Antibody response to BTN162b2 mRNA vaccination in naïve versus SARS-CoV-2 infected subjects with and without waning immunity

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Abstract

We profiled antibody responses in a cohort of recipients of the BTN162b2 mRNA vaccine who were either immunologically naïve (n=50) or had been previously infected with SARS-CoV-2 (n=51). Of the previously infected, 25 and 26 were infected during the first and second pandemic waves in Italy, respectively; the majority of those from the first wave had corresponding waning immunity with low to undetectable levels of anti-S antibodies and low anti-N antibodies. We observed in recipients who had been previously infected that spike-specific IgG and pseudovirus neutralization titers were rapidly recalled by a single vaccine dose to higher levels than those in naïve recipients after the second vaccine dose, irrespective of waning immunity. In all recipients, a single vaccine dose was sufficient to induce a potent IgA response that was not associated with serum neutralization titers.

As we write, four COVID-19 vaccines have been authorized for use by FDA and/or EMA, and additional vaccine candidates are under evaluation. The authorized vaccines are based on the use of mRNA [1] or adenoviral vectors [2] that induce the expression of the SARS-CoV-2 spike protein. Apart from the Johnson & Johnson adenovirus-based vaccine that requires only a single dose, all other vaccines are based on a double dose regimen to maximize their efficacy.

Increasingly available anti-SARS-CoV-2 antibody and virus-specific T cell data support a strategy that previously infected (P.I.) vaccine recipients have sufficient immune response from only one vaccine dose;[3-6] this would have significant impact on global vaccine supply. Spike-specific IgG antibody levels and ACE2 antibody binding inhibition responses elicited by a single vaccine dose in individuals with prior SARS-CoV-2 infection (P.I.) were similar to those seen after two doses of vaccine in

individuals without prior infection (naïve) [7]. In another study, the antibody titers of recipients with preexisting immunity were 10 to 45-fold higher than naive recipients at the same time points after the first vaccine dose of vaccine, and no increases in antibody titers were observed in P.I. recipients who received the second vaccine dose [8]. Similarly, Bradley et al reported that after the first vaccine dose, recently infected P.I. recipients had higher titers of antibodies to the S1 and S2 subunit and the receptor-binding domain (RBD) of the spike protein compared to naïve recipients who had received the two doses of vaccine [9]. Interestingly, at baseline, naïve recipients exhibited a significant level of reactivity to the S2 subunit, suggesting a pre-existing cross-reactive response to common coronavirus infections [9]. In another study, antibody binding to a trimeric spike protein and live-virus neutralization assays performed on a cohort of volunteers who received one dose of an mRNA vaccine showed a rapid response in P.I. recipients with antibody titers raising at 7 days and reaching a peak 10-14 days post-vaccination, a kinetic that was significantly faster than that observed in naïve individuals [10]. In addition, two of these studies reported that vaccine reactogenicity was more prominent in P.I. individuals after the first dose but similar between the two groups after the second dose [7,8]. A cautionary tale for the use of a two doses regimen in P.I. individuals was raised by Levi et al who considered the possibility of antibody-dependent enhancement [11] or antigen exhaustion as a result of an over-boosting of immune responses [12]. The lack of an established correlate of protection against disease and/or infection adds further complexity. The emergence of SARS-CoV-2 variants is also a concern, and Stamatatos et al. highlighted the importance of two dose regimens in both naïve and P.I. individuals to achieve cross-variant neutralizing antibodies [13]. An additional component of the immune response to SARS-CoV-2 that could influence the outcome of vaccination is the presence in most

SARS-CoV-2 naïve individuals of variable levels of pre-existing immunity to spike protein epitopes that are shared with other common human coronaviruses (hCoVs); [14,15] these have been suggested to be potentially protective or pathogenic and may shape the kinetic and potency of the immune response to the vaccine [16-19].

