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1 **Development and validation of an enzyme immunoassay for detection and quantification of SARS-**
2 **CoV-2 salivary IgA and IgG**
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4
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21 **Running title:** Development of salivary antibody assays to SARS-CoV-2

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22 **Footnote page**

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39 **ABSTRACT**

40 Oral fluids offer a non-invasive sampling method for the detection of antibodies. Quantification
41 of IgA and IgG antibodies in saliva allows studies of the mucosal and systemic immune response after
42 natural infection or vaccination. We developed and validated an enzyme immunoassay (EIA) to detect
43 and quantify salivary IgA and IgG antibodies against the prefusion-stabilized form of the SARS-CoV-2
44 spike protein. Normalization against total antibody isotype was performed to account for specimen
45 differences, such as collection time and sample volume. Saliva samples collected from 187 SARS-CoV-
46 2 confirmed cases enrolled in 2 cohorts and 373 pre-pandemic saliva samples were tested. The
47 sensitivity of both EIAs was high (IgA: 95.5%; IgG: 89.7%) without compromising specificity (IgA:
48 99%; IgG: 97%). No cross reactivity with seasonal coronaviruses was observed. The limit of detection
49 for SARS-CoV-2 salivary IgA and IgG assays were 1.98 ng/mL and 0.30 ng/mL, respectively. Salivary
50 IgA and IgG antibodies were detected earlier in patients with mild COVID-19 symptoms than in severe
51 cases. However, severe cases showed higher salivary antibody titers than those with a mild infection.
52 Salivary IgA titers quickly decreased after 6 weeks in mild cases but remained detectable until at least
53 week 10 in severe cases. Salivary IgG titers remained high for all patients, regardless of disease severity.
54 In conclusion, EIAs for both IgA and IgG had high specificity and sensitivity for the confirmation of
55 current or recent SARS-CoV-2 infections and evaluation of the IgA and IgG immune response.

56

57

58 **Key words**: SARS-CoV-2, salivary antibodies, mild disease, severe disease

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59 **INTRODUCTION**

60 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of the
61 coronavirus disease 2019 (COVID-19) pandemic, is a Betacoronavirus related to Severe Acute
62 Respiratory Syndrome Coronavirus (SARS-CoV) and Middle East Respiratory Syndrome Coronavirus
63 (MERS-CoV) [1-3]. As of July 30, 2021, SARS-CoV-2 infections has caused more than 200 million
64 cases worldwide, and an estimated 4.2 million deaths. The clinical spectrum of SARS-CoV-2 infection
65 ranges from asymptomatic infection to symptomatic disease [4]. The high proportion of asymptomatic
66 individuals not only results in a high transmission rate, but also suggests differences in the host immune
67 response compared to other coronaviruses [5]. Since the duration of immunity to SARS-CoV-2 dictates
68 the overall course of the pandemic as well as post-pandemic strategies, a comprehensive understanding
69 of the relationship between systemic and mucosal antibody responses becomes important. Because the
70 oral and nasal cavities are considered main sites for SARS-CoV-2 entry and replication, locally
71 produced mucosal antibodies may protect against infection. Therefore, saliva samples can be used as a
72 non-invasive tool for virus detection as well as measuring the immune response (mucosal and systemic)
73 [6] .

74 Salivary antibody levels can be 100 to 1000-fold lower than serum levels [7]. Salivary IgG is
75 mainly derived from serum by leakage across capillaries and enters saliva through gingival crevices. At
76 mucosal membranes, IgA is the main immunoglobulin class and is found most often in the secretory
77 form (sIgA). Within 2-3 weeks after onset of disease, SARS-CoV-2–specific IgG antibodies can be
78 detected in saliva, persist for at least 9 months, and show high correlation with serum antibody levels in
79 most COVID-19 patients [8-11]. Salivary IgA antibodies, on the contrary, rapidly increase 1 week after
80 onset of disease, become undetectable 4-5 weeks later, and show a moderate correlation with serum
81 levels [8, 9]. Saliva provides a non-invasive collection method, easy to implement in remote areas and
82 community settings without a need for extensive training. These features, while additionally evaluating
83 both mucosal and systemic immune responses, make salivary antibody testing an ideal approach to
84 evaluate population immunity, transmission, asymptomatic infections, and vaccine performance.
85 We previously demonstrated the value of saliva-based antibody assays to evaluate immune responses
86 mounted against norovirus [12]. In this manuscript, we describe the development and validation of an
87 enzyme immunoassay (EIA) to quantitatively evaluate the presence of SARS-CoV-2–specific IgA and
88 IgG antibodies in saliva and describe the salivary immune response to SARS-CoV-2 mounted in
89 different cohorts of infected patients.

90

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MATERIALS AND METHODS

Saliva samples

A total of 333 saliva samples were collected from 187 participants who had tested positive for SARS-CoV-2 by real-time reverse transcription polymerase chain reaction (rRT-PCR) [13] or antigen test in two cohorts **(Figure 1)**. In cohort I, 235 samples were collected from a) 113 participants at a single time point and b) 32 participants on a weekly basis for 4-5 weeks (n=122) after diagnosis. In cohort II, 98 saliva samples were collected from 42 participants with either asymptomatic (n=8), mild (n=29) or severe disease (n=5) at different times after the onset of disease (range 0-203 days). Disease severity was defined according to the World Health Organization criteria [4] and clinical data were obtained using a standardized questionnaire. In addition, 373 pre-pandemic archived samples collected between 2009-2010 were included as negative controls [12].

Specimen collection and processing

Saliva was collected at least 30 minutes after consumption of food or liquids. Pre-pandemic archived saliva samples were collected using the Oracol saliva collection device, processed, and stored at -80°C according to the manufacturer's instructions (Malvern Medical Developments, Worcester, UK). Cohort I samples were collected using the Oracol S14 collection device by gently rubbing the swab along the gumline around the entire mouth for approximately 1 minute. This collection device specifically harvests gingival crevicular fluid, which resembles serum composition [14]. Saliva samples collected by the Oracol swabs were separated by centrifugation (10 min at 1500 x g), transferred to the attached microtube (10 µl -200 µl), and stored at -80°C until analysis. For cohort II, participants were asked to cough deeply and spit into a collection cup containing virus isolation media (PBS plus 2% FBS, Gentamicin, Amphotericin B). Saliva samples were clarified by centrifugation (10 min at 3000 x g), aliquoted and stored at -80°C until analysis. All samples collected during the pandemic were inactivated by gamma radiation (2 x 10^6 rads) prior to testing [15]. Samples were initially tested for SARS-CoV-2–specific salivary IgA. If enough sample volume (≥ 100µl) was available, samples were tested for SARS-CoV-2–specific salivary IgG.

