Available from: http://dx.doi.org/10.1038/s41586-020-2349-y

32. Edridge AWD, Kaczorowska J, Hoste ACR, Bakker M, Klein M, Loens K, et al. Seasonal coronavirus protective immunity is short-lasting. Nat Med [Internet]. 2020;26(11):1691–3. Available from: http://dx.doi.org/10.1038/s41591-020-1083-1

33. Folkhälsomyndighetens. Folkhälsomyndighetens veckorapporter om covid-19. [Internet]. 2020. p. June-November. Available from: https://www.folkhalsomyndigheten.se/folkhalsorappo
Figure legends

**Figure 1.** Measurement of IgG and IgA to Spike-f (soluble trimeric form of the spike glycoprotein stabilized in the pre-fusion conformation) and NC-C (nucleocapsid C-terminal fragment) of SARS-CoV2 in saliva of convalescent patients (Cohort 1). (A) Multiplex assay measured signal scores on indicated immunoglobulins to Spike-f and NC-C in the pre-COVID-19 control samples (Ctrl, n=35), and convalescent patient samples at indicated month post infection (n=74). The data is expressed in median fluorescence index (MFI) and plotted using dot plots where each dot is one individual sample. Horizontal bars denote the mean and vertical lines represent standard error. Mann-Whitney U test for significance was performed (B) Spearman correlation analysis with coefficient indicated for respective antibody specificity pairs. n.s = not significant.

**Figure 2.** SARS-CoV-2 specific IgG and IgA in saliva of undiagnosed study participants (Cohort 2) measured by same method as in Figure 1. Samples were subgrouped by participant-reported COVID-19-like symptoms during the 14 day to three months prior to the sampling (Cohort 2). (A) Multiplex assay measured signal scores on indicated immunoglobulins to Spike-f and NC-C in pre-COVID-19 control samples (Ctrl, n=35), and participants in Cohort 2 reporting no or yes to recent COVID-19 like symptoms (n=146). The data is expressed in median fluorescence index (MFI) and plotted using dot plots where each dot is one individual sample. Horizontal bars denote the mean and vertical lines represent standard error. Mann-Whitney U test for significance was performed (B) Spearman correlation analysis with coefficient indicated for respective antibody specificity pairs. n.s = not significant.
Figure 3. Stability tests of saliva samples subjected to heat (HT), 1% Triton-X-100 (Triton), and indicated time in room temperature. (A) SARS-CoV2 specific IgG and IgA reactivities in convalescent saliva samples (Pos) or pre-pandemic saliva samples (Neg) were tested either untreated, after HT at 56°C for 30 min, or after Triton inactivation for 60 min. Reactivities to Spike-f and respective NC-C antigens are shown as box plots with each dot representing one single sample. (B) Convalescent saliva samples (blue) or pre-pandemic saliva samples (grey) were aliquoted and placed at room temperature (22°C) at indicated time points, prior to freezing and subsequent measurement of SARS-CoV-2-specific IgG to spike or nucleocapsid.
Table 1. Specificity and sensitivity of single antigen or combination antigen in detecting SARS-CoV-2 IgG in convalescent saliva (1-9 month PSO) and pre-pandemic saliva.

| Antigen | Host     | Convalescent (N=74) | Pre-pandemic (N=23)* |
|---------|----------|---------------------|----------------------|
|         | Sensitivity [%] | Pos | Neg | Specificity [%] | Pos | Neg |
| Spike-f | HEK      | 88  | 65  | 9       | 100 | 0   |
| S1      | CHO      | 62  | 46  | 28      | 100 | 0   |
| NC-C    | E. coli  | 66  | 49  | 25      | 100 | 0   |
| Spike-f | HEK      | 62  | 46  | 28      | 100 | 0   |
| S1      | CHO      | 66  | 49  | 25      | 100 | 0   |
| NC-C    | E. coli  | 57  | 42  | 32      | 100 | 0   |
| Spike-f | HEK      | 72  | 53  | 21      | 100 | 0   |
| S1      | CHO      | 72  | 53  | 21      | 100 | 0   |
| NC-C    | E. coli  | 72  | 53  | 21      | 100 | 0   |

*Another 12 independent pre-pandemic saliva samples were used to establish assay cut-offs.

* One sample shows intensity signal at cutoff level.
Table 2. Demographic characteristics of convalescence samples of Cohort 1, grouped by the time-points of post symptom onset (PSO) at which the samples were taken.

| Parameters                          | Convalescent saliva samples (n=74) |
|-------------------------------------|-------------------------------------|
|                                     | < 3 months (n=9) | 3-8 months (n=33) | 9 months (n=32) |
| Gender (F:M)                        | 08:01 | 23:10 | 06:26 |
| Age (years) median (range)          | 59 (48-67) | 49 (20-63) | 57 (45-78) |
| Hospitalization status (%)          |                                   |
| o Never hospitalized                | 66.7 | 94 | 97 |
| o Hospitalized for only isolation purpose | 11  | 3  | 3  |
| o Hospitalized due to COVID-19 symptoms | 22  | 3  | 0  |
| Days PSO (Mean | SD)     | 55 | 20 | 120 | 41 | 273 | 11 |
Table 3. Cohort 1: Salivary antibodies to Spike-f or NC-C over time concurs with serum positivity in clinically validated SARS-Cov-2 antibody diagnostics

| Convalescence (mo) | Serum Ab SARS CoV-2 | Saliva IgG | Saliva IgA |
|--------------------|---------------------|------------|------------|
|                    |                     | Spike-f    | NC-C       | Spike-f    | NC-C       |
| <3                 | 88.9%               | 100.0%     | 88.9%      | 55.6%      | 22.2%      |
| 3-8                | 90.9%               | 84.8%      | 69.7%*     | 12.5%***   | 3.1%**     |
| 9                  | 96.9%               | 87.5%      | 56.2%***   | 9.7%***    | 6.5%***    |

Note: *, ** and *** indicate p<0.05, p<0.01 and p<0.0001 respective compared to clinical SARS-Cov-2 serum antibody diagnosis, determined by N-1 Chi-squared test (Campbell I, Statistics in medicine, 2007, Richardson JTE, Statistics in medicine, 2011). Ab (antibody). Spike-f (spike foldon), NC-C (nucleocapsid c-terminal chain).
Fig 2.

A. Undiagnosed IgG

B. Undiagnosed IgA

Spearman r: Undiagnosed
Fig. 3

A. IgG: [Graph showing distribution of IgG levels for Spike-f and NC-C]

IgA: [Graph showing distribution of IgA levels for Spike-f and NC-C]

B. IgG Spike-f [Graph showing IgG levels over time (0h, 4h, 7h, 9h)]

IgG NC-C [Graph showing IgG levels over time (0h, 4h, 7h, 9h)]