Here we report data from a serological profile of a cohort of 101 naïve and P.I. recipients who received both doses of Pfizer-BioNtech BNT162b2 mRNA vaccine. We took advantage of the availability of two different sub-groups of P.I. recipients who experienced a SARS-CoV-2 infection during the first wave (Spring 2020) and the second wave (Autumn 2020) of the pandemic in Northern Italy to investigate the different effects of vaccination in recipients with recent-active or past-waning immunity. Antibody levels were measured at three time points: prior to first vaccination (T0), prior to second vaccination (T1), and three weeks after the second vaccination (T2). As the BNT162b2 vaccine is expected to elicit only IgG-S antibodies, we also tested all subjects for the presence of IgG-nucleocapsid (IgG-N) antibodies to identify recipients with past infection. The IgG-N antibody test is also a reliable marker of enduring immunity to SARS-CoV-2 [20] and as such can be used to monitor waning immunity. Since natural infection with SARS-CoV-2 is often followed by a rapid rise in IgG antibodies that can occur concomitantly or even before the appearance of IgM antibodies [14,21-23] we tested whether a similar pattern would follow vaccination by measuring both IgG antibodies specific for the RBD of the spike protein (IgG-S(RBD)) and IgM spike-specific antibodies (IgM-S).

IgA have also been implicated in protective immunity to SARS-CoV-2, but, to the best of our knowledge, there are no available data on the IgA response following vaccination and no potential association between vaccine-induced IgA response and serum virus-neutralizing activity. During natural SARS-CoV-2 infection, IgA

responses precede IgG responses [24-26]. Whether this is also the case after vaccination is unknown. We therefore tested all sera for the presence of IgG-N, IgM-S, IgG-S(RBD), and IgA-S as well as for the presence of virus-neutralizing activity as measured in a pseudovirus neutralization assay.

We enrolled 101 healthcare workers with (P.I.) or without (naïve) preexisting immunity to SARS-CoV-2. Of the 51 P.I. vaccinees, 25 had been infected during the first wave and 26 during the second wave. All subjects received the first vaccine dose (BNT162b2 mRNA, Pfizer-BioNTech) in January 2021. The two groups were homogeneous in age and sex (Table 1).

IgG-N antibody testing was negative in all naïve recipients and positive in 24/51 (47%) P.I. recipients (Fig. 1A). A single subject, originally classified as naïve, who resulted negative at baseline but highly positive at T1 and T2 for the presence of IgG-N was excluded from the analysis. In the P.I group, at baseline, 7/25 (28%) and 17/26 (65%) of those who were infected during the first and second waves, respectively, were positive for IgG-N antibodies, consistent with a trend toward waning immunity in recipients infected during the first wave (Fig. 2A and Table 2).

IgM-S antibodies measured before vaccination (T0) showed a similar pattern with 8/25 (32%) and 16/26 (61%) testing positive at baseline in the first and second wave P.I. recipients, respectively (Fig. 2B and Table 2). Following vaccination, 27/50 (54%) naïve recipients became IgM-S positive after the first dose, and 29/49 (59%, 20 of whom were already positive after the first dose) were positive after the second dose with no significant increase in antibody titers compared to baseline (Fig. 1B and Table 2). In P.I. recipients from the first or second wave, there was no significant difference in the IgM-S response after vaccination (Fig. 2B and Table 2).