Enzyme immunoassay (EIA) for detecting SARS-CoV-2-specific IgA and IgG in saliva

Convalescent sera from 3 SARS-CoV-2 patients with IgA and IgG antibodies against SARS-CoV-2 spike protein were used for the initial assay development. The pre-fusion stabilized ectodomain

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123 of SARS-CoV-2 spike (S) that was used in the assays was obtained from in suspension adapted HEK-
124 293 cells as described previously [16]. Antigen concentrations ranging from 0.125-1.00 µg/ml in PBS
125 and 1:1,000 to 1:20,000 diluted horse radish peroxidase (HRP)-conjugated goat anti-human IgA or IgG
126 were initially tested. Positive and negative controls (SARS-CoV-2 convalescent serum and pre-
127 pandemic saliva samples, respectively), as well as blank controls (only blocking buffer) were also
128 included in each run. All volumes were 100 µl per well, except where indicated. All washes were
129 performed 3 times with 250 µl of PBS/0.05% Tween-20 (PBST) using a BioTek 405 plate washer. All
130 dilutions were prepared in blocking buffer (5% (w/vol) powdered milk/PBST). All incubations were
131 carried out for 1 h at 37°C except where indicated. All concentrations and incubation times were
132 optimized to maximize the optical density (OD) difference between pre-pandemic negative samples and
133 SARS-CoV-2 convalescent sera.

134 Immunol 2 HB flat 96 well-plates (Fisher Scientific) were coated with 100 µl SARS-CoV-2
135 spike protein (0.5 µg/ml in PBS, row A to D, positive coated wells) or PBS (row E-H, negative coated
136 wells) and incubated overnight at 4°C in a humidified chamber. Plates were washed, blocked with 200
137 µl /well blocking buffer for 2 h at 37°C. After blocking, plates were washed three times and 4-fold serial
138 dilutions (1:10-1:160) of each saliva sample were added to both antigen and PBS coated wells. Plates
139 were incubated, washed, and bound antibodies were detected using HRP-conjugated goat anti-human
140 IgA (Sera Care) diluted 1:4,000 in blocking buffer or anti-IgG (Sera Care), diluted 1:16,000. After
141 incubation, plates were washed again before adding 100 ul of 3,3′,5,5′-Tetramethylbenzidine (TMB)
142 substrate (Sera Care). The colorimetric reaction was stopped 5 min later by adding 100 µl Stop solution
143 (Sera Care). The plates were read at 450 nm and 630 nm using an Epoch2 instrument (BioTek). After
144 background correction, ODs were calculated (OD_{450nm} -OD_{630nm}) and the adjusted OD value (OD value
145 positive coated well minus OD value negative coated well) was determined.

146

147 **Enzyme immunoassay (EIA) for detecting total IgA and IgG in saliva**

148 For the detection of total IgA, Immunol 2 HB flat 96 well-plates (Fisher Scientific) were coated
149 with goat anti-human IgA (α-chain) (0.5 µg/ml in PBS, rows A to F, positive coated wells) or PBS 1x
150 (rows G-H, negative coated wells) and incubated overnight at 4°C in a humidified chamber. Plates were
151 washed and blocked with blocking buffer (200 µl /well) for 2 h at 37°C. Three dilutions (1:1,280,
152 1:5,120 and 1:20,480) of each saliva sample were prepared. A standard curve for IgA was prepared by
153 serial dilution of purified human IgA (Sigma-Aldrich). After incubation, plates were washed and saliva
154 samples were added to rows A-D. Purified human IgA dilutions were added to both positive coated (anti

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155 IgA, rows E-F) and negative coated (PBS, rows G-H) wells. Plates were incubated, washed, and bound
156 antibodies were detected using 1:4,000 diluted HRP-conjugated goat anti-human IgA (Sera Care). After
157 incubation, plates were washed again before adding TMB substrate (Sera Care). The colorimetric
158 reaction was stopped 5 min later by adding Stop solution (Sera Care).
159 The same plate design and steps were used for IgG. Plates were coated with goat anti-human IgG
160 (γ-chain) at 0.5 μg/ml in PBS. A standard curve for IgG was prepared by serial dilutions of purified
161 human IgG (Sigma-Aldrich) and bound antibodies were detected using 1:16,000 diluted HRP-
162 conjugated goat anti-human IgG (Sera Care).
163
164 **Sensitivity and specificity**
165 The sensitivity (ability to identify samples with antibodies to SARS-CoV-2), and the specificity
166 (ability to identify samples without antibodies to SARS-CoV-2) were defined as the values for which
167 there is 95% probability that the estimated value can be obtained [17]. For IgA EIA validation, 373
168 SARS-CoV-2 rRT-PCR negative saliva samples (pre-pandemic) collected from healthy adults (2009-
169 2010) and 44 saliva samples from SARS-CoV-2 rRT-PCR-confirmed cases were used. Similarly, for
170 validation of the IgG assay, 373 pre-pandemic saliva samples and 68 saliva samples from confirmed
171 cases were used. Saliva samples were collected ranging from 0 to 63 days after diagnosis.
172
173 **Limit of detection**
174 The limit of detection (LOD) was defined as the lowest predicted value for which there is 95%
175 probability that an estimated value can be obtained. To determine the LOD for the IgA (or IgG) EIA, 5
176 IgA (or IgG) positive SARS-CoV-2 saliva samples were 4-fold serially diluted (1:10-1:10,240). The
177 adjusted OD values were extrapolated from the linear portion of the IgA (or IgG) standard curve. The
178 LOD was determined from the lowest concentration of antibody above the cut off value (mean of the
179 adjusted OD values for the pre-pandemic samples + 3SD).
180
181 **Analytical specificity**
182 To evaluate potential cross reactivity between antibodies against the spike protein of SARS-
183 CoV-2 and other human coronaviruses, convalescent serum from patients positive for the common
184 human coronaviruses [HuCoV 229 (n=2), HuCoV NL-63 (n=1), HuCoV OC43 (n=7) and HuCoV
185 HUK1 (n=1)] were included.
186

