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Cross-neutralization of distant coronaviruses correlates with Spike S2-specific antibodies from immunocompetent and immunocompromised vaccinated SARS-CoV-2-infected patients

Sara V Patel
Boston College
Brooke M Leeman
Boston College
Patricia J Botros
Boston College
Joanna Folta
Boston College
Dhiman Shahid
Boston College
Anya I Rocque
Boston College
Andrew S Joyal
Boston College
Joseph A Vecchio
Boston College
Eliza Passell
Massachusetts General Hospital
Dessie Tien
Massachusetts General Hospital
Zahra Reynolds
Massachusetts General Hospital
Karry Su
Massachusetts General Hospital
Tammy D Vyas
Massachusetts General Hospital
Jatin M Vyas
Massachusetts General Hospital
Emory Abar
Massachusetts General Hospital

Mamadou Barry
Massachusetts General Hospital
Andrew Alexandrescu
Massachusetts General Hospital
Zachary Wallace
Massachusetts General Hospital
Jeffrey M DaCosta
Boston College
Manish C Choudhary
Brigham and Women's Hospital
Trevor Tamura
Brigham and Women's Hospital
Gregory Edelstein
Brigham and Women's Hospital
Yijia Li
Brigham and Women's Hospital
Rinki Deo
Brigham and Women's Hospital
Jeffrey A Sparks
Brigham and Women's Hospital
Julie Boucau
Ragon Institute of MGH, MIT and Harvard
Owen Glover
Ragon Institute of MGH, MIT and Harvard
Amy Barczak
Ragon Institute of MGH, MIT and Harvard
Jacob Lemieux
Massachusetts General Hospital
Mark J Siedner
Massachusetts General Hospital
Jonathan Z Li
Brigham and Women's Hospital
Ismael Ben Farouck Fofana
fofanai@bc.edu

Boston College

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1 **Cross-neutralization of distant coronaviruses correlates with Spike S2-specific antibodies**
2 **from immunocompetent and immunocompromised vaccinated SARS-CoV-2-infected**
3 **patients**
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6 Sara V. Patel[1‡], Brooke M. Leeman[1‡], Patricia J. Botros[1], Joanna Folta[1], Dhiman Shahid[1], Anya
7 I. Rocque[1], Andrew S. Joyal[1], Joseph A. Vecchio[1], Eliza Passell[2], Dessie Tien[2], Zahra Reynolds[2],
8 Karry Su[2], Tammy D. Vyas[2], Jatin M. Vyas[2], Emory Abar[2], Mamadou Barry[2], Andrew
9 Alexandrescu[2], Zachary Wallace[2], Jeffrey M. DaCosta[1], Manish C. Choudhary[3], Trevor
10 Tamura[3], Gregory Edelstein[3], Yijia Li[3], Rinki Deo[3], Jeffrey A. Sparks[3], Julie Boucau[4], Owen
11 Glover[4], Amy Barczak[4], Jacob Lemieux[2], Mark J. Siedner[2], Jonathan Z. Li[3], Ismael Ben Fofana[1]
12
13 1: Biology Department, Boston College, 140 Commonwealth Avenue, Chestnut Hill, MA
14 2: Massachusetts General Hospital, Harvard Medical School, Cambridge, MA, USA
15 3: Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA.
16 4: Ragon Institute of MGH, MIT, and Harvard, Boston, MA, USA.
17
18 [‡] These authors contributed equally to this work
19 [*] Authors for correspondence: ismael.fofana@bc.edu
20
21
22 As of May 2023, the public health emergency of COVID-19 was lifted across the globe.
23 However, SARS-CoV-2 infections continue to be recorded worldwide. This situation has been
24 attributed to the ability of the virus to evade host immune responses including neutralizing

antibody-derived Immunity. The vast majority of antibody escape mutations have been associated with the S1 subunit of the spike protein, especially the Receptor Binding Domain (RBD) but also the N-terminal Domain (NTD). The other region of the spike, the S2 subunit, is the most conserved region amongst coronaviruses. We hypothesized that S2-specific antibody responses are suboptimal in vaccinated and SARS-CoV-2 infected patients resulting in an ineffective neutralization of distant coronaviruses. Here, we analyzed S2-specific antibody responses SARS-CoV-2-infected individuals, including a mixed cohort of those with and without immunosuppression and prior vaccination. We found that S2-specific antibody responses are generally lower than S1-specific antibody responses. Furthermore, we observed in immunocompetent individuals that S1 and S2-specific antibody responses are both positively correlated with Wuhan, Omicron, SARS-CoV and W1V1-CoV pseudovirus neutralization. Among the immunocompromised patients, S1-specific antibody responses were rarely correlated with pseudovirus neutralization in contrast to S2-specific antibody responses which frequently correlated with pseudovirus neutralization. These data highlight the potential of the S2-subunit as an ideal target for induction of cross-neutralizing antibody immunity against divergent coronaviruses.

43 **Introduction**

44 Coronavirus Disease of 2019 (COVID-19) emerged in late 2019 in Wuhan (China) [1] and
45 rapidly expanded across the globe with the World Health Organization (WHO) declaring a
46 global health pandemic in March 2020 [2]. A number of vaccine candidates were developed
47 including Pfizer-BioNTech, Moderna with emergency use authorization granted in the United
48 States under a 1-year period [3]. Millions of people were immunized worldwide and many lives
49 were saved [4]. In the US the public health emergency of COVID-19 expired in May, 2023 [5].
50 However, the virus has continued to evolve and circulate across the globe with occurrences of
51 variants of concern including the two major waves of the Delta and Omicron variants ongoing
52 [6]. This raises a concern for vaccine-induced protection amongst the most vulnerable sections of
53 the population, especially the immunocompromised [7-10].

54

55 The titer of neutralizing antibodies against SARS-CoV-2 has been identified as a predictor of
56 COVID-19 disease severity [11]. COVID-19 disease severity has also been associated with high
57 levels of SARS-CoV-2 antibodies in other studies which did not always discriminate between
58 binding antibody titers and neutralization potency [12, 13]. SARS-CoV-2 ability to evade
59 antibody responses through S1 region mutations including the receptor binding domain has been
60 described [11, 14-17]. It is noteworthy that SARS-CoV-2 ability to evade antibody neutralization
61 is not limited to the RBD [18]. Nucleotide deletions were observed in the N-terminal Domain
62 (NTD) of the S1-subunit of the spike protein leading to escape from neutralizing antibody
63 responses [18].

64 The ability of SARS-CoV-2 to escape antibody responses and the well-established importance of
65 neutralization antibody responses to disease progression and survival has enhanced the concerns

66 for immunocompromised individuals [7-10]. Here, we measured the levels of antibody responses
67 targeting the S1 and S2 regions of the spike from serum of vaccinated individuals with non-
68 severe COVID-19, including those with and without immunosuppression. We then analyzed
69 correlations between neutralization of wild-type Wuhan, Omicron BA.1, SARS-CoV and W1V1-
70 CoV pseudoviruses. We found that S1-specific antibody responses are more abundant while S2-
71 specific antibody responses are suboptimal for efficient neutralization of distant coronaviruses,
72 SARS-CoV and W1V1-CoV. Nonetheless, we found that S1- and S2-specific antibody levels
73 were generally correlated with pseudovirus neutralization for both immunocompetent and
74 immunocompromised patients. A more restrictive pattern emerged with the
75 immunocompromised for whom S2-specific antibody levels distinctively correlated with
76 Omicron, SARS-CoV and W1V1-CoV pseudovirus neutralization.
77
78
79 **Results**
80 Study specimens were tested from individuals enrolled in the POSITIVES study (Post-
81 vaccination Viral Characteristics Study), a prospective observational cohort study that enrolls
82 ambulatory persons with acute COVID-19. Specimens were collected between January 2021 and
83 May 2023 [19]. SARS-CoV-2 infection was confirmed by RT-PCR. Eight of the 87 participants
84 were non-vaccinated and one participant received a single dose of the Johnson & Johnson
85 COVID-19 vaccine. All other participants received between 2 and 5 doses of either the Moderna
86 or Pfizer/BioNTech COVID-19 mRNA vaccines. All 3 vaccines at the time of this analysis were
87 based on a Wuhan spike trimer formulation. Table 1 presents the demographic and clinical
88 characteristics of the participants.