IgG-S(RBD) were detectable after the first vaccine dose in 49/50 (98%) naïve recipients but with very low titers that were boosted by the second vaccine dose resulting in a highly significant increase ($p<0.0001$) (Fig. 1D and Table 2). Forty-six/51 (90%) P.I. recipients showed low IgG-S(RBD) titers at baseline (Fig. 1D and Table 2), and the first vaccine dose induced a strong increase in IgG-S(RBD) reaching levels that were 12-fold higher than titers observed in naïve subjects after the first dose (naïve T1: 1673±1787; P.I. T1: 20131±18990; $p<0.0001$) and 1.9-fold higher when comparing P.I. and naïve recipients after the second dose (naïve T2: 19551±10941; P.I. T2: 37607±22895; $p=0.4583$, ns), whereas it was similar when comparing P.I. recipients after the first dose to naïve recipients after the second dose (naïve T2 19551±10941; P.I. T1: 20131±18990, $p>0.9999$, ns). The second vaccine dose in P.I. recipients did not result in a significant increase in antibody titers consistent with data reported by other authors [7-9,27]. Comparison of P.I. recipients infected during the two waves did not show any significant difference in the responses to the first and second vaccine dose (Fig. 2D and Table 2), although there was an unexpectedly higher (although not statistically significant) response to the first dose in those infected during the first wave compared with those infected during the second wave, with recipients showing binding titers as high as 80000 AU/ml.

The pseudovirus neutralization assay (TCID50) essentially mirrored the results of the IgG-S(RBD) assay with 47/50 (94%) naïve recipients showing a weak but positive score (>120 for TCID50) after the first dose and 49/49 (100%) after the second dose, but with a highly significant ($p<0.0001$) increase in neutralizing titers (Fig. 1E). In P.I. recipients, we observed the same pattern seen in the IgG-S(RBD) assay with an efficient boost of neutralizing antibodies after the first dose ($p<0.0001$) and no further increase after the second dose (Fig. 1E).

A correlation analysis between the IgG-S(RBD) and the pseudovirus neutralization assay (TCID50) confirmed a strong association between serum IgG-S(RBD) and neutralizing titers (Fig. 3A) consistent with other reports showing a major role played by RBD-specific antibodies in virus neutralization [28]. Results of the two waves P.I. cohorts also mirrored those obtained with the IgG-S(RBD) and evidenced a surprisingly, although not statistically significant, higher virus-neutralization response in P.I. recipients of the first wave compared with the second, with TCID50 titers as high as 1/4000 (Fig. 2E). These data are strongly suggestive of the persistence of memory B cell responses that can be rapidly recalled by a single vaccine dose after 9-10 months from primary infection even in the absence of detectable serum IgG-S(RBD) antibodies. It is also conceivable that natural infection with SARS-CoV-2 may prime the immune system to produce antibody specificities other than RBD that can be readily recalled by a single dose of vaccine.

The potential implication of cross-reactive immunity to other coronaviruses in the response to vaccination is supported by an unexpected feature that emerged from our data: the unconventional isotype pattern observed in both naïve and P.I recipients. In the naïve recipients, after the first dose, when the canonical primary immune response is expected to generate IgM first followed by IgG, only 27/50 (54%) recipients were positive for IgM-S with no further increase after the second dose (29/49; 59%), whereas 49/50 (98%) and 49/49 (100%) naïve recipients scored positive for IgG-S(RBD) after the first and second dose respectively (Table 2). Twenty-three/50 (46%) naïve recipients showed an IgG-S(RBD) positive test but were negative for IgM-S, 27/50 (54%) were positive for both IgG-S(RBD) and IgM-S, and none were positive for IgM-S and negative for IgG-S(RBD) (Table 3). This isotype pattern is consistent

with that of an anamnestic response sustained by memory B cells specific for spike epitopes shared with other common human coronaviruses [14,15].

