Neutralising reactivity against SARS-CoV-2 B.1.617.2 (Delta) variant by vaccination status and pre-exposure

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Abstract

In February and March 2020, one of the first Italian clusters of SARS-CoV-2 infection was detected in the municipality of Vo’. Positive subjects were followed up at 2 and 9 months post-infection with different immuno-assays and a micro-neutralisation test. Here we report on the results of the third serosurvey conducted in the same population in June 2021, 15 months post-infection, when we tested 61% of the infected individuals (n=76). Antibodies against the spike (S) antigen significantly decreased (P<0.006, Kruskal-Wallis test) among unvaccinated subjects (n=35) and increased (P<0.0001) in vaccinated individuals (n=41), whereas those against the nucleocapsid (N) decreased in the whole cohort. From the comparison with two control groups (naïve Vo’ inhabitants (n=20) and healthcare workers (HCW, n=61)), subjects vaccinated post exposure (hybrid immunity) had higher antibody levels (P<0.0001) than subjects vaccinated when naïve. Two doses of vaccine elicited stronger anti-S antibody response than natural infection (P<0.0001). Finally, the neutralising reactivity of sera against the B.1.617.2 (Delta) was lower than compared to the B.1 strain (median 1:320 versus 1:1280 1/dil, P<0.0001, and 1:640 versus 1:2560 1/dil, P=0.0014, after one or two vaccine doses, respectively), although subjects with hybrid immunity maintained neutralising titres above 1:40 1/dil.

Introduction

Understanding the extent and duration of protection developed upon natural SARS-CoV-2 infections and vaccination is a current research priority. Evidence suggests that more than 90% of COVID-19 patients seroconvert after natural infection and develop variable levels of neutralising antibodies \(^1\)–\(^3\), and demonstrates that the currently EMA and FDA approved vaccines induce humoral and cellular immunity in most individuals \(^4\)–\(^7\). However, antibody titres have been reported to wane over time\(^8\). Although memory B cells and cellular immunity can offer a quick and potent response in case of re-exposure to the virus\(^9\), preventing re-infections\(^10\)–\(^15\) and offering long-term protection regardless of the presence of antibody-escaping mutations\(^16\)–\(^18\), the interplay between antibody and cellular immunity, and the variation of naturally- and vaccine-acquired protection, remain to be fully characterised and understood. From an immunological perspective, there can be significant differences in the immune response generated by vaccines in individuals who were not exposed to SARS-CoV-2 before vaccination, and in subjects who recovered from a naturally acquired infection (so called ‘hybrid immunity’). Recent studies have reported of increased potency of ‘hybrid immunity’, with viral antigen persistence in some tissues being hypothesised as a potential mechanism driving the process of memory B and T cell maturation, resulting in an increased affinity against viral antigens.

Long term immunity against SARS-CoV-2 infection is jeopardized by the continuous evolution of the virus, which has led to the emergence of new strains with increased transmissibility or capable of partially
escaping the immune protection elicited from infection and vaccination, thus posing further challenges for
epidemic control. The most widespread variants currently circulating are called Delta plus strains\textsuperscript{19,20} and
belong to a group of sub-lineages of the B.1.617.2 variant of concern (VOC), which emerged in India in
October 2020\textsuperscript{21} and rapidly spread across the globe. To date, monitoring viral evolution is a central
component of the epidemiological surveillance implemented in several countries to inform situation
awareness and detect, new variants in local and global populations. While some VOCs with key changes in
the spike protein were demonstrated to have a reduced susceptibility to neutralising antibodies\textsuperscript{22–25}, there
is no firm evidence that B.1.617.2 and its descendant sub-lineages have increased neutralisation
resistance\textsuperscript{26}, although some contrasting results emerged\textsuperscript{27}. To investigate the interaction of natural
immunity and vaccination in inducing protective immunity, in June 2021 we conducted a serological and
viral neutralization study on a highly characterized cohort of subjects infected during the first wave, back in
February 2020. This study follows on from the previous serosurveys conducted in the same population at
two and nine months after the initial SARS-CoV-2 outbreak\textsuperscript{8,28}, and provides unique longitudinal data on the
magnitude, neutralizing ability, and persistence of the antibody response against the spike (S) and
nucleocapsid (N) antigens in unvaccinated pre-exposed subjects as well as vaccinated pre-exposed and
naïve subjects, against both a B.1 SARS-CoV-2 strain circulating at the start of the pandemic and the
currently circulating B.1.617.2 strain.