89

90 ***Validity of modified soluble S2 as ELISA antigens***

91 We first sought to measure antibody levels against soluble S1 and S2 antigens. The amino-acid
92 sequences corresponded to the wild-type Wuhan and the Omicron B.1.1.529 variant (also known
93 as the Omicron BA.1 variant and hereafter referred to as Omicron were compared to the soluble
94 Wuhan S2 and Omicron S2 mutated for solubility by the manufacturer (Acro Biosystem). The
95 analysis also included the S1 and S2 subunits corresponding to SARS coronavirus (SARS-CoV)
96 and the bat SARS-Like coronavirus W1V1 (W1V1-CoV).

97 The soluble Wuhan S2 and Omicron S2 antigens were similar and clustered to their
98 corresponding wild-type Wuhan S2 and Omicron S2 subunits (Supplementary Figure 1). In
99 general, comparison of S1 and S2 subunits confirmed the similarities between Wuhan and
100 Omicron and their isolation from the distant relatives SARS-CoV and W1V1-CoV
101 (Supplementary Tables 1-3).

102

103 ***S1-specific antibody titers surpassed S2-specific antibody levels***

104 Antibody levels were measured by ELISA using the S1 and S2 antigens from the Wuhan and
105 Omicron strains in the form of soluble proteins (Figure 1). S1-Wuhan-specific antibody levels
106 (mean=0.23) were significantly higher than S2-Wuhan-specific antibody levels (mean=0.13)
107 (paired t-test; $P < 0.001$) (Figure 1a). The same pattern was seen for Omicron, with the S1-
108 Omicron-specific antibody levels (mean=0.28) significantly higher than S2-Omicron-specific
109 antibody levels (mean=0.07) (paired *t*-test; $P < 0.001$) (Figure 1a). Overall, S1-Omicron-specific
110 antibody levels were the highest while S2-Omicron-specific antibody levels were the lowest
111 (Figure 1a).

112

113 ***Binding antibody titers were higher with vaccinated groups compared to the non-vaccinated***
114 ***group, with no significant differences among the booster groups (2-5 doses)***

115 Samples were subsequently analyzed according to the vaccination status and number of doses for
116 comparison of S1- and S2-specific antibody levels. Vaccinated participants (1-5 doses; n=79)
117 presented higher antibody titers than non-vaccinated ones (0 doses; n=8) (Figure 1b). The 1-dose
118 vaccination group had only 1 participant, which reduced the power of statistical analyses
119 involving this group. The remaining vaccination groups (doses 2-5) had significantly higher
120 antigen binding compared to non-vaccinated participants (linear mixed model with Tukey HSD
121 post-hoc test; $P < 0.01$). However, no significant differences in antibody levels were observed in
122 comparisons between the multiple booster groups (2 to 5 doses; Figure 1b).

123 Inter-group analyses were followed up with intra-group analyses, in which paired t-tests
124 were used to test if participants had higher antibody levels for S1- or S2-specific antibodies. For
125 groups with either 0 or 1 dose, no significant differences were found since low sample size
126 reduced statistical power (Figure 1b). For groups with 2-5 doses, all tests found significantly
127 higher antibody levels in S1 compared to S2-specific antigens. For example, in participants that
128 received 3 doses (n=39), S1-Wuhan antibody levels (mean=0.24) were significantly higher than
129 S2-Wuhan levels (mean=0.13) (paired *t*-test; $P < 0.001$), and also S1-Omicron antibody levels
130 (mean=0.33) were significantly higher than S2-Omicron levels (mean=0.07) (paired *t*-test; $P <$
131 0.001) (Figure 1b).

132

133 ***Pseudovirus neutralization was higher with Wuhan and Omicron compared to SARS-CoV and***
134 ***W1V1-CoV***

135 Sera from all participants (n=87) were evaluated for neutralization capacity of several
136 coronaviruses. We used a previously published pseudovirus-based neutralization assay [11, 14,
137 20] with pseudoviruses corresponding to the Wuhan and Omicron variants, as well as the distant
138 relatives SARS-CoV and W1V1-CoV [21].

139 Highest serum antibody neutralization concentrations were observed with Wuhan
140 pseudovirus (Figure 2). Nine of the samples required a higher serum starting dilution of 20x and
141 3 samples necessitated a higher serum starting dilution of 30x for the crossing of the 50%
142 neutralization levels in order to generate the NT50 values (Supplementary Figure 2).
143 Overall, strong serum antibody neutralization concentrations were observed against the Omicron
144 pseudovirus, but these were significantly lower when compared to the Wuhan pseudovirus
145 neutralization ($\log_{10}$ NT50+1 transformation; Wuhan mean=3.63, Omicron B1.1.259 mean=3.37;
146 linear mixed model with Tukey HSD post-hoc test; $P < 0.01$, Figure 2a).

147 Lower serum neutralization concentrations were observed with the more distantly related
148 SARS-CoV ($\log_{10}$ NT50+1 transformation; mean=2.40) and W1V1-CoV (mean=2.39)
149 pseudoviruses. Wuhan pseudovirus neutralization concentrations were significantly higher than
150 those observed with both SARS-CoV (linear mixed model with Tukey HSD post-hoc test; $P <$
151 0.001) and W1V1-CoV (linear mixed model; $P < 0.001$) (Figure 2a). Similar results were
152 recovered when comparing Omicron pseudovirus neutralization concentrations to those with
153 SARS-CoV and W1V1-CoV (linear mixed model with Tukey HSD post-hoc test; $P < 0.001$;
154 Figure 2a). No significant differences were observed between SARS-CoV and W1V1-CoV
155 pseudovirus neutralization ($P > 0.05$; Figure 2a).
156

157 ***Vaccination significantly improved pseudovirus neutralization potency***

158 Vaccinated participants (1-5 doses; n=79) presented higher neutralization concentrations
159 compared to non-vaccinated participants (0 doses; n=8) (Figure 2b). Neutralization was lowest in
160 the 0-dose group (log10 NT50+1 transformation; mean=1.94), and the means of all other dose
161 groups were significantly higher. This was found even for the comparison to the 1-dose group
162 that included only one participant (mean=3.47; linear mixed model with Tukey HSD test; $P <$
163 0.05). Neutralization was further elevated in participants that had boosters (2-dose mean=3.10; 3-
164 dose mean=3.05; 4-dose mean=2.99; 5-dose mean=3.08). Comparisons of the non-vaccinated (0-
165 dose) to these groups with boosters (2-5 dose groups) were all highly significant (linear mixed
166 model with Tukey HSD test; $P < 0.001$; Figure 2b). Intra-group analyses (i.e., within those with
167 the same number of vaccine doses) did not find differences among pseudovirus neutralization for
168 those within the 0-dose and 1-dose groups. Within groups that had multiple doses (2-5 doses),
169 pseudovirus neutralization was significantly higher for Wuhan and Omicron compared to SARS-
170 CoV and W1V1-CoV (linear mixed model with Tukey HSD test; all $P < 0.05$; Figure 2b).
171
172 ***No significant correlation was observed between antibody responses and the number of days***
173 ***from the last dose***
174 We hypothesized that the time since last immunization could impact antibody responses. We first
175 investigated if the number of days after the last vaccine dose influenced the level of antibody
176 responses in the vaccinated participants (Supplementary Figure 3). The single dose participant
177 was at 262 days after his immunization while, as expected, the group with 2 doses had the most
178 extended period (mean=393) followed by 3 doses (mean=201.1), 4 doses (mean=148.8) and 5
179 doses (mean=134.9) (Supplementary Figure 3a). Significant differences were only observed in
180 comparisons with the 2-dose and the 3-, 4-, and 5-dose groups (one-way ANOVA with Tukey

181 HSD test; $P < 0.001$; Supplementary Figure 3a). However, no significant correlations were
182 observed between the number of days from the last vaccine and S1- or S2-specific antibody
183 levels (Pearson's correlation; $P > 0.05$; Supplementary Figure 3b). The potential impact of the
184 number of days after the last vaccine dose on the pseudovirus neutralization concentrations
185 produced similarly low correlation coefficients, with the one significant result of a significant
186 negative correlation between number of days since last dose and neutralization of SARS-CoV
187 (Pearson's correlation; $P < 0.05$; Supplementary Figure 3c).
188
189 ***S1- and S2-specific antibody titers generally correlated positively with pseudovirus***
190 ***neutralization potency***
191 We next evaluated the relationship between antigen-specific antibody titers and neutralization
192 capacity. Linear correlations between pseudovirus neutralization concentrations and S1- or S2-
193 specific Wuhan/Omicron antibody levels were analyzed for all participants (n=87). Separate
194 analyses were done for each pseudovirus (Wuhan, Omicron, SARS-CoV and W1V1-CoV),
195 resulting in 16 comparisons.
196 There was a predominantly positive correlation between neutralization capacity and
197 antibody titers, with 15 of the 16 analyses resulting in a positive correlation coefficient (Figure
198 3). Furthermore, 12 or the 16 analyses had significantly positive coefficients (Pearson's
199 correlation; $P < 0.05$). Antibody correlations with the Wuhan pseudovirus were the weakest, with
200 correlations between -0.08 and 0.17 and no significant results (Figure 3a). For the remaining
201 pseudoviruses, significant positive correlations were recovered in all analyses (Figure 3b-d). The
202 highest correlation coefficients were found in analyses with W1V1-CoV, with r=0.53 for S2-
203 Omicron and r=0.51 for S2-Wuhan (both $P < 0.001$; Figure 3d).