At baseline, IgA-S were detected in none of the naïve recipients, in 18/25 (72%) of the P.I. recipients who were infected during the first wave, and in 22/26 (84%) of those infected during the second wave (Fig. 1C and Table 2). The first vaccine dose resulted in a significant increase ($p<0.0001$) in IgA-S titers in both naïve and P.I. recipients (Fig. 1C). The second dose further boosted the IgA-S titers in naïve recipients ($p<0.0001$) but led to a significant reduction in P.I. recipients ($p=0.0099$). The decline in IgA titers after the second vaccination in P.I. recipients was not related to the time from infection, as it was observed in both subjects infected during the first and second wave and most likely represents a response to the vaccination (Fig. 2C). A correlation analysis between the IgA-S and IgG-S(RBD) titers at baseline and T1 and T2 revealed in naïve subjects the appearance of high IgA titers after the first vaccine dose followed by a significant increase in IgG-S(RBD) titers only after the second dose (Fig. 3B). In P.I. recipients, the first and second dose boosted both IgA-S and IgG-S(RBD) titers (Fig. 3B). We did not observe a correlation between IgA-S titers and virus-neutralization titers (TCID50) both in naïve and P.I. recipients (Fig. 3C). On the other hand, the early increase in IgA after the first dose that preceded the increase in IgG-S(RBD) titers after the second vaccine dose is consistent with what has been observed during natural SARS-CoV-2 infection where IgAs precede IgGs [25,26,29]. The presence of IgA-S in a significant proportion of P.I. recipients at baseline is also consistent with the slower waning of IgA compared to IgG observed in convalescent patients [30].

We next examined the influence of gender in the IgG-S(RBD) and IgA-S responses to vaccination. In the naïve recipients, there were no differences in the kinetic and size

198 of IgG-S(RBD) responses between males and females. In contrast, in the P.I group,
199 males responded to the vaccine by producing higher titers of IgG-S(RBD) than
200 females ($p<0.05$) (Fig. 4A). This difference was reflected by the neutralization assay,
201 which showed higher neutralizing titers in males than in females (Fig.4B). The
202 differences between the two groups were significant in male and female naïve IgA-S
203 responses at T1 ($p<0.05$) and T2 ($p<0.01$) (Fig.4C).
204 Taken together these data show that: 1) immunologically naïve recipients react to the
205 first dose of vaccine with a low-titer IgG-S(RBD) response that is then boosted by the
206 second dose; 2) in previously infected recipients one dose of vaccine is sufficient to
207 induce antibody titers that are higher than those observed in naïve recipients after the
208 second vaccine dose; 3) there is a good correlation between IgG-S(RBD) titers and
209 virus neutralizing titers, confirming that the IgG-S(RBD) testing is a proxy for virus
210 neutralization; 4) recipients who were infected with SARS-CoV-2 during the first
211 pandemic wave exhibit a rapid response to vaccination measured as both IgG-S(RBD)
212 binding titers and virus neutralizing titers; 5) in 46% of naïve recipients, IgG-S(RBD)
213 appear in the absence of IgM-S as if the response to vaccination was influenced by
214 previous antigen exposures; 6) in naïve recipients IgA-S appear before IgG-S(RBD)
215 after the first vaccine dose and are further boosted by the second dose; 7) in
216 previously infected recipients, IgA show a rapid, high titer response to the fist vaccine
217 dose that is followed by a decline with the second dose; 8) there is no correlation
218 between IgA-S antibody titers and virus neutralization; 9) neutralization titers and
219 IgG-S(RBD) were higher in previously infected male recipients compared to female
220 but not in naïve recipients.
221 Our findings expand on previous studies that indicated higher levels of anti-S
222 antibodies at baseline and after a single mRNA vaccine dose in previously infected