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187 **Data analysis**

188 Descriptive statistics for continuous variables are presented as median plus interquartile ranges
189 (IQR). For categorical variables, n (%) was used for descriptive statistics and differences were evaluated
190 by Chi-Square or Fisher's exact test. Non-parametric data from more than 2 groups were compared by
191 Kruskal-Wallis test. Receiver operating characteristics (ROC) analysis was used to evaluate sensitivity
192 and specificity of each assay when establishing a cut off for positivity. Each saliva sample was tested at
193 2 different dilutions. To quantify total and SARS-CoV-2 specific antibodies, adjusted OD values from
194 serially diluted purified human IgA (or IgG) were plotted against concentration and fit to a sigmoidal 4
195 parameter logistic model. Total and SARS-CoV-2 -specific salivary IgA (or IgG) titers were
196 extrapolated from the linear portion of the IgA (or IgG) standard curve. To account for participant
197 differences (e.g., severity of illness, immunocompetency, collection time, antibody secretion levels),
198 SARS-CoV-2 specific IgA or IgG were normalized to 100 µg of total IgA or IgG, respectively. When
199 virus-specific antibodies could not be detected, but total antibodies were detected, SARS-CoV-2-
200 specific IgA (or IgG) per 100 µg of total IgA (or IgG) were arbitrarily assigned as half the lower limit of
201 detection, based on the standard curve of purified human IgA [18]. Statistical analysis was performed
202 using GraphPad Prism version 8.0 (GraphPad Software, San Diego, California USA,
203 www.graphpad.com), and *p*-values <0.05 were considered significant.

204

205 **Ethics statement and Disclaimer**

206 This activity was reviewed by CDC and was conducted consistent with applicable federal law and CDC
207 policy (See, e.g., 45 C.F.R. part 46.102(l)(2), 21 C.F.R. part 56; 42 U.S.C. §241(d); 5 U.S.C. §552a; 44
208 U.S.C. §3501 et seq.). The study was approved by the Institutional Review Boards of the Oregon Health
209 & Science University (IRB# 21230) (cohort II). Participants that were cognitively or decisionally
210 impaired were excluded. Written informed consent was obtained from each participant. The findings and
211 conclusions in this article are those of the authors and do not necessarily represent the official position
212 of the Centers for Disease Control and Prevention.

213

214 **RESULTS**

215 We developed and validated EIAs for the quantitative detection of SARS-CoV-2-specific
216 salivary IgA and IgG antibodies in saliva. The final assay conditions are summarized in Table 1. Pre-
217 pandemic saliva (negative for SARS-CoV-2 antibodies) and convalescent serum from COVID-19
218 patients were used for the initial standardization of both EIAs. All reagent concentrations [SARS-CoV-2

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219 spike protein, anti- human IgA (α-chain) or IgG (γ-chain), HRP- labeled secondary antibody, purified
220 human IgA and purified human IgG concentration for standard curves] and incubation times were
221 optimized to reduce background, obtain maximum anti-SARS-CoV-2 specific signal, and to maximize
222 the optical density difference between pre-pandemic negative samples and SARS-CoV-2 convalescent
223 sera.
224

225 **Clinical sensitivity and specificity**

226 A dilution of 1:10 of true negative (pre-pandemic) and true positive (SARS-CoV-2 rRT-PCR–
227 confirmed cases) saliva samples resulted in OD values with no background signal in the negative coated
228 wells (PBS coat). Adjusted OD values for both SARS-CoV-2-specific IgA and IgG antibodies from true
229 positive samples were significantly higher in saliva from rRT-PCR confirmed cases than from pre-
230 pandemic samples (**Figure 2A**, p< 0.0001). ROC analysis was applied to determine the cut off value that
231 maximizes sensitivity and specificity (**Figure 2B and C).** For the IgA EIA, the assay specificity
232 improved from 91.5% (95% CI: 88.2%-93.9%) to 97.3% (95% CI: 95.0%-98.5%), when the cut off
233 value increased from one to three standard deviations (SD) above the mean ($\bar{x}$) of the pre-pandemic
234 samples, whereas the sensitivity remained at 95.5% (95% CI: 84.9%-99.2%). For the IgG EIA, a
235 significant increase of the specificity from 97.9% (95% CI: 96.6%-99.3%) to 98.9% (95% CI: 97.3%-
236 99.6%) (p<0.05) was achieved when the cut off value was modified from $\bar{x} + SD$ to $\bar{x} + 3SD$. Although
237 the sensitivity slightly decreased from 91.2% (95% CI: 82.1%-95.9%) to 89.7% (95% CI: 80.2%-
238 94.9%), this difference was not significant (p=0.0625). Based on these data we used a cut off value of
239 $\bar{x} + 3SD$ of the adjusted OD values from pre-pandemic saliva samples for both the IgA and IgG EIAs
240 assays. The overall diagnostic accuracy was 98.3% (95% CI:95.6%-100%) and 99.1 (95% CI:98.0%-
241 100%) for the IgA and IgG assays, respectively (Table 1).
242

243 **Limit of detection**

244 For both IgA and IgG antibody assays, a sigmoidal four-parameter logistic curve was fitted to the
245 resulting adjusted OD values to yield a standard curve of antibody concentration versus OD **(Figure
246 2D)**. The lower limit of detection for the assays was 1.98 ng/ml for SARS-CoV-2 IgA antibodies and 0.3
247 ng/ml for SARS-CoV-2 IgG antibodies. At the upper limits of quantification, above which the detectors
248 on the plate reader for total IgA and IgG antibodies are saturated, samples were tested at higher dilutions
249 (< 1:1,280) to fit within the linear portion of the sigmoidal curve, without compromising the detection of
250 antibodies against SARS-CoV-2. We tested 3 dilutions for SARS-CoV-2 and total antibodies. (Table 1).

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251 At 1:10 dilution, 42 saliva samples tested positive for SARS-CoV-2 IgA whereas at 1:40 and 1:160
252 dilutions, 16/42 (38%) and 25/42 (59%) false negatives were detected. For total IgA antibodies, 10/42
253 (24%) saliva samples were above the upper limit of quantification at 1:1,280 dilution, whereas at
254 1:5,024 dilution 42/42 (100%) were detected and 1:20,480 dilution 2/42 (5%) false negative were
255 detected. At 1:10 dilution, 60 saliva samples tested positive for SARS-CoV-2 IgG, whereas at 1:40 and
256 1:160, 33/60 (55%) and 40/60 (67%) false negatives were detected. For total IgG antibodies, 12/60
257 (20%) saliva samples were above the upper limit of quantification at 1:1,280 dilution, whereas at
258 1:5,024 dilution 60/60 (100%) were detected and 1:20,480 dilution 7/60 (12%) false negative were
259 detected **(Figure 2E).** Based on these data we chose to test each saliva sample at 1:10 and 1:40 dilution
260 for SARS-CoV-2 antibodies and 1:1,280 and 1:5,120 dilution for total antibodies.
261