Results

Serum reactivity to spike (S) and nucleocapsid (N) antigens

In June 2021, 76 subjects infected by SARS-CoV-2 in February/March 2020 (as defined by the ground truth
definition, see Methods) were tested with the same methods applied in the previous surveys (Methods)
(Fig. 1). Overall, all 76 (100%, 95% Confidence Interval (CI) 95.3-100%) individuals tested positive to at least
one assay, with 9 (11.8%, 95% CI 5.6-21.3%) being positive to all three of them. As observed in our previous
surveys, in June 2021 we observed strong differences in the proportion of positive subjects depending on
the assay used, with 11.8% (9 out of 76, 95% CI 5.6-21.3%), 80.3% (61 out of 76, 95% CI 69.5-88.5%), and
93.4% (71 out of 76, 95% CI 85.3-97.8%) testing positive at the 15 months follow up for Abbott, DiaSorin
and Roche, respectively. In June 2021, neutralising titres greater than 1:40 were found in 56.6% (43 out of
76, 95% CI 44.7-67.9%) of subjects. Of additional 61 volunteering subjects, who took part in the June 2021
survey and were not identified as infected in February/March 2020 according to our ground truth
definition, 3 had a positive swab between May and December 2020, and 8 showed positivity to at least two
different serological assays and were excluded from the analyses; the remaining 50 subjects were used as a
naïve control group.
**Fig. 1. Description of the study.** a) Flow chart illustrating the study design, which focuses on the subjects who were found to be positive early in the pandemic (from February to May 2020, according to the ground-truth definition). The serosurveys were conducted in Vo’ on three time points, 1–3 May 2020, 28–29 November 2020, and 5 June 2021. b) Timeline of the surveys conducted in the study area since the start of the SARS-CoV-2 epidemic in Vo’.

Impact of vaccination on antibody reactivity
On 5th June 2021, 53.9% (41 out of 76, 95% CI 42.1-65.5%) of the participants previously infected by SARS-CoV-2 according to the ground truth definition had received at least one dose of vaccine at least seven days before testing. As expected, vaccination had a strong impact on S-targeting antibody levels (Fig. 2) but not on those directed against the N antigen (Fig. 3). All vaccinated subjects showed reactivity against the S antigen, had a neutralising titre greater than 1:40 (41 out of 41 for both DiaSorin and neutralisation, 95% CI 91.4-100%) (Fig. 2b, 2d), and they still showed reactivity against the N antigen either when using Abbott (22.0%, 9 out of 41, 95% CI 10.6-37.7%) or Roche (92.7%, 38 out of 41, 95% CI 80.1-98.4%) assays (Supplementary Fig. 1b and 1d). In the unvaccinated group the serum reactivity against the S antigen was significantly lower compared to the vaccinated subjects, with positivity rates of 57.1% (20 out of 35, 95% CI 39.3-73.7%) and 5.7% (2 out of 35, 95% CI 0.7-19.2%) for DiaSorin and microneutralisation assays, respectively (Fig. 2a-d). Among 50 naïve subjects, 37.0% (20 out of 54, 95% CI 24.3-51.3%) had received at least one dose of vaccine at least seven days before testing. Of vaccinated naïve subjects, 0% (0 out of 20, 95% CI 0.0-16.9%), 95.0% (19 out of 20, 95% CI 75.1-99.9%), 0% (0 out of 20, 95% CI 0.0-16.9%), and 25% (5 out of 20, 95% CI 8.7-49.1%) were positive to Abbott, DiaSorin, Roche and neutralisation, respectively, whereas 8.8% (3 out of 34, 95% CI 1.9-23.7%), 35.3% (12 out of 34, 95% CI 19.8-53.5%), 2.9% (1 out of 34, 95% CI 0.1-15.3%), and 0% (0 out of 34, 95% CI 0.0-10.3%) of the unvaccinated and naïve (as of February/March 2020) subjects showed positivity to the same tests. These latter among naïve unvaccinated subjects are most likely false positives, since the percentages are in line with our previous positive predictive values estimates for the different assays and the positivity to one test is never confirmed by any of the others.
Fig. 2. Anti-S antibody titres and dynamics in vaccinated and unvaccinated subjects with pre-exposure to SARS-CoV-2. a-d) Observed antibody titres in unvaccinated and vaccinated subjects exposed to SARS-CoV-2 and tested in May 2020, November 2020 and June 2021 by DiaSorin (vaccinated n=38, P < 0.0001 from November 2020 to June 2021; unvaccinated n=33, P = 0.0041 from November 2020 to June 2021) and micro-neutralisation assays (vaccinated n=38, P < 0.0001 from November 2020 to June 2021; unvaccinated n=32, P = 0.0053 from November 2020 to June 2021). The horizontal line represents the median, the vertical line represents the 95% confidence intervals. e-h) Observed individual-level paired antibody titres in subjects exposed to SARS-CoV-2 and tested in May 2020, November 2020 and June 2021. In June 2021, 60.6% (20 out of 33 unvaccinated subjects, 95% CI 42.1-77.1%) and 6.3% (2 out of 32 unvaccinated individuals, 95% CI 0.8-20.8%) had antibodies more than 15 months post infection according to DiaSorin and micro-neutralisation, respectively. Subjects with increasing titres are coloured in green, while subjects with a negative result in June 2021 are presented in red. i-j) Estimated antibody decay rate distributions calculated among the unvaccinated subjects exposed to SARS-CoV-2 in February/March 2020 and tested in May 2020, November 2020 and June 2021. We estimated a median half-life of 214 (95% CI 168-288) days and 174 (95% CI 146-202) days for the antibodies detected by the DiaSorin and micro-neutralisation assays,
respectively. Asterisks indicate *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Statistical significance of antibody levels was evaluated by Kruskal-Wallis test.