223 individuals compared with those without prior infection, suggesting that a second
224 vaccine dose does not offer P.I. recipients a substantially greater benefit over a single
225 dose in antibody neutralization. The availability of two sub-groups of recipients who
226 had been infected during the first and second waves of the pandemic in Italy gave us
227 the additional opportunity to evaluate the effects of one dose versus two doses of
228 vaccination in the context of a past-waning immunity and compare it with that of
229 recent-active immunity. The lower IgG-N and IgG-S(RBD) antibody titers observed
230 at baseline in the first wave P.I. vaccines compared to the second wave P.I vaccines
231 (Fig. 2A) gave us confidence that the two cohorts were in two different stages of post-
232 infection immunity. However, when the IgA-S antibody titers were considered, no
233 substantial differences were observed between the two cohorts at baseline, indicating
234 that serum IgAs may be a better marker of long-lasting immunity and that
235 immunological memory may be more long-lasting than what previously estimated on
236 the basis of IgG antibody levels alone. This was further substantiated by the surprising
237 rapid recall of high IgG-S(RBD) and virus neutralizing titers observed in first wave
238 P.I. recipients even in individuals with absent or very low serum IgG-S(RBD) levels.
239 Although not statistically significant, the response observed in the first wave P.I.
240 recipients was greater than that observed in the second wave P.I. recipients, probably
241 as a result of the persistence of memory B cell responses that can be rapidly recalled
242 by a single dose and consistent with recent data on the appearance in convalescent
243 patients of memory B cells with a turnover time of 6 months that express antibodies
244 with increased somatic hypermutations, neutralizing breadth, and potency [31]. The
245 possibility that natural infection with SARS-CoV-2 may prime the immune system to
246 produce antibody specificities other than RBD, further expanding the antibody
247 repertoire induced by vaccination, should also be considered. Our study did not

address the epitope specificities of vaccination-induced antibodies, and additional studies addressing the fine specificities of vaccine-induced antibodies are warranted.

A priming effect of previous exposures to common human coronaviruses on the immune response to the vaccine is suggested by our findings in the cohort of naïve recipients showing IgG-S(RBD) antibodies in the absence of IgM-S antibodies, an isotype pattern typical of anamnestic responses. Antibodies that cross-neutralize SARS-CoV-1, SARS-CoV-2, and other coronaviruses have been described that bind conserved epitopes of the hACE2 binding site showing extensive conservation among the SARS-like coronaviruses [32]. It remains to be determined whether the BTN162b2 mRNA vaccine is capable of eliciting these types of antibodies.

Our findings on the more rapid and potent IgA response compared with IgG responses to the first vaccine dose parallel those in SARS-CoV-2 infected patients, where IgA antibodies that bind to SARS-CoV-2 are produced rapidly after infection and remain elevated in the plasma for at least 40 days after the onset of symptoms [33]. We did not see a significant association between serum IgA and virus-neutralizing activity post-vaccination, which, in contrast, has been reported in COVID-19 patients [28]. However, it is plausible that the types of IgA antibodies elicited by intramuscular vaccination may differ from the compartmentalized, mucosal immune response to natural infection. Accordingly, SARS-CoV-2 specific plasma IgA monomers have been shown to be two times less potent than IgG equivalents and, in contrast, IgA dimers, the principal type of antibody in the nasopharynx, are 15 times more potent against the same target as IgA monomers.[33]

Our data clearly show a rapid and potent induction of IgG-S(RBD) after a dose of vaccine in individuals with past-waning immunity. Although a clear correlate of protection from COVID-19 has not yet been identified, the recent findings by Zohar

and colleagues that, notwithstanding equivalent IgM and IgA immunity to the virus observed in different disease severity levels, rapid and potent IgG class switching is associated with survival [34] provide an additional argument in support of the use of a single-dose vaccine regimen in individuals with prior SARS-CoV-2 infections. Limitations of our study include the design (cross-sectional), the limited sample size, the use of only one type of vaccine (Pfizer-BioNTech BNT162b2), the lack of information on T-cell responses, and neutralization response against emerging SARS-CoV-2 variants of concern.

Methods

The sera of 101 healthcare workers with and without pre-existing immunity for SARS-CoV-2 (as per former nasal swab positivity) who received their first vaccine dose (BNT162b2 mRNA, Pfizer-BioNTech) in January 2021 were analyzed. Samples had been collected and stored in the University of Verona biobank (Ethics Committee approval prot. N. 1538) and in Tropica Biobank of the IRCCS Sacro Cuore Don Calabria Hospital (Ethics Committee approval prot. N. 50950). All participants signed informed consent.