262 **Analytical specificity**

263 To evaluate the potential cross reactivity with antibodies against seasonal coronaviruses (229E,
264 HKU1, OC43, NL63), convalescent serum samples from confirmed seasonal coronavirus patients were
265 tested for the presence of SARS-CoV-2 IgA and IgG **(Figure 2F).** At the lowest serum dilution (1:100),
266 both SARS-CoV-2 IgA and IgG adjusted OD values were significantly lower in seasonal α-coronavirus
267 (0.032±0.021 and 0.045±0.01), and seasonal β-coronavirus (0.058±0.024 and 0.044±0.014) serum than
268 SARS-CoV-2 serum (0.742±0.133 and 0.841±0.203) ($p<0.0001$), suggesting that our assays show no
269 cross-reactivity with antibodies against seasonal coronaviruses.
270

271 **Kinetic and magnitude of SARS-CoV-2 specific antibody response in saliva after infection.**

272 Cohort I yielded 205 saliva samples from 118 SARS-CoV-2 confirmed cases available to be
273 tested for SARS-CoV-2-specific salivary IgA and 166 saliva samples for which enough saliva was
274 available were also tested for IgG **(Figure 1)**. The mean time between SARS -CoV-2 diagnosis to saliva
275 collection was 22.9 days (median 20; IQR: 14-31.75; range: 2–65 days). Overall, 86.4% (102/118) of
276 participants had measurable salivary antibodies for one (IgA: 19.5%; n=23/118; IgG: 20.3%, n=24/118)
277 or both isotypes (46.6%, 55/118). Conversely, 13.6% (16/118) participants tested negative for both
278 SARS-CoV-2 salivary IgA and IgG. During the first week after diagnosis, the positivity rate for both
279 IgA and IgG was 28.6% (2/7 samples). For IgA, positivity rate rapidly increased to 62.1% at week 2,
280 peaked at week 4 (68.6%) and decreased to 0% at week 9 and week 10. At week 3, the positivity rate for
281 IgG was 80%, peaked at 100% at week 8 and remained positive to the end of the study (week 10)
282 **(Figure 3A)**. The median amount of SARS-CoV-2-specific IgA was 0.1 (IQR:0.1-49.44) ng/100 µg of

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283 total IgA at week 1 and increased to 7.95 (IQR: 0.1-25.74) ng/100 µg of total IgA at week 3 (IQR: 0.1-
284 25.74). After fluctuating at week 4 and week 6, the IgA levels became undetectable at week 9. The
285 salivary IgG titers increased between week 1 (median: 0.1; IQR:0.1-14.32) and week 5 (median: 123.4;
286 IQR:39.32-269.5), increased to 162.9 (IQR:31.93-322.9) ng/100 µg of total IgG at week 6 and then
287 remained stable throughout the follow-up period. Compared to week 1 significant differences between
288 IgG levels were observed at week 5 ($p<0.05$) and week 6 ($p=0.01$) **(Figure 3B)**.

289 In the cohort II, 85 saliva samples from 42 rRT-PCR-positive participants who were either
290 asymptomatic (n=8), had mild (n= 29) or severe (n= 5) clinical COVID-19 disease symptoms were
291 tested for IgA and IgG antibodies **(Table 2 and Figure 1)**. In the group of patients with mild symptoms
292 the mean time between onset of disease to sample collection was 146.8 days (median: 178; IQR: 81.25-
293 200.8; range 1-208 days) and 25.6 days (median: 18; IQR: 14.5-40.5; range:12-60 days) in the group
294 with severe symptoms.

295 Overall, for cohort II, the positivity rate for IgA showed a clear pattern with increasing values
296 during week 1-3, a peak at week 4, whereas the positivity rate for IgG was less defined. **(Figure 3C).**
297 For patients with mild disease symptoms, the positivity rate for IgA and IgG was 33.3% at week 1 after
298 onset of symptoms. The IgA titer rapidly increased and peaked at week 4 (100%), sharply decreased to
299 0% at week 9 and remained negative until week 30. The positivity rate for IgG peaked at 50% at week 3,
300 fluctuated for several weeks and returned to 0% 30 weeks after onset of disease. In patients with severe
301 clinical symptoms, the positivity rate for IgA and IgG were consistently higher than in mild cases at
302 each time point. The positivity rate for IgA peaked at week 3 (100%) then slowly decreased to baseline
303 at week 10 whereas IgG peaked (80%) at week 5 and remained elevated until the end of the study (week
304 10).

305 In cohort II, salivary IgA levels in patients with severe clinical symptoms peaked at week 3
306 (median:346; IQR: 128.2-855.9) and remained positive for the entire study (10 weeks), whereas salivary
307 IgG titers peaked at week 4 (median: 810.5; IQR: 598.2-1778) and remained positive until week 10. In
308 patients with mild disease, salivary IgA titers peaked later, at week 5 (median: 143.6; IQR: 9.94-277.3)
309 and became undetectable at week 7, while salivary IgG antibodies peaked earlier at week 3 (median:
310 507.9; IQR: 58-957). Although with fluctuating values, salivary IgG titers remained detectable until
311 week 36 after onset of disease. In asymptomatic participants (n=8), salivary IgA titers were detected 1
312 week earlier than IgG. Overall, during the first 6 weeks after onset of symptoms, salivary IgA and IgG
313 titers were higher in patients with severe symptoms compared to patients with mild symptoms **(Figure**
314 **3D).**

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DISCUSSION

We report the development and validation of in-house EIAs to quantitatively assess the presence of SARS-CoV-2-specific IgA and IgG in saliva and showed its value to describe the salivary immune response after natural SARS-CoV-2 infection. Both the IgA and IgG assays were highly accurate, sensitive, and specific. The sensitivity of both assays was high (IgA: 95.5%; IgG: 89.7%) without compromising specificity (IgA: 99%; IgG: 97%). Other published studies have shown high sensitivity for IgG (88-98.4%), but low (17-59%) for IgA, with a high specificity for both isotypes (96-100%) [8-11]. Difference in sensitivity between our and other IgA assays could be explained by the assay platform, type of sample, or collection time after infection. The limit of detection of 1.98 ng/ml for SARS-CoV-2 IgA and 0.3 ng/ml for SARS-CoV-2-specific IgG suggest that both assays are suitable for the detection of salivary antibodies in samples collected early after infection up to several weeks after recovery.