204 Although S1-specific antibody levels were higher compared to their S2-specific
205 counterparts (i.e., higher y-intercepts), the correlation between neutralization and antibody
206 production was mostly higher in S2- compared to S1-specific analyses. For example, for the
207 Omicron pseudovirus the correlation coefficient was higher for S2-Wuhan ($r = 0.36$) compared
208 to S1-Wuhan ($r = 0.27$), as well as for S2-Omicron ($r = 0.35$) compared to S1-Omicron ($r =$
209 0.25) (Figure 5b). This pattern was found for 6 of the 8 possible S1- vs S2-specific comparisons.
210
211 ***Antibody responses observed with the immunocompetent participants were generally higher***
212 ***than that of the immunocompromised participants***
213 Since the POSITIVES cohort includes immunocompetent as well as immunocompromised
214 participants, we evaluated the impact of immunocompromised status on coronavirus-specific
215 humoral immunity. In this sub-study, 17 of the 87 samples were obtained from
216 immunocompromised participants. Binding (Figure 4a) and neutralizing (Figure 4b) antibody
217 titers were overall lower for the immunocompromised, although no comparison reached
218 statistical significance.
219
220 ***Antibody levels and pseudovirus neutralization were correlated more strongly in***
221 ***immunocompetent patients than in the immunocompromised group***
222 Correlations between antibody levels and pseudovirus neutralization titers were analyzed
223 separately for immunocompromised and immunocompetent groups. In each case, S1- and S2-
224 specific Wuhan and Omicron antibodies were tested with each pseudovirus in a separate
225 analysis. For the immunocompromised group (Figure 5), 12 of the 16 correlations were positive,
226 but only two tests were significant. For the W1V1-CoV pseudovirus, there was a significant

227 positive correlation between neutralization and S2-Wuhan (Pearson's correlation; $r = 0.65$; $P <$

228 0.01) and S1 Omicron (Pearson's correlation; $r = 0.59$; $P < 0.05$) antibody levels.

229 Correlations between neutralization and antibody levels were considerably stronger in the

230 immunocompetent group (Figure 6) which had a larger sample size (n=70) than the

231 immunocompromised (n=17). Here, 15 of the 16 correlations were positive, with 10 being

232 significantly so. For the Wuhan pseudovirus there were no significant correlations with S1- and

233 S2-specific antibody levels (Pearson's correlation; $P > 0.05$; Figure 6a). For the Omicron

234 pseudovirus there were significant positive correlations for S2-Wuhan (Pearson's correlation; $r =$

235 0.36; $P < 0.01$) and S2-Omicron ($r = 0.39$; $P < 0.001$) antibody levels (Figure 6b). For both

236 SARS-CoV and W1V1-CoV pseudoviruses, significant positive correlations were found in all

237 tests (Figure 6c-d). As in the analyses with all samples, the same general pattern of higher

238 correlations between neutralization and antibody production for S2- compared to S1-specific

239 counterpart was found when only analyzing immunocompetent participants.

240

241 ***Increasing the number of booster doses significantly improved antibody responses for***

242 ***immunocompetent, but not immunocompromised, participants***

243 The impact of booster doses on the levels of antibody responses was examined separately for the

244 immunocompromised and immunocompetent participants (Supplementary Figure 4). All the

245 immunocompromised participants received at least one vaccine dose so group comparisons were

246 limited to only vaccinated participants (Supplementary Figure 4a). Although higher S1- and S2-

247 specific antibody levels were observed for 3-, 4-, and 5-dose groups, the means of different dose

248 groups were not significantly different from each other (linear mixed model with Tukey HSD

249 test; $P > 0.05$; Supplementary Figure 4a). In analyses within each dose group of

250 immunocompromised participants, significantly higher antibody levels were found in the 4-dose
251 S1- vs S2 Omicron (paired t-test; $P < 0.05$), the 5-dose S1- vs S2-Wuhan (paired t-test; $P <$
252 0.05), and the 5-dose S1- vs S2-Omicron (paired t-test; $P < 0.001$), comparisons (Supplementary
253 Figure 4a).

254 In the immunocompetent group, multiple doses of vaccines promoted higher antibody
255 production than the non-vaccinated while no significant difference was observed between the
256 multiple doses (doses 2-5; Supplementary Figure 4b). There were no immunocompetent
257 participants that received only one dose, but those receiving 2, 3, 4, and 5 doses all had higher
258 mean antibody levels compared to non-vaccinated immunocompetent participants (linear mixed
259 model with Tukey HSD test; $P < 0.001$). There were no statistical differences among mean
260 antibody levels in the 2-, 3-, 4-, and 5-dose groups ($P > 0.05$). Within each immunocompetent
261 dose group, paired *t*-tests were used to evaluate if S1- and S2-specific antigens had equal means.
262 For the 0-dose group there were no significant differences, but for most comparisons in the other
263 dose groups there were higher antibody levels toward the S1-specific antigen compared to its S2-
264 specific counterpart (6 of 8 comparisons; $P < 0.01$).

265

266 ***Increasing the number of booster doses did not significantly improved neutralization titers***
267 ***observed with the immunocompromised participants***

268 We also separately examined the impact of booster doses on pseudovirus neutralization for the
269 immunocompromised and immunocompetent participants and recovered similar results
270 (Supplementary Figure 5). Mean neutralization levels among the dose groups did not differ
271 significantly (linear mixed model with Tukey HSD test; $P > 0.05$; Supplementary Figure 5a).
272 Neutralization was also similar across the different pseudoviruses for within dose group

273 comparisons with three exceptions: Wuhan vs SARS-CoV in the 3-dose group, Wuhan vs SARS-
274 CoV in the 5-dose group, and Wuhan vs W1V1-CoV in the 5-dose group (Supplementary Figure
275 6a).

276 In contrast again, an increased number of vaccine doses promoted a stronger response in
277 the immunocompetent group (Supplementary Figure 5b). Those receiving 2, 3, 4, and 5 doses all
278 had higher neutralization levels compared to non-vaccinated immunocompetent participants
279 (linear mixed model with Tukey HSD test; $P < 0.001$). There were no statistical differences
280 among mean neutralization levels in the 2-, 3-, 4-, and 5-dose groups ($P > 0.05$). Within each
281 immunocompetent dose group, linear mixed models with patient ID as a random effect were
282 used to compare Wuhan, Omicron, SARS-CoV, and W1V1-CoV pseudovirus neutralization
283 levels. For all within-dose group comparisons of vaccinated (2-5 doses) immunocompetent
284 participants (linear mixed model with Tukey HSD test; $P < 0.001$; Supplementary Figure 6b).
285
286

287 **Discussions**

288 As of May 2023, COVID-19-related health emergency restrictions were lifted across the globe
289 [5, 22]. However, SARS-CoV-2 infections continue to be recorded worldwide [6]. This situation
290 has been attributed to the ability of the virus to evade host immune responses including
291 neutralizing antibody-derived immunity. The vast majority of antibody escape mutations have
292 been associated with the S1 subunit of the spike protein especially the Receptor Binding Domain
293 (RBD) but also the N-terminal Domain (NTD) [14-16, 18]. The other region of the spike, the S2
294 subunit, is the most conserved region amongst coronaviruses [23]. We hypothesized that S2-