**Fig. 3. Anti-N antibody titres and dynamics in subjects with pre-exposure to SARS-CoV-2.**

a-b) Observed antibody titres in subjects exposed to SARS-CoV-2 and tested in May 2020, November 2020 and June 2021 with Abbott (n=65, P = 0.0095 from November 2020 to June 2021) and Roche assays (n=65, P = 0.0073 from November 2020 to June 2021). The horizontal line represents the median, the vertical line represents the 95% confidence intervals. c-d) Observed individual-level paired antibody titres in subjects exposed to SARS-CoV-2 and tested in May 2020, November 2020 and June 2021. In June 2021, 13.8% (9 out of 65 subjects, 95% CI 6.5-24.7%) and 95.4% (62 out of 65 subjects, 95% CI 87.1-99.0) resulted positive to Abbott and Roche assays respectively, more than 15 months post infection. Subjects with increasing titres are coloured in green, and subjects with a negative result in June 2021 are presented in blue. e-f) Estimated antibody decay rate distribution calculated among subjects exposed to SARS-CoV-2 in February/March 2020 and tested in May 2020, November 2020, and June 2021. We estimated a median half-life of 115 (95% CI 105-126) days and 179 (95% CI 146-255) days for the antibodies detected by the Abbott and Roche
Antibody dynamics in vaccinated and unvaccinated subjects

Among vaccinated subjects, independently of the number of doses received, we found a significant increase of both DiaSorin and neutralisation titres (Wilcoxon matched-pairs signed rank test \( p < 0.0001 \) for both cases), with all subjects showing an increasing trend (except three individuals who already had the maximum amount of antibodies quantifiable by DiaSorin); on the contrary, in the unvaccinated group, antibodies directed against the S antigen decreased significantly, as measured by DiaSorin and neutralisation (Wilcoxon matched-pairs signed rank test \( p < 0.0001 \) for both cases) (Fig. 2).

The serum reactivity against the N antigen progressively decreased with time irrespectively of the utilised assay in the whole cohort (Fig. 3, Wilcoxon matched-pairs signed rank test \( p < 0.001 \) for both Abbott and Roche assays) and among vaccinated and unvaccinated individuals separately (Wilcoxon matched-pairs signed rank tests \( p < 0.001 \)) (Supplementary Fig. 1). Nonetheless, we observed a significant difference between anti-N antibody titres detected in June 2021 between vaccinated and unvaccinated individuals (Mann Whitney test \( P = 0.0003 \) and \( P = 0.0005 \) for Roche and Abbott, respectively) (Supplementary Fig. 2).