Overall, we detected SARS-CoV-2-specific IgA and IgG responses as early as 1 week after onset of disease or diagnosis when disease data were not available. The IgA positive rate decreased to zero after 10 weeks, whereas IgG positivity rate remained high for at least up to 30 weeks. Similarly, data from previous cross-sectional studies showed detectable SARS-CoV-2 -specific IgA and IgG levels in saliva 2–4 weeks after onset of symptoms, with only IgG response antibodies persisting beyond 60 days [8, 9]. A different study reporting results from single saliva samples collected <3 to 9 months after onset of disease showed a consistently high IgG positivity, but a significant decrease of IgA [10]. In a longitudinal study including 95 participants, the mean time from disease onset to IgG detection in saliva was 9-11 days and IgG antibodies remained detectable until day 90 [19].

In cohort II, when no saliva sample was collected during the first week after onset of disease, we assumed that participants were negative for both SARS-CoV-2-specific IgA and IgG. Salivary IgA levels peaked 3 weeks after onset of disease and remained elevated until at least week 10 in participants with severe disease symptoms. Conversely, in patients with mild disease salivary IgA levels were transient, peaked late at week 5 and returned to baseline levels at week 7. Participants with severe disease showed a later (week 4) peak for IgG than those with mild disease, and both remained positive until the end of the study. These initial increases in salivary IgA and IgG were similar in asymptomatic individuals, although the number of samples available after 1 week was limited. Our data agree with the study by Pisanic *et al.*, where salivary IgA and IgG antibodies reached a peak at 3 weeks post infection, but only IgG remained above baseline levels for at least 60 days after onset of symptoms [9]. Another

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347 group found similar IgG and IgA kinetics in serum from mild and severe cases [20]. Several studies
348 reported higher correlation for IgG than IgA when testing paired serum and saliva samples [8, 9],
349 leading to question the advantage of testing IgA in saliva. Early SARS-CoV-2 humoral immune
350 responses are dominated by IgA antibodies which remained detectable in saliva for a longer time than in
351 serum (days 49 to 73 post-symptoms). IgA antibodies also contribute to virus neutralization to a greater
352 extent compared to IgG, although they circulate for shorter time than IgG [21]. Given the less invasive
353 collection method, and the transient presence of IgA in contrast to long-lasting IgG, testing for IgA in
354 saliva might allow for a better understanding of the timing of infection.

355 Our study has several limitations. First, data on the onset and severity of disease was not always
356 available. Second, collection of saliva samples did not always start on day 0 after onset of disease, not
357 all participants provided samples at the same time points, and a limited number of longitudinal samples
358 from asymptomatic individuals were available. Third, due to low sample volume, IgG testing could not
359 be performed on all saliva samples. Fourth, saliva samples were not screened for the presence of
360 antibodies against SARS-CoV-1, MERS-CoV or seasonal coronaviruses. We did test convalescent sera
361 positive for seasonal coronavirus and showed no cross-reactivity. Others have shown some cross
362 reactivity with SARS-CoV-1 and MERS-CoV which will be relevant only if these viruses circulate in
363 the same population tested with our assay for antibodies against SARS-CoV-2. Finally, paired serum
364 samples to correlate the immune response were not available; therefore, correlation between saliva and
365 serum could not be evaluated.

366 In summary, we developed and validated EIAs that are sensitive and specific to detect SARS-
367 CoV-2 specific IgA and IgG antibodies in saliva. Significant fluctuations of salivary IgA and IgG
368 antibodies levels were observed after infection. Detection of salivary antibodies may serve as an easy-to-
369 employ screening method for population and transmission studies as well as evaluation of vaccine
370 response, especially when collection of blood is challenging or not feasible.

371

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380

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436 **Table 1:** Parameter optimization and assay validation

Parameter	**Salivary IgA**	**Salivary IgG**
Antigen (SARS-CoV-2 spike protein) concentration	0.5 µg/ml	0.5 µg/ml
Immunoglobulin Isotype capture (anti-IgA or IgG) concentration	0.5 µg/ml	0.5 µg/ml
Immunoglobulin Isotype standard curve range	0.8 -2,000 ng/ml	0.4 -1,000 ng/ml
Saliva sample dilution	1:10 to 1:160 (SARS-CoV-2) 1:1,280 to 1:20,480 (Total Ig isotype)	
Secondary antibody (HRP-conjugate) concentration	1:4,000	1:16,000
Substrate development system	3,3′,5,5′-Tetramethylbenzidine (TMB)	
Validation		
Diagnostic accuracy		
Area under curve (AUC) (95% CI)	0.9833 (0.9564-1.000)	0.9909 (0.9797-1.000)
Std. Error	0.01370	0.005694
P value	<0.0001	<0.0001
Sensitivity (%) (95% CI)	95.5 (84.9-99.2)	89.7 (80.2-94.9)
Specificity (%) (95% CI)	97.3 (95.0-98.5)	98.9 (97.3-99.6)
Limit of detection	1.98 ng/ml	0.3 ng/ml

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438 **Table 2:** Demographics and clinical characteristics of the SARS-CoV-2 patients from cohort II

Characteristic	Asymptomatic (n=8)	Mild disease [a] (n=29)	Severe disease [ab] (n=5)	Total [b] (n=42)
Age (y), median (IQR)[c]	44.5 (25.2-49.7)	62.0 (44.0.-65.5)*	63(49.0-74.0)*	56.0(43.5-65)
Sex(male/female)	2/6	10/19	2/3	14/28
SARS-CoV-2 infection severity, n, (%)[d]				
Ambulatory				
No limitation of activities	8(100)	-	-	8 (19.0)
Limitation of activities	-	22 (75.9)	-	22(52.4)
Hospitalized				
No oxygen therapy	-	1(3.4)	-	1 (2.4)
Oxygen therapy [e]	-	6(20.7)	-	6 (14.3)
Non-invasive ventilation	-	-	3(60.0)	3 (7.1)
Intubation (mechanical ventilation)	-	-	-	-
Ventilation +additional organ support.	-	-	2(40.0)	2(4.8)
Level of care at saliva sampling, n, (%)				
Outpatient	8(100)	22(75.9)	-	30(71.4)
Hospitalized	-	7(24.1)	5(100)	12 (28.6)**
Symptoms				
Fever >=100.4 F	-	16(55.2)	1(20.0)	17(40.5)
Chills	-	9(31.0)	1(20.0)	10 (23.8)
Weakness	-	15(51.7)	3(60.0)	18 (42.9)
Muscle aches	-	12(41.4)	1(20.0)	13 (31.0)
Runny nose	-	8(27.6)	1(20.0)	9 (21.4)
Sore throat	-	9(31.0)	-	9 (21.4)
Cough	-	23(79.3)	3(60.0)	26 (61.9)
Shortness of breath	-	21(72.4)	5(100.0)	26 (61.9)
Nausea	-	5(17.2)	-	5 (11.9)
Vomiting	-	-	-	-
Headache	-	11(37.9)	1(20.0)	12 (28.6)
Diarrhea	-	5(17.2)	2(40.0)	7 (16.7)
Abdominal Pain	-	4(13.8)	-	4 (9.5)
Rash	-	-	-	-
Other	-	19(65.6)	1(20.0)	20 (47.6)