The SARS-CoV-2 IgG-N assay and the SARS-CoV-2 IgM-S assay (Abbott, Ireland) are chemiluminescent microparticle immunoassays (CMIA) used to detect IgG antibodies to the nucleocapsid protein and IgM antibodies to the spike protein, respectively, of SARS-CoV-2 in human serum. The automated assay was performed according to the manufacturer's procedure, using the ARCHITECT I System (Abbott). The resulting chemiluminescent reaction was measured as a relative light unit (RLU) by the system optics. The RLU of the sample (S) was automatically compared with the RLU of a specific calibrator I, resulting in an assay index (S/C).

As per manufacturer's instructions, the interpretation of the results were as follow: index (S/C)<1.4 = negative, index (S/C)≥1.4 = positive for IgG-N, and index (S/C)<1 = negative, index (S/C)≥1 = positive, for IgM-S.

Serum samples were tested for the presence of SARS-CoV-2 IgA using the Anti-SARS-CoV-2 ELISA kit (EUROIMMUN Medizinische Labordiagnostika AG, Germany). The assay detects IgA antibodies in serum binding the S1 domain of the SARS-CoV-2 spike protein. Samples were tested and analyzed as recommended by the manufacturer, and results reported as a ratio based on sample O.D. divided by the O.D. of the calibrators. Antibodies were considered undetectable (negative result) if the ratio was less than 0.8, borderline (inconclusive) between 0.8 and 1.1, and positive if greater 1.1.

The SARS-CoV-2 IgG II Quant assay (Abbott, Ireland) is a CMIA used for the quantitative measure of IgG-S(RBD) antibodies (including neutralizing Abs) in human serum. The automated assay was performed according to the manufacturer's procedure, using the ARCHITECT I System (Abbott). Results were reported as Arbitrary Unit (AU)/mL, according to the following interpretation: AU/mL<50 = negative, AU/mL≥50 = positive. According to the WHO International Standard for anti-SARS-CoV-2 immunoglobulin binding antibody units (BAU), the AU/mL are converted into BAU by the equation: AU/mL* 0.142 = BAU/mL.

Lentiviral particles pseudotyped with SARS-CoV-2 spike were produced in 10 cm plates seeded the day before with 3 million HEK293T cells in 10 ml of complete DMEM, supplemented with 10% FBS. Cells were transfected using the Calcium Phosphate technique with 15 μg of an Env-defective SIV-Mac239 provirus construct expressing GFP in place of Nef [35] and 1.5 μg PCDNA3.1 expression vector encoding the WT SARS-CoV-2 spike (reference sequence Wuhan-Hu-1, accession number

YP_009724390) with a truncation of the C-terminal 19 amino acids. Supernatants containing pseudotyped virions were harvested 48 hours post-transfection, filtered through a 0.45-µm filter, and frozen at -80°C until used. Sera neutralization titers were assayed on Huh-7 cells engineered to overexpress the SARS-CoV-2 receptor ACE2 upon stable transduction with a lentiviral expression vector. Target cells were seeded on 384-well tissue culture plates one day before neutralization. Virus inoculum was adjusted to produce no more than 10% of monolayer transduction to ensure a linear working range of the assay. Sera dilutions were added to target cells using an acoustic dispenser (Beckman Echo 650) to reach the indicated dilution in DMEM with 10% FBS. Pseudotyped virus was then added to wells using a Tecan Evo® 200 liquid handler. After 48 hours, transduction was assessed by calculating the percentage of GFP-expressing cells upon nuclei counterstaining with Hoechst 33342 and measuring using the High Content Molecular Device Image Xpress® Micro Confocal. Each serum dilution was evaluated in triplicate. Neutralization was measured by calculating the residual transduction activity of the pseudovirus considering the untreated sample as 100%. Fitted sigmoidal curves and IC50 were obtained using Prism (Graphpad) with the least square variable slope method and using the normalized dose response protocol.