295 specific antibody responses are suboptimal in vaccinated and SARS-CoV-2 infected patients
296 resulting in an ineffective neutralization of distant coronaviruses.
297
298 Homology between the spike proteins of wild-type Wuhan and the distant SARS coronavirus
299 (SARS-CoV) and the coronavirus of bat origin (W1V1-CoV) have been previously reported at
300 75.6% and 76.5% [21], respectively. Booster doses induced efficient neutralization of the wild-
301 type Wuhan and the Omicron variants [21]. Our data were in accordance with these findings;
302 booster immunizations provided higher neutralization potency against Wuhan and the Omicron
303 variant while the distant relatives W1V1-CoV and SARS-CoV were less sensitive to serum
304 antibodies obtained from patients who received Wuhan-trimer based immunogens [21]. This
305 weak neutralization of distant coronaviruses was observed despite the S2 region of Wuhan and
306 Omicron sharing an almost 90% identity with SARS-CoV and W1V1-CoV. We correctly
307 predicted that S2-specific antibody levels are suboptimal in vaccinated COVID-19 patients as
308 confirmed by the lower S2-specific antibody levels in comparison with S1-specific antibody
309 levels.
310
311 Furthermore, we evaluated the benefit of booster immunization on antibody responses in
312 immunocompromised individuals. Even though we found elevated levels of spike-specific
313 antibody responses in immunocompromised individuals, these levels were lower than that
314 observed among immunocompetent individuals. The presence of significantly lower neutralizing
315 antibody titers for an immunocompromised patient have been previously observed [16]. A
316 contrasting result has also been reported with similar levels of S1-specific antibodies observed
317 between immunocompetent counterparts and a group of 584 immunocompromised patients with

318 hematologic cancers who received a third COVID_19 mRNA vaccine booster [24]. Moreover,
319 the benefit of a third vaccine booster was highlighted in another study which found elevated
320 humoral immune responses in immunocompromised children who had earlier received a second
321 booster vaccine [25].
322
323 More recently, the immunocompromised were found to have diminished SARS-CoV-2-specific
324 humoral by comparison with the immunocompetent in a large cohort of 56 immunocompromised
325 participants and 184 non-immunocompromised participants [10]. Our data are in accordance
326 with these later findings. We found lower neutralization antibody potency for
327 immunocompromised individuals versus immunocompetent.
328
329 **Limitations**
330 Our study is limited by modest sample sizes, particularly among the immunocompromised sub-
331 group, which may affect our ability to differentiate antibody responses between groups.
332 Another limitation to our study is the absence of samples from vaccinated but non-infected
333 participants. We were unable to discriminate between vaccine induced-immunity and natural
334 immunity following SARS-CoV-2 infection in the current study which only measured
335 spike/spike subunit-specific antibody responses. Future studies should include the measurement
336 of anti-nucleocapsid titers, as this protein is not included in Wuhan spike trimer-based mRNA
337 vaccine considered in this study.
338
339 In summary, we found that S1-specific antibody levels were higher in vaccinated and infected
340 COVID-19 patients. We also found that both S1- and S2- specific antibody responses generally

341 correlated with the four coronaviruses (SARS-CoV-2 Wuhan, Omicron, SARS-CoV and W1V1-

342 CoV). Among the immunocompromised, the most distinctive result was obtained with S2-

343 Wuhan-specific antibody levels which correlated with the neutralization capacity against

344 Omicron, SARS-CoV and W1V1-CoV.

345

346 **Perspectives**: Our data raises the hope that S2-targeting immunogens can successfully eliminate

347 the spread of SARS-CoV-2 variants [26-28]. The data encourages further efforts towards the

348 development of S2-targeting immunogens as universal vaccines for the eradication of SARS-

349 CoV-2 and the prevention of future excursion of coronaviruses into human populations.

350

351

352 **Methods**

353 **Study enrollment and sample collection.** Serum samples were obtained from the prospective

354 Post-Vaccination Viral Characteristics Study (POSITIVES) between January 2021 and May

355 2023. These samples were collected between 14 days and 48 days from the first PCR test with a

356 median of 20 days, an interquartile range of 7 days and 75% of samples being under 24 days.

357 Here, we consider the timing of the last dose in our analysis of the impact of the vaccine type,

358 Moderna or Pfizer/BioNTech COVID-19 mRNA. The study has been described in full detail

359 previously [10, 19]. In summary individuals in the Mass General Brigham medical record

360 system with confirmed COVID-19 infection were recruited by phone to join the study.

361 Consenting individuals provided nasal swabs for estimation of virologic decay and blood at

362 enrollment and days 14, 180, and 360. Demographic (sex, age and ethnicity) and vaccination

363 status were obtained from participant reports and medical record abstraction. The

364 immunocompromised individuals in this study have been previously described [10] and they
365 included non-severe immunocompromised participants as well as severe immunocompromised
366 participants [10]. The severe immunocompromised were individual with severe-hematological
367 malignancy/transplant patients (S-HT) and severe autoimmune patients (S-A, participants with
368 autoimmune condition receiving B-cell targeting agents or B cell deficiency) as previously
369 categorized and (NS) [29, 30].
370
371 **Ethics declaration.** This study was approved by the Institutional Review Boards of Boston
372 College (IRB Protocol Number # 21.115.01e) and Mass General Brigham (IRB# 2021P000812).
373 Informed consent was obtained from all participants. The authors confirm that all research was
374 performed in accordance with relevant guidelines/regulations.
375
376 **Cell Lines.** The 2 cell lines used in this study, Human Kidney Embryonic cells (HEK293T) and
377 HEK293T cells engineered to express the Angiotensin Convertase Enzyme 2 (293T-ACE2) have
378 previously been described [20, 31]. HEK293T-ACE2 cells were a gift from Dr. Huihui Mou and
379 Dr. Michael Farzan (SCRIPPS Research Institute, Florida, USA).
380
381 **SARS-CoV-2-specific antibody measurement by ELISA.**
382 **ELISA *Antigens***
383 Soluble SARS-CoV-2 spike S1 and S2 corresponding to the Wuhan and Omicron (B.1.1.529)
384 variants were obtained from Acro Biosystem. Wuhan version of SARS-CoV-2 spike S1 protein,
385 His Tag (Acro Biosystem catalog # S1N-C52H3) contains AA Val 16 - Arg 685 (Accession #
386 QHD43416.1). Wuhan version of SARS-CoV-2 spike S2 protein, His Tag (Acro Biosystem

387 catalog # S2N-C52H5) contains AA Ser 686 – Pro 1213 16 - Arg 685 (Accession #
388 QHD43416.1). Omicron/BA.1 version of SARS-CoV-2 Spike S2, His Tag (Acro Biosystem
389 catalog # S2N-C52Hf) contains AA Ser 686 - Pro 1213 (Accession # QHD43416.1 (N764K,
390 D796Y, N856K, Q954H, N969K, L981F, F817P, A892P, A899P, A942P, K986P, V987P).
391 Mutations are identified on the SARS-CoV-2 Omicron variant (Pango lineage: BA.1; GISAID
392 clade: GRA; Nextstrain clade: 21K). SARS-CoV-2 spike S1, His Tag (B.1.1.529/Omicron)
393 (S1N-C52Ha) contains AA Val 16 - Arg 685 (Accession # QHD43416.1 (A67V, HV69-70del,
394 T95I, G142D, VYY143-145del, N211del, L212I, ins214EPE, G339D, S371L, S373P, S375F,
395 K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H,
396 T547K, D614G, H655Y, N679K, P681H)). The spike mutations are identified on the SARS-
397 CoV-2 Omicron variant (Pango lineage: B.1.1.529; GISAID clade: GR/484A; Nextstrain clade:
398 21K).

399 ***S2 Antigen modification for solubility***

400 Proline substitutions (F817P, A892P, A899P, A942P, K986P, V987P) were introduced in both
401 spike S2 (Wuhan and Omicron versions) by the manufacturer (Acro Biosystem) in order to
402 prevent the formation of aggregates in the course of protein production.