Considering the subjects tested across all serosurveys conducted in May 2020, November 2020, and June 2021, the median half-life of the antibodies detected by Abbott, DiaSorin, Roche, and neutralisation are of 115 days (95% CI 105-126), 214 days (95% CI 168-288), 179 days (95% CI 146-255), and 174 (95% CI 146-202) respectively.

Correlation between two DiaSorin assays and neutralisation

We assessed in parallel the performance of two DiaSorin tests, the first version targeting antibodies against the S1/S2 antigen and the updated version containing a full trimeric spike antigen. The two assays showed a strong correlation (Spearman’s \( r = 0.820 \), 95% CI 0.670-0.906) (Supplementary Fig. 3) and concordance (Supplementary Table 1). We estimated a conversion factor between the two assays of 3.190 (95% CI 3.061-3.319, \( P \) value < 0.0001) by linear regression, and found high correlation between the antibody levels measured by the DiaSorin assays and the neutralising titres (DiaSorin S1/S2 vs neutralisation: Spearman’s \( r = 0.857 \), 95% CI 0.729-0.928; DiaSorin TrimericS vs neutralisation: Spearman’s \( r = 0.752 \), 95% CI 0.551-0.870; all \( P \) values are significant, \( P < 0.0001 \)) (Supplementary Fig. 3).

Hybrid immunity provides higher anti-S antibody and neutralisation titres than vaccination in naïve subjects

We investigated the impact of past SARS-CoV-2 exposure to the humoral immune response induced by vaccination as measured by anti-S antibodies and neutralisation titres. Comparing the antibody titres of
subjects from the Vo’ cohort (n = 20) vaccinated when naïve to vaccinated individuals post exposure (n = 41) we observed significantly higher titres in previously exposed individuals (Mann Whitney test, P < 0.0001). Two vaccine doses reduced the observed difference in antibody titres between subjects vaccinated when naïve and subjects vaccinated post exposure (Fig. 4a, Kruskal-Wallis test, P = 0.01). Neutralisation and anti-S titres observed in pre-exposed vaccinated subjects after one and two vaccine doses were statistically comparable (Kruskal-Wallis test, P = 1 for both DiaSorin and neutralisation). Similar trends were observed when comparing the group of vaccinated subjects previously exposed to SARS-CoV-2 with an independent cohort of healthcare workers (HCW, n = 61) vaccinated when naïve from the complex operational unit (U.O.C.) of Microbiology and Virology of Padua University Hospital (Kruskal-Wallis test, P < 0.0001 for both DiaSorin and neutralisation)(Fig. 4b, 4d).

**Fig. 4. Antibody levels in vaccinated naïve and vaccinated pre-exposed individuals according to DiaSorin and micro-neutralisation assays.** a-b) Observed antibody levels measured by DiaSorin assays in vaccinated naïve and pre-exposed individuals with at least one dose of vaccine (Mann Whitney test, P < 0.0001) and with one or two doses of vaccine (Kruskal-Wallis test, vaccinated naïve versus pre-exposed subjects after one vaccine dose, P < 0.0001; after two vaccine doses, P = 0.01; vaccinated naïve HCW versus pre-exposed subjects after two vaccine doses, P < 0.0001). c-d) Observed neutralising antibody titres measured by a micro-neutralisation assay in vaccinated naïve and pre-exposed individuals with at least one dose of vaccine (Mann Whitney test, P < 0.0001) and with one or two doses of vaccine (Kruskal-Wallis test, vaccinated naïve...
versus pre-exposed subjects after one or two vaccine doses, $P < 0.0001$; vaccinated naïve HCW versus pre-exposed subjects after two vaccine doses, $P < 0.0001$). Asterisks indicate *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. GT: ground truth, infected Vo’ population; HCW: healthcare workers.