Statistical analysis

P values were calculated using the non-parametric two-tailed unpaired Kruskal-Wallis test (Fig. 1 and Fig. 2), the two-sided Spearmen rank-correlation test (Fig. 3), the Wilcoxon matched-pairs signed ranked test (Fig. 4), and the chi-squared test (Table 1) using SPSS (version 22, SPSS Inc.) and Prism 9 (GraphPad Software, LLC).

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Requests for data may be directed to the corresponding authors. Data limitations are designed to ensure patients and participant confidentiality.

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Author Contributions:

L.D.C. designed the study, enrolled patients, analyzed data and discussed results; M.T.V. designed the study, collected samples and clinical data; Z.B. and C.P. designed the study, collected data, managed data and critically revised the manuscript; M.P. and S.A. performed and analyzed pseudovirus neutralization assays; S.M. , S.F., D.A. L.M., N.T. and S.S.L. participated in data collection and analysis; A.M., J.B., V.L.V., G.D. and E.C. collected samples and clinical data; L.L. analyzed and

discussed data and critically revised the manuscript; A.B. analyzed and discussed data, drafted and wrote the manuscript; D.Z. designed and coordinated the study, analyzed data and results, prepared figures and tables, drafted and wrote the manuscript.

Competing Interests Statement

All authors declare no competing financial interests.

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Tables

Table 1. Characteristics of the study population

	Naïve (50)	**P.I. (51)**	**p**
Males/Females	13/37	19/32	p=0.23 (ns)
Age (yrs)	43±13	46±12	p=0.21 (ns)
Fever after 1st dose (%)	7.7	0	p=0.40 (ns)
Discomfort after 1st dose (%)	26.9	22.2	p=0.78 (ns)
Fever after 2nd dose (%)	38.5	33.3	p=0.79 (ns)
Discomfort after 2nd dose (%)	53.8	66.7	p=0.51 (ns)

P.I: Previously infected; ns: not significant

Table 2. Antibody and neutralization assays (positives/total and percent)

Test	Time	Naïve	P.I.	1st wave	2nd wave
IgG-N	T0	0/48 (0%)	24/51 (47.1%)	7/25 (28%)	17/26 (65.4%)
	T1	0/50 (0%)	23/51 (45.1%)	7/25 (28%)	16/26 (61.5%)
	T2	0/49 (0%)	21/49 (42.9%)	6/25 (24%)	14/24(58.3%)
IgM-S	T0	0/48 (0%)	24/51 (47.1%)	8/25 (32%)	16/26 (61.5%)
	T1	27/50 (54%)	23/51 (45.1%)	8/25 (32%)	15/26 (57.7%)
	T2	29/49 (59.2%)	15/49 (30.6%)	5/25 (20%)	10/24 (41.7%)
IgG-S(RBD)	T0	0/48 (0%)	46/51 (90.2%)	23/25 (92%)	23/26 (88.5%)
	T1	49/50 (98%)	50/51 (98%)	25/25 (100%)	25/26 (96.2%)
	T2	49/49 (100%)	49/49 (100%)	25/25 (100%)	24/24 (100%)
IgA-S	T0	0/49 (0%)	40/51 (78.4%)	18/25 (72%)	22/26 (84.6%)
	T1	44/50 (88%)	51/51 (100%)	25/25 (100%)	26/26 (100%)
	T2	49/49 (100%)	49/49 (100%)	25/25 (100%)	24/24 (100%)
TCID50	T0	2/48 (4.2%)	46/51 (90.2%)	23/25 (92%)	23/26 (88.5%)
	T1	47/50 (94%)	51/51 (100%)	25/25 (100%)	26/26 (100%)
	T2	49/49 (100%)	50/50 (100%)	25/25 (100%)	25/25 (100%)