403 ***ELISA Procedure***

404 ELISA was performed as previously described [11, 20, 32]. Briefly, 96-well Nunc MaxiSorp
405 ELISA plates (Thermo Scientific) were coated with viral antigens (Wuhan S1, Wuhan S2,
406 Omicron S1 or Omicron S2) diluted in carbonate-bicarbonate buffer to a concentration of 1
407 mg/mL before incubation for 1 h at room temperature. Plates were washed with a buffer
408 consisting of 50 mM Tris (pH 8.0) (ThermoFisher), 140 mM NaCl (MilliporeSigma), and 0.05%
409 Tween-20 (ThermoFisher). Next, plates were incubated with a blocking buffer consisting of 1%

410 BSA (MilliporeSigma), 50 mM Tris (pH 8.0), and 140 mM NaCl for 30 min at room
411 temperature. The plates were washed 1-3 times after blocking. Serum samples were diluted
412 1:100 with a dilution buffer consisting of 1% BSA, 50 mM Tris (pH 8.0), 140 mM NaCl, and
413 0.05% Tween-20. After sample addition, plates were incubated at 37°C for 30 minutes followed
414 by washing, 5 times. Serum IgG levels were detected by addition of HRP-conjugated anti-
415 human-IgG purchased from ThermoFisher (catalog # 62–8420) and diluted (1:4,000). The plates
416 were incubated for 30 min at room temperature. After the washes, TMB substrate
417 (ThermoFisher) was added to each plate for 10 min and the reaction was terminated with TMB
418 stop solution (Southern Biotech). Data were acquired by spectrophotometry at 450 nm using a
419 Victor X5 microplate reader (Perkin Elmer).
420
421 **SARS-CoV-2 Pseudovirus Production.** Pseudovirus production and titration have previously
422 been described [11, 20, 32]. The plasmids obtained from Addgene were gifted by Dr. Alejandro
423 Balazs. A group of 4 plasmids - pHAGE-CMV-luc2-IRES-ZsG-W (Addgene plasmid # 164432),
424 pRC-CMV-Rev1b (Addgene plasmid # 164443), pHDM-Tat1b (Addgene plasmid # 164442),
425 pHDM-Hgpm2 (Addgene plasmid # 164441) - were used for production of all pseudovirus
426 variants. Only the plasmid corresponding to the spike differed for the 4 viruses. The plasmids
427 pTwist-SARS-CoV-2 Δ18 (Addgene plasmid # 164436), pTwist-SARS-CoV-2 Δ18 B.1.1.529
428 (Addgene plasmid # 1789907), pTwist-W1V1-CoV Δ18 (Addgene plasmid # 164439), and
429 pTwist-SARS-CoV Δ18 (Addgene plasmid # 169465) were used for production of the Wuhan,
430 Omicron, SARS-CoV and W1V1-CoV pseudoviruses, respectively. A total of 5 plasmids were
431 therefore used for each of the 4 pseudoviruses with the spike expression plasmid being the only
432 variable. On the day before transfection, 12-15 million HEK293 T cells were seeded in T175

433 (ThermoFisher) in presence of 25 ml of DMEM10. Before transfection, culture media was
434 replaced with a fresh 25 ml DMEM10. The transfection was performed with GenJet (SignaGen
435 Laboratories) according to the manufacturer's recommendations. Twenty-four hours later,
436 transfection media was replaced with fresh DMEM10 and culture supernatant containing
437 secreted pseudoviruses was harvested 5 days post-transfection and cleared using a 0.45 µm
438 Nalgene syringe filter (ThermoFisher). The pseudovirus preparation was divided into 1 ml
439 aliquots per cryovial and stored at -80ºC.

440

441 **SARS-CoV-2 Pseudovirus Titration.** Titration of pseudovirus preparations has been previously
442 described [11, 20, 32]. Here, 293T-ACE2 cells (10^4 cells/well) were seeded in 100 µl of
443 DMEM10 into 96-well black/clear bottom plates purchased from ThermoFisher (catalog #
444 165305). For titration, 50 µl of 2x serially diluted pseudovirus preparation were added to
445 corresponding wells. Control (background) wells received 50 µl of DMEM10. On the fifth day,
446 Pseudovirus infectivity was quantified by luciferase assay using the previously described in-
447 house luciferin buffer [11, 33]. Assay plates were read using a Victor X5 microplate reader
448 (Perkin Elmer).

449

450 **SARS-CoV-2 Pseudovirus neutralization assay.** Pseudovirus neutralization has previously
451 been described [11, 20, 32]. All reagents, cells, virus and serum were added in a single
452 streamline with incubation and assay readout in the same plate, ThermoFisher 96-well
453 black/clear bottom plates. A luciferase readout of 30,000 luminescence rate units (LRU) was
454 targeted as viral input with a 5-day incubation period. Patients' sera were diluted with DMEM10
455 starting at 10-fold dilution and performing 3-time serial dilutions (from 1/10 to 1/21870). A

456 starting dilution of 20x (1/20 to 1/43740) and 30x (1/30 to 1/65610), when necessary, were
457 applied to the samples for which the 10-fold dilutions were insufficient to cross the 50%
458 neutralization mark. Fifty µl of pseudovirus preparations were added onto the diluted sera and
459 the mixtures were incubated for 1 hour at 37ºC before addition of HEK293T-ACE2 cells (10^4
460 cells/well) prepared in 50 µl of DMEM10. Background wells containing cells-only were
461 prepared while cells plus virus-only (no sera) were prepared as positive controls corresponding
462 to 100% assay readout. The plates were incubated at 37ºC, 5% CO2 and 70% humidity for 5
463 days. Following transduction, cells were lysed and luciferase assay performed as previously
464 described [11, 20, 33]. Fifty microliters of luciferin buffer containing 20 mM Tris-HCl
465 (ThermoFisher), 100 mM EDTA (ThermoFisher), 1 mM $MgCl_2$ (ThermoFisher), 26.5 mM
466 $MgSO_4$ (ThermoFisher), 17 mM dithiothreitol (Goldbio), 250 mM Adenosine-5'-Triphosphate
467 (Goldbio), 750 mM D-luciferin (Goldbio), were added to the well and incubated for 5 minutes
468 with agitation before luminescence was quantified within 30 minutes of buffer addition using a
469 Victor X5 microplate reader (Perkin Elmer). Neutralization curves were analyzed using
470 GraphPad prism. Neutralizing antibody responses (NT50) were calculated by taking the inverse
471 of the 50% inhibitory concentration value for each sample. Of note, the inverse serial dilution
472 number was multiplied by two to obtain the final NT50 values because (diluted) sera were
473 further diluted with equal volumes of pseudovirus during the serum-virus incubation step.
474
475 **Statistical analysis.** Graphpad Prism 9 (v9.3.1) was used to analyze neutralization data and
476 determine the 50% neutralization titer (NT50). R (v4.2.1) was used for all other statistical
477 analyses. Antibody binding means were analyzed using *t*-tests when comparing two groups (e.g.,
478 S1- vs S2-specific binding), with a paired test when the same patients were sampled in both

479 groups. Neutralizing antibody titer means were compared using a one-way ANOVA when all
480 samples were independent, and linear mixed models with patient ID as a random effect when the
481 same participant was measured for multiple antigens/pseudoviruses. Due to considerable skew in
482 NT50 values, in these analyses these values were transformed (log10 NT50+1). For significant
483 ANOVA and linear mixed models, differences among groups were identified with the post-hoc
484 Tukey HSD test. Linear correlations between antibody binding and neutralization were analyzed
485 using Pearson's correlation test. Across all tests an alpha of 0.05 was used to determine statistical
486 significance.

487

488 **Data availability**

489 Data presented in this study are available by request to the corresponding author.

490

491

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493

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579

580

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587

588 **Author contributions**

I.B.F., J.L., M.S., J.D. and J.L. contributed to the study design, data interpretation and writing of the manuscript. S.P. and B.L. helped with writing of the manuscript. S.P., B.L., P.B., J.F., D.S., A.R., A.J., J.V., E.P., D.T., Z.R., K.S., T.V., J.V., E.A., M.B., A.A., Z.W., J.D., M.C., T.T., G.E., Y.L., R.D., J.S., J.B., O.G., A.B., J.L., M.S., J.Z.L, and I.B.F. contributed to the laboratory data generation and analysis. I.B.F., J.Z.L. and J.D. contributed to statistical analysis and writing of the manuscript. J.L., M.S., and J.Z.L. contributed to clinical data collection and analysis. All authors reviewed and approved the manuscript.