[a] Disease severity (mild vs severe) was defined according to the World Health Organization Classification [4]

[b] Categorical values were compared using Fisher exact test or Chi-square for more than 2 groups. Continues variable were compared by Kruskal-Wallis test. * $p<0.05$; ** $p<0.001$

[c] Interquartile range

[d] Clinical spectrum of SARS-CoV-2 infection according to World Health Organization Classification [4]

[e] Oxygen by mask or nasal prong

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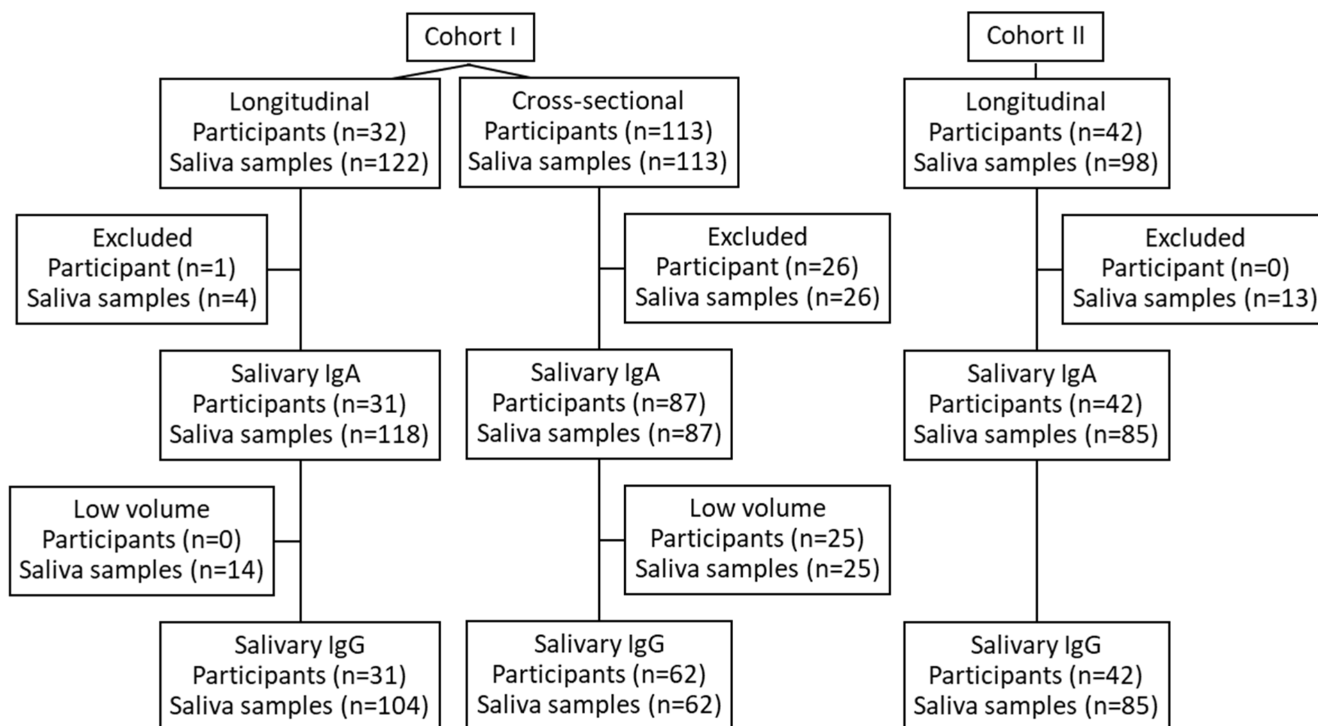

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442 **Figure 1: Specimen collection and testing.** A total of 333 saliva samples were collected from 187
443 participants who had tested positive for SARS-CoV-2 by rRT-PCR or antigen test. In cohort I, 113
444 participants provided a single sample and 32 participants provided samples on a weekly basis for 4-5
445 weeks after diagnosis. In cohort II, 42 participants provided saliva samples at different times after onset
446 of diseases.

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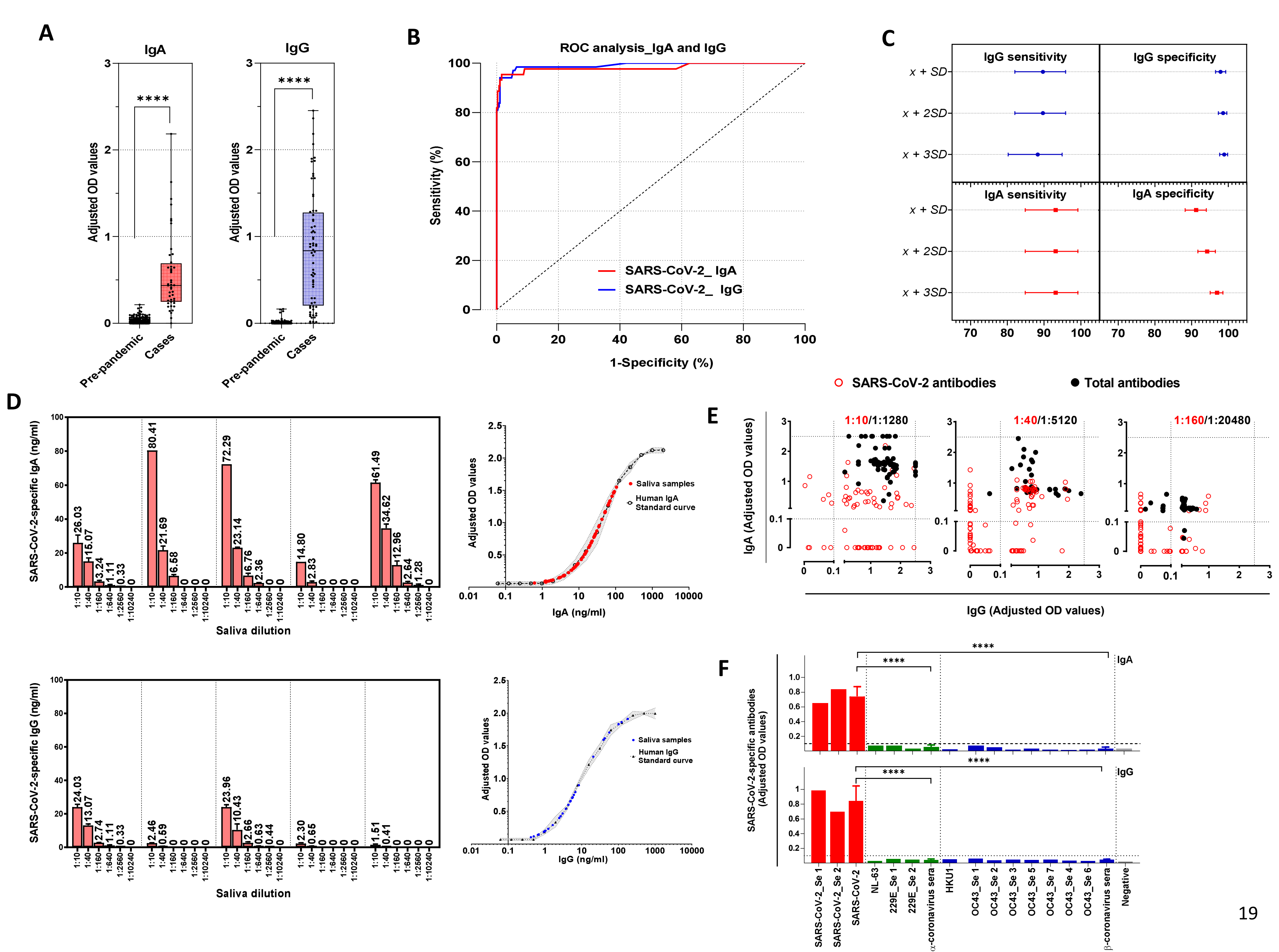