P.I.: Previously Infected

Table 3. Number and percent of IgM-S and IgG-S(RBD) positive subjects

		Naïve			P.I.			1st wave			2nd wave		
IgM	**IgG**	**T0**	**T1**	**T2**	**T0**	**T1**	**T2**	**T0**	**T1**	**T2**	**T0**	**T1**	**T2**
+	+	0 (0%)	27 (54%)	29 (59.2%)	24 (47.1%)	22 (43.1%)	15 (30.6%)	8 (32%)	8 (32%)	5 (20%)	16 (61.5%)	14 (53.8%)	10 (41.7%)
+	-	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (2%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (3.8%)	0 (0%)
-	+	1 (2.1%)	23 (46%)	20 (40.8%)	22 (43.1%)	28 (54.9%)	34 (69.4%)	15 (60%)	17 (68%)	20 (80%)	7 (26.9%)	11 42.3%)	14 (58.3%)
-	-	47 (97.9%)	0 (0%)	0 (0%)	5 (9.8%)	0 (0%)	0 (0%)	2 (8%)	0 (0%)	0 (0%)	3 (11.5%)	0 (0%)	0 (0%)

P.I.: Previously Infected

Figure legends

Fig. 1: analysis of the antibody response profile at the time of first vaccination (T0), second vaccination (T1), and 3 weeks after the boost (T2) in naïve and previously infected (P.I.) recipients. Median values with the interquartile range are displayed. The horizontal dot lines indicate the limit of each assay, according to the manufacturer's instructions. Panel A: IgG for the SARS-CoV-2 nucleocapsid protein N; panel B: IgM for the spike glycoprotein; panel C: IgA for the spike glycoprotein; panel D: IgG for the receptor-binding domain (RBD) of the spike; panel E: pseudoviruses neutralization assay, expressed as median tissue culture infectious dose (TCID50). Sample sizes are reported in Table 2. P values were calculated using the non-parametric, two-tailed unpaired Kruskal-Wallis test. Differences were considered significant if $p<0.05$.

Figure 2: analysis of the antibody response profile at the time of first vaccination (T0), second vaccination (T1) and 3 weeks after (T2) in subjects infected during the first (orange dots) and the second (red dots) COVID-19 wave. Median values with the interquartile range are displayed; the horizontal dot lines indicate the limit of each assay, according to the manufacturer's instructions. Panel A: IgG for the SARS-CoV-2 nucleocapsid protein N; panel B: IgM for the spike glycoprotein; panel C: IgA for the spike glycoprotein; panel D: IgG for the receptor-binding domain (RBD) of the spike; panel E: neutralization assay, expressed as median tissue culture infectious dose (TCID50). Sample sizes are reported in Table 2. P values were calculated using the non-parametric, two-tailed unpaired Kruskal-Wallis test. Differences were considered significant if $p< 0.05$.

Figure 3: analysis of the correlation at time of first vaccination (T0), second vaccination (T1), and 3 weeks after (T2) in naïve (top row) and previously infected (P.I.) recipients (bottom row). Panel A: correlation between IgG-S(RBD) and neutralization (TCID50); panel B: correlation between IgG-S(RBD) and IgA; panel C: correlation between neutralization (TCID50) and IgA-S. Sample sizes are reported in Table 2. The correlation was calculated using the two-sided Spearmen rank-correlation test.

Figure 4: antibody response in naïve and previously infected (P.I.) male (blue dots) and female (red dots) subjects following vaccination. Panel A: IgG-S(RBD) titers at the time of first vaccination (T0, n. males/females 12/36 for naïve, 19/32 for P.I.), second vaccination (T1, n. males/females 13/37 for naïve, 19/32 for P.I), and 3 weeks after the boost (T2, n. males/females 13/36 for naïve, 19/30 for P.I); panel B: neutralization activity expressed as TCID50 (n. males/females: T0 12/36, T1 13/37, T2 13/36 for naïve, T0 19/32, T1 19/32, T2 19/31 for P.I.); panel C: IgA-S antibody titers (n. males/females: T0 12/37, T1 13/37, T2 13/36 for naïve, T0 19/32, T1 19/32, T2 18/31 for P.I.). P-values were calculated using the Wilcoxon matched-pairs signed ranked test and considered significant if $p<0.05$.