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Competing interests

The authors declare no competing interests. J.Z.L has consulted for Abbvie and received a grant from Merck but none of these activities impacted nor influenced the design, performance and conclusions of this study.

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Table 1. Demographic and clinical characteristic of patients

Table 1. Demographic and clinical characteristic of patients

	Total	0 Doses	1 Dose	2 Doses	3 Doses	4 Doses	5 Doses
Patients (n=87)							
Female (n=60), %	68.976	5.747	0	8.046	34.483	10.345	10.345
Male (n=27), %	31.034	3.448	1.149	2.299	10.345	8.046	5.747
Last Shot, %							
Johnson & Johnson (n=1), %	1.149	0	1.149	0	0	0	0
Pfizer (n=40), %	45.977	0	0	6.897	24.138	6.897	8.046
Moderna (n=38), %	43.678	0	0	3.448	20.69	11.494	8.046
None (n=8), %	9.195	NA	NA	NA	NA	NA	NA
Race %							
White (n=69), %	79.31	6.897	1.149	6.897	36.782	13.793	13.793

Asian (n=2), %	2.299	0	0	0	2.299	0	0
Black or African American (n=6), %	6.897	0	0	1.149	2.299	2.299	1.149
Others and unknown (n=10), %	11.494	2.299	0	2.299	3.448	2.299	1.149

624

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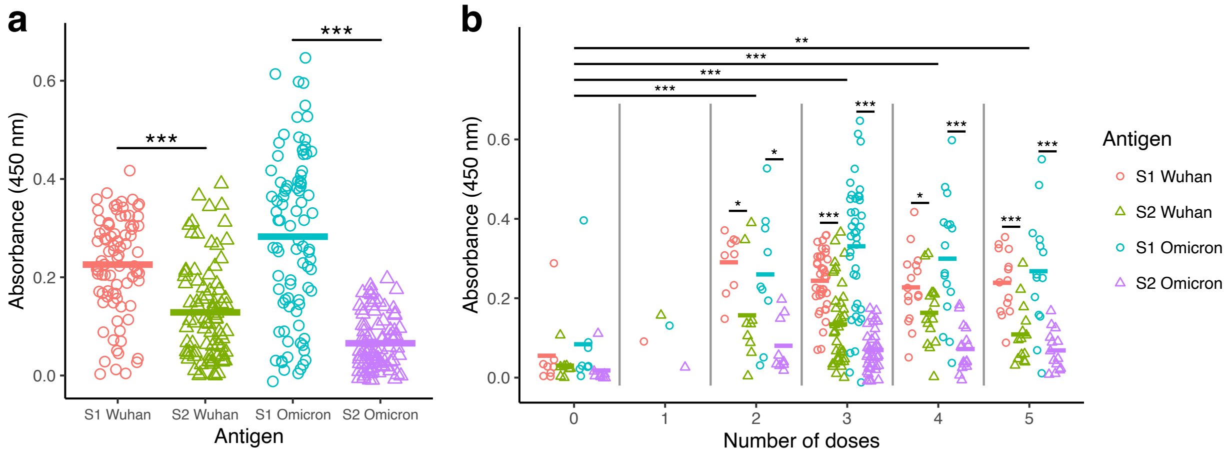


626

627 **Figure 1: High S1-specific and low S2-specific antibody levels were observed with**

628 **vaccinated and SARS-CoV-2-infected individuals.**

629 Binding antibody levels were measured using serum obtained from 87 study participants. Serum

630 binding antibody titers were measured by ELISA using S1-Wuhan, S2-Wuhan, S1-Omicron, and

631 S2-Omicron as antigens. Antibodies were detected using secondary anti-human IgG-HRP

632 conjugated. Absorbance was determined at 450 nm. (a) S1- and S2-specific antibodies levels

633 from all participants. Paired *t*-tests were used to compare antibody absorbance for S1 vs S2

634 regions of Wuhan and Omicron antigens. (b) S1- and S2-specific antibodies levels from all

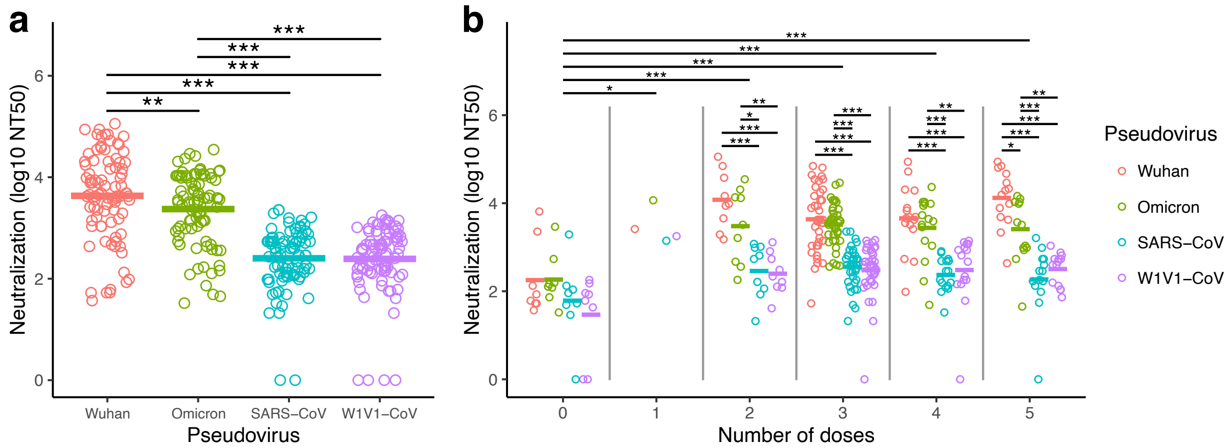
635 participants according to the number of vaccine doses. For each dose number, paired *t*-tests were

636 used to compare antibody absorbance for S1 vs S2 regions of Wuhan and Omicron antigens.

637 Means for each number of doses were compared using a linear mixed model with individual

638 patient identification as a random effect. Statistical significance was defined as $*P < 0.05$, $**P <$

639 0.01, and $***\ P < 0.001$.



640

641 **Figure 2: Highest antibody neutralization concentrations were observed against Wuhan**

642 **and Omicron pseudoviruses.**

643 Neutralizing antibody levels were measured using serum obtained from 87 study participants.

644 Neutralization concentrations were reported as a 50% neutralization titer (NT50). Values were

645 transformed due to skew in the data (log10 of NT50+1 transformation). (a) Neutralization titers

646 for Wuhan, Omicron, SARS-CoV, and W1V1-CoV pseudoviruses. Titer means of pseudoviruses