Two vaccine doses in naïve individuals trigger higher anti-S antibodies and neutralising titres than natural infection

Using the conversion factor calculated to convert the results of the old DiaSorin S1/S2 assay into the new DiaSorin trimericS assay, we compared the antibody response after natural infection with the response to vaccination, roughly two months after the immune stimulus. Vo’ subjects exposed to SARS-CoV-2 in February/March 2020 and tested in May 2020 showed lower anti-S antibody levels with respect to both naïve subjects from Vo’ (Kruskal-Wallis test, $P < 0.0001$) and HCW (Mann-Whitney test, $P < 0.0001$) after two doses of vaccine (Fig. 5a and 5b). A similar trend was observed for neutralising antibody titres, although the difference is significant only between exposed subjects and vaccinated HCW (Mann-Whitney test, $P = 0.0002$) (Fig. 5c and 5d).

**Fig. 5.** Anti-S antibody levels and neutralisation titres induced by vaccination and natural infection.

Observed antibody levels in Vo’ unvaccinated individuals pre-exposed to SARS-CoV-2 infection, Vo’ subjects and HCW subjects vaccinated when naïve, according to (a) DiaSorin S1/S2 (Kruskal-Wallis test,
unvaccinated pre-exposed versus vaccinated when naïve after one dose of vaccine, \( P = 1 \), or two doses of
vaccine, \( P < 0.0001 \), (b) DiaSorin TrimericS (Mann-Whitney test, unvaccinated pre-exposed versus HCW
subjects vaccinated when naïve, \( P < 0.0001 \)) (c-d) and micro-neutralisation (Kruskal-Wallis test,
unvaccinated pre-exposed versus vaccinated when naïve after one dose of vaccine, \( P = 0.001 \), or two doses
of vaccine, \( P = 1 \); Mann-Whitney test, unvaccinated pre-exposed versus HCW subjects vaccinated when
naïve, \( P = 0.0002 \)) assays. Asterisks indicate *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \), ****\( p < 0.0001 \). GT: ground
truth, infected Vo’ population; HCW: healthcare workers.

**Association analysis**

Among infected unvaccinated subjects, we observed no significant differences in the antibody titres by
symptom occurrence, hospitalisation, sex, age-group and BMI. In the Vo’ cohort, we observed no
statistically significant difference in the number of vaccinated and not vaccinated subjects by infection
status (according to the baseline ground truth definition), symptom occurrence and sex.

**Neutralisation reactivity of Delta VOC**

The sera obtained from vaccinated individuals pre-exposed to SARS-CoV-2 infection and subjects
vaccinated when naïve were tested in a micro-neutralisation assay against the B.1.617.2 variant, to assess
the neutralising ability of the humoral immunity mounted upon vaccination. Lower neutralising titres
against the B.1.617.2 than compared to the B.1 strain were observed in individuals vaccinated after natural
exposure, both after one and two vaccine doses (Kruskal-Wallis test, \( P < 0.0001 \) and \( P = 0.0014 \),
respectively), although all but one subject maintained a neutralisation titre ≥1:40 (1/dil) (20 out of 20 after
one dose of vaccine, 100%, 95% CI 83.2-100%, 19 out of 20 after two doses of vaccine, 95%, 95% CI 75.1-
99.9%) (Fig. 6a). The decrease in neutralisation caused by the B.1.617.2 variant was observed also in
unvaccinated individuals previously exposed (Fig. 6b) (Mann Whitney test, \( P = 0.0002 \), but in a context
where most of them displayed low neutralising titres also against the B.1 strain (33 out of 35 (94.3%, 95% CI
80.8-99.3%) and 34 out of 35 (97.1%, 95% CI 85.1-99.9%) subjects with neutralising titres below 1:80 (1/dil)
threshold against the B.1 and B.1.617.2 variants, respectively). A similar but non-significant trend was
present in subjects vaccinated when naïve (Fig. 6c) (Kruskal-Wallis test, \( P = 1 \) and \( P = 0.15 \) after one or two
vaccine doses, respectively).
Fig. 6. Neutralisation titres against the B.1 and B.1.617.2 SARS-CoV-2 variants in individuals vaccinated naïve or pre-exposed and unvaccinated pre-exposed subjects. a-b) Neutralising antibody titres against B.1 and B.1.617.2 SARS-CoV-2 variants among pre-exposed a) vaccinated and b