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448 **Figure 2: Development and validation of enzyme immunoassays for the detection of salivary IgA and IgG against SARS-CoV-2.**
449 Saliva samples were collected from SARS-CoV-2 confirmed cases (IgA, n=44; IgG, n=68). Pre-pandemic samples were collected between
450 2009-2010 (n=373). Saliva samples were diluted 1:10 and added to a 96-well plate precoated with SARS-CoV-2 S antigen**.** **(A)** Sample
451 adjusted OD values for SARS-CoV-2 IgA and IgG. **(B)** Receiver operating curves for each assay were constructed with data from SARS-
452 CoV-2 confirmed cases, as well as pre-pandemic samples. The optimal cut off value to differentiate cases from controls was set as the
453 maximum Youden's index (sensitivity + specificity - 1). **(C)** Sensitivity (95% CI) and specificity (95%CI) for three different cut off values
454 [mean $(\overline{x})$ of the pre-pandemic samples plus standard deviations (*SD*)] were considered for each antibody isotype. **(D)** To assess the limit of
455 detection for each isotype, non-linear regression was performed using 5 saliva samples serially diluted in 2 independent experiments.
456 Adjusted OD values were extrapolated from IgA or IgG standard curve for each sample and dilution. SARS-CoV-2 specific antibody
457 concentrations (ng/ml) are indicated on top of each bar. **(E)** Saliva sample dilution evaluation. Saliva samples from SARS-CoV-2 confirmed
458 cases (n=66) were tested for total (dilutions 1:1,280 to 1:20,480, black dots) and SARS-CoV-2 (dilutions 1:10 to 1:160, red dots) antibodies.
459 False negative rates were calculated for each dilution. The dotted line represents the calculated cutoff value (OD 0.1) discriminating between
460 positive and negative samples, and the upper limits of quantification (OD 2.5) above which detectors on the plate reader are saturated. **(F)**
461 Specificity of antigens against a panel of seasonal coronavirus convalescent serum. SARS-CoV-2 S antigen was tested against convalescent
462 serum from confirmed SARS-CoV-2 cases (red), seasonal coronavirus cases prior to pandemic (green and blue) and pre-pandemic negative
463 samples (gray) for IgG and IgA. The horizontal dotted line represents the calculated cutoff value discriminating between positive and negative
464 samples based on ROC analysis in (C).

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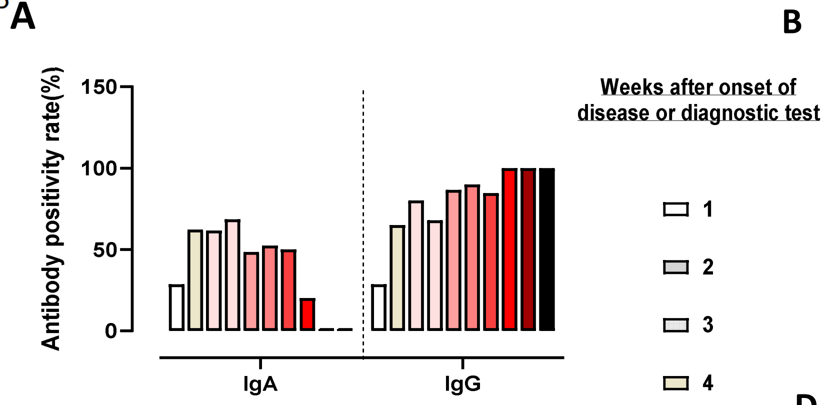