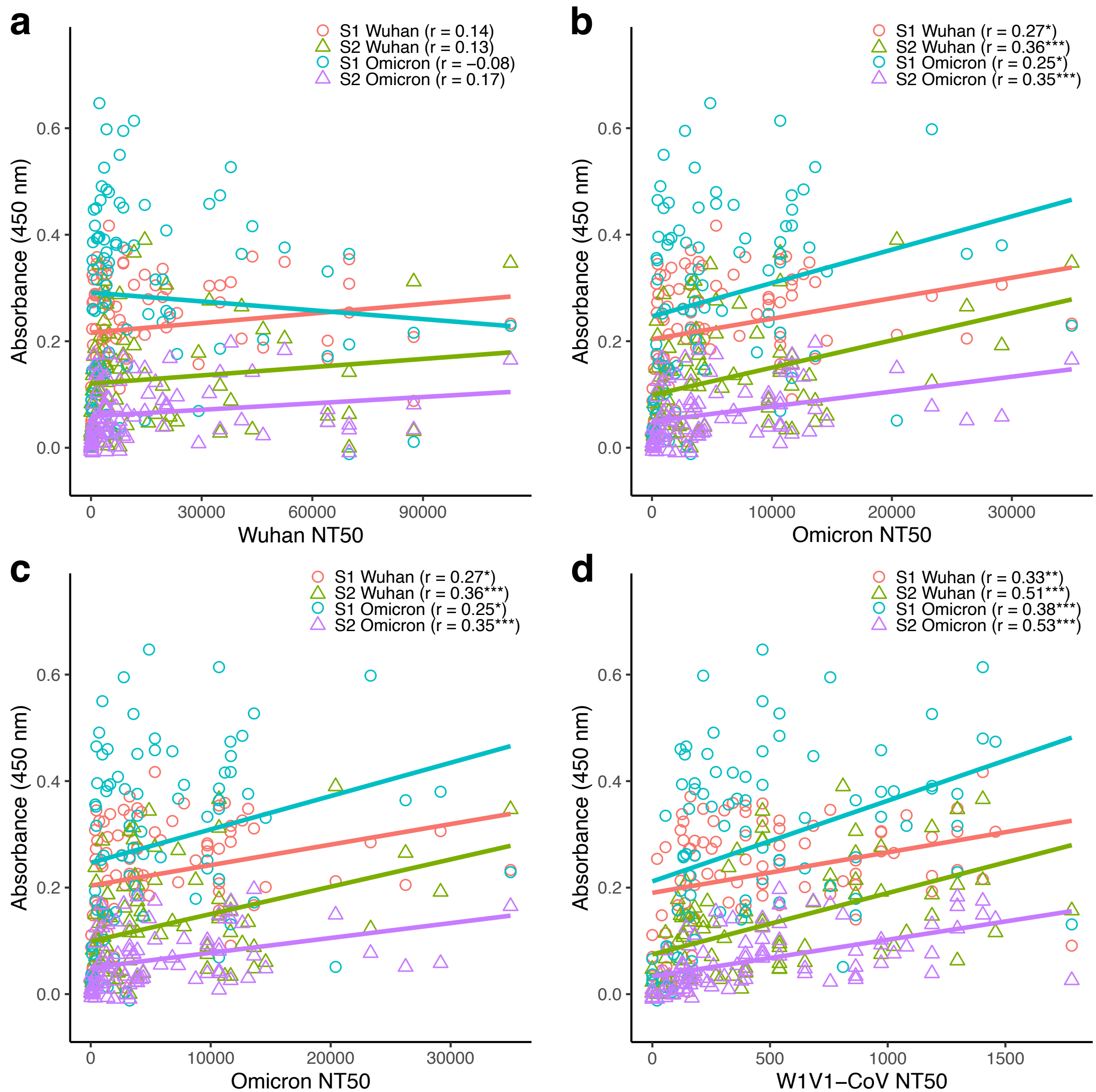
647 were compared using a linear mixed model with individual patient identification as a random

648 effect. (b) Neutralization titers analyzed according to the number of vaccine doses. Similar linear

649 mixed models were used to compare values within and among the number of doses. Statistical

650 significance was defined as *$P < 0.05$, **$P < 0.01$, and *** $P < 0.001$.

651



652

653 **Figure 3: Antibody titers were predominantly positively correlated to pseudovirus**

654 **neutralization.**

655 Binding (measured by ELISA) and neutralizing (measured as 50% neutralization titer; NT50)

656 antibody levels were compared for the 87 study participants. (a) S1-Wuhan, S2-Wuhan, S1-

657 Omicron and S2-Omicron specific levels vs Wuhan pseudovirus NT50. (b) S1-Wuhan, S2-

658 Wuhan, S1-Omicron, and S2-Omicron specific levels vs Omicron pseudovirus NT50. (c) S1-

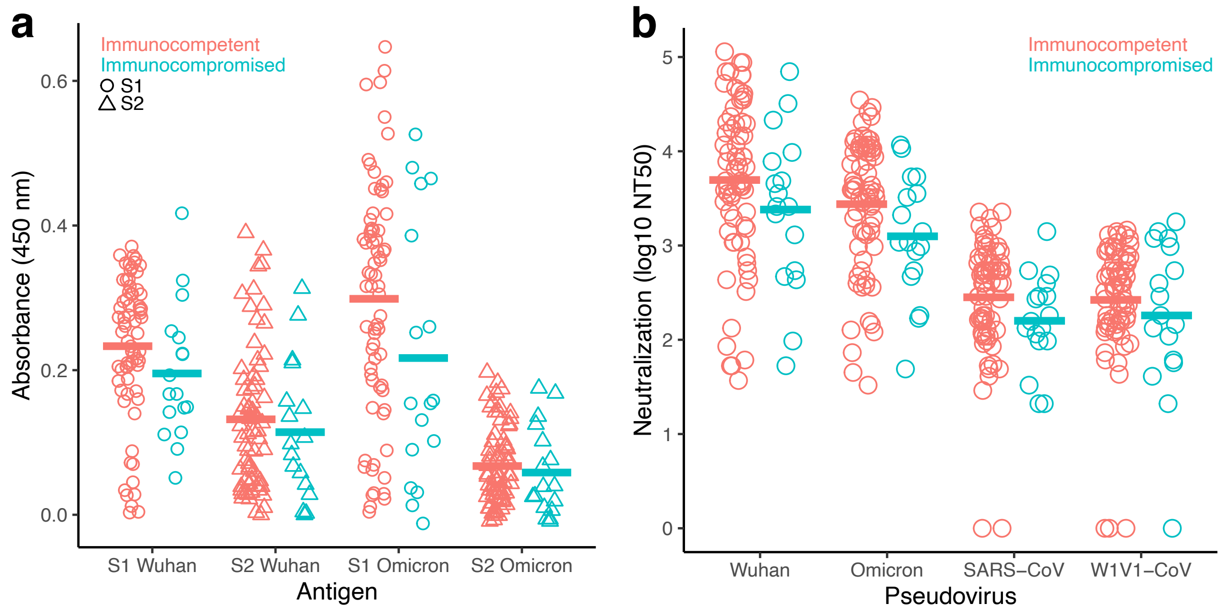
659 Wuhan, S2-Wuhan, S1-Omicron, and S2-Omicron specific levels vs SARS-CoV pseudovirus

660 NT50. (d) S1-Wuhan, S2-Wuhan, S1-Omicron, and S2-Omicron specific levels vs W1V1-CoV

661 pseudovirus NT50. Correlations were analyzed using Pearson's correlation, with statistical

662 significance defined as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

663

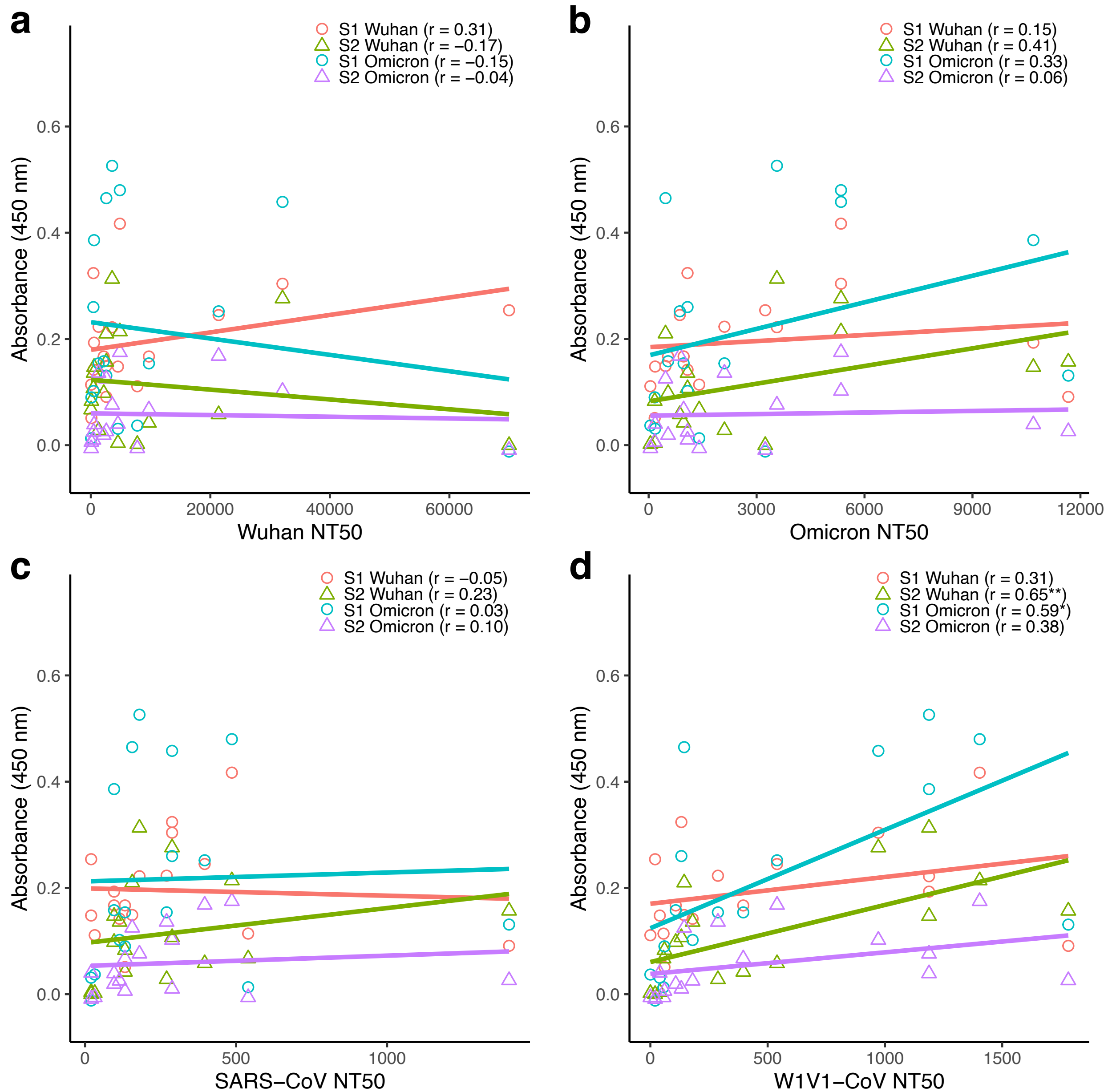


664

665 **Figure 4: Lower, but not significant, binding and neutralization antibody levels were**
666 **observed with the immunocompromised individuals.**

667 Binding and neutralizing antibody levels compared for 70 immunocompetent and 17
668 immunocompromised study participants. NT50 values were transformed due to skew in the data
669 (log10 of NT50+1 transformation). (a) S1- and S2-specific antibody levels measured by ELISA
670 using S1-Wuhan, S2-Wuhan, S1-Omicron, and S2-Omicron as antigens. (b) Serum antibody
671 neutralizing concentrations (50% neutralization titer; NT50) were determined against Wuhan,
672 Omicron, SARS-CoV and W1V1-CoV pseudoviruses. For each measurement,
673 immunocompetent and immunocompromised means were analyzed using a *t*-test. Statistical
674 significance was defined as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

675



676

677 **Figure 5: Antibody titers were generally positively, but not significantly, correlated to**

678 **pseudovirus neutralization in immunocompromised participants.**

679 Binding (measured by ELISA) and neutralizing (measured as 50% neutralization titer; NT50)

680 antibody levels were compared for the 17 immunocompromised participants. (a) S1-Wuhan, S2-

681 Wuhan, S1-Omicron, and S2-Omicron specific levels vs Wuhan pseudovirus NT50. (b) S1-

682 Wuhan, S2-Wuhan, S1-Omicron, and S2-Omicron specific levels vs Omicron pseudovirus

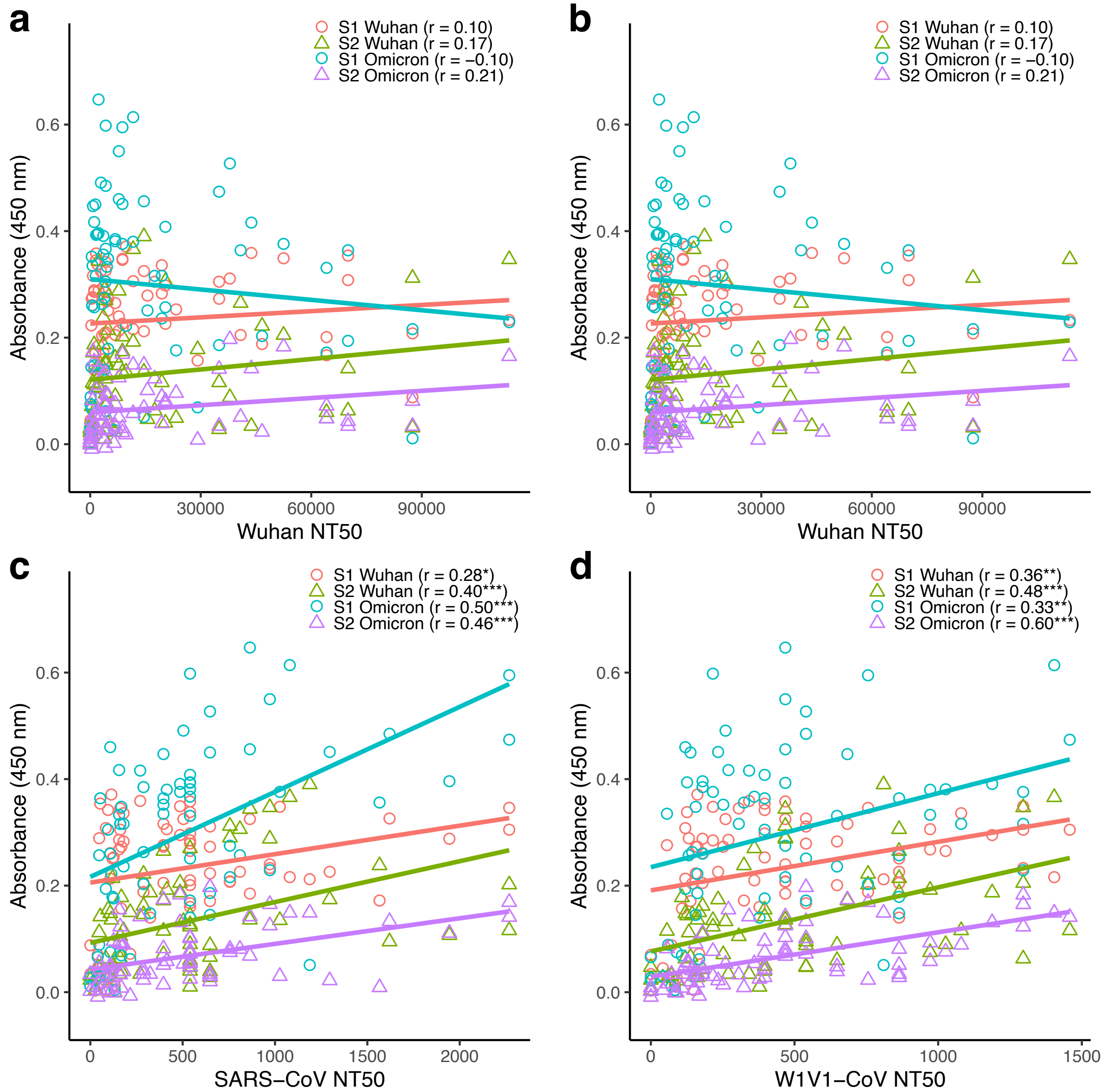
683 NT50. (c) S1-Wuhan, S2-Wuhan, S1-Omicron, and S2-Omicron specific levels vs SARS-CoV

684 pseudovirus NT50. (d) S1-Wuhan, S2-Wuhan, S1-Omicron, and S2-Omicron specific levels vs

685 W1V1-CoV pseudovirus NT50. Correlations were analyzed using Pearson's correlation, with

686 statistical significance defined as *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

687



688

689 **Figure 6: Antibody titers were predominantly positively and significantly correlated to**

690 **pseudovirus neutralization in immunocompetent participants.**

691 Binding (measured by ELISA) and neutralizing (measured as 50% neutralization titer; NT50)

692 antibody levels were compared for the 70 immunocompetent participants. (a) S1-Wuhan, S2-

693 Wuhan, S1-Omicron, and S2-Omicron specific levels vs Wuhan pseudovirus NT50. (b) S1-

694 Wuhan, S2-Wuhan, S1-Omicron, and S2-Omicron specific levels vs Omicron pseudovirus

695 NT50. (c) S1-Wuhan, S2-Wuhan, S1-Omicron, and S2-Omicron specific levels vs SARS-CoV

696 pseudovirus NT50. (d) S1-Wuhan, S2-Wuhan, S1-Omicron, and S2-Omicron specific levels vs

697 W1V1-CoV pseudovirus NT50. Correlations were analyzed using nonparametric Spearman

698 correlation on GrapPad prism. Correlations were analyzed using Pearson's correlation, with

699 statistical significance defined as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

Supplementary Files

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- Pateletal2024SupplementaryIBF241122.pdf