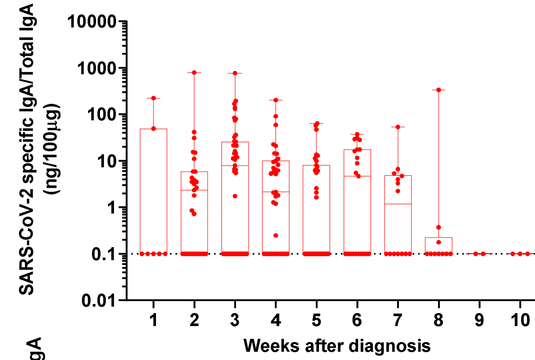

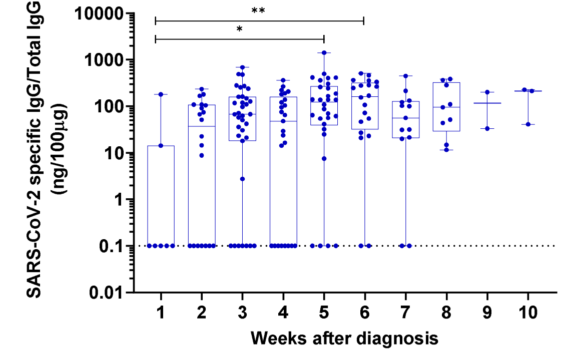

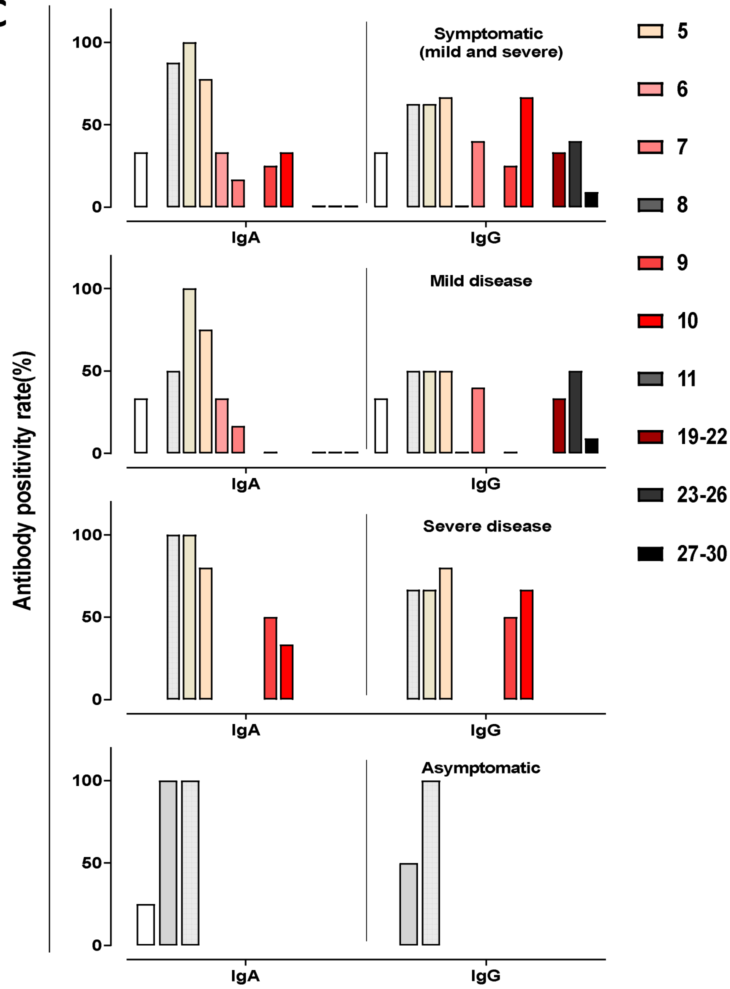

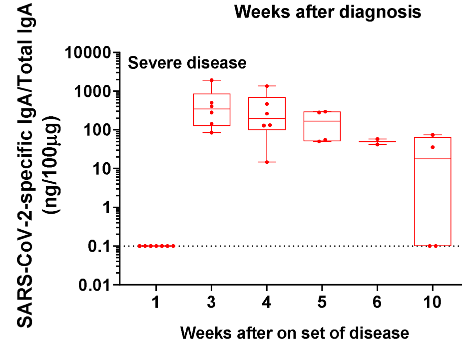

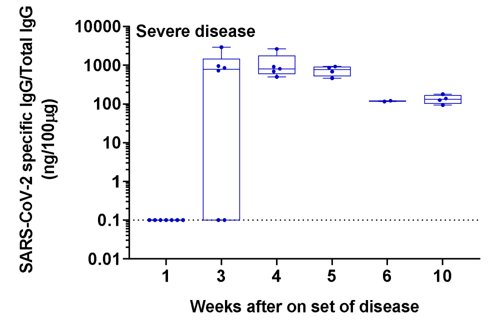

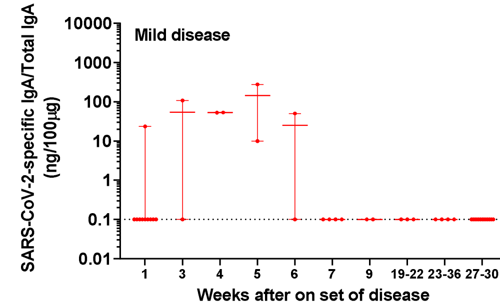

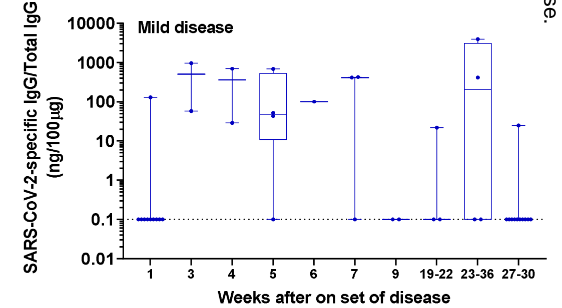

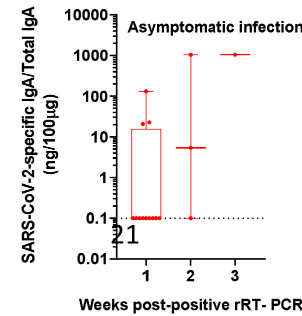

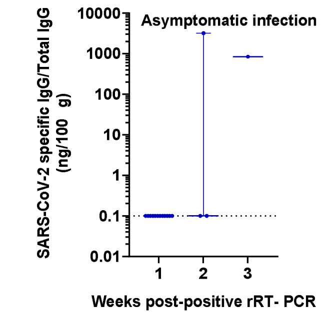

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466 **Figure 3: Kinetics and magnitude of SARS-CoV-2 specific salivary antibody response after infection.** Positivity rate after **(A)** positive
467 diagnostic test in cohort I (118 and 93 participants for IgA or IgG, respectively) or **(C)** onset of disease in cohort II (42 participants). Empty
468 column indicates no sample available. Positive rate equal 0% is indicated by an underscore. **(B and D)** SARS-CoV-2-specific salivary
469 antibody levels in COVID-19 patients. SARS-CoV-2 salivary IgA and IgG levels after a positive diagnostic test on day 0 (B, Cohort I, 205
470 and 166 samples for IgA and IgG, respectively) or on set of disease (D, Cohort II, 85 saliva samples for IgA and IgG). SARS-CoV-2
471 antibodies were normalized to 100 µg of total salivary IgA or IgG, respectively to account for differences among participants, collection time
472 and secretion levels. When SARS-CoV-2 specific salivary antibodies were not detected, but total antibodies (IgA or IgG, respectively) were
473 present, normalized SARS-CoV-2 salivary antibody levels were arbitrarily assigned to 0.1 for visualization purpose (dotted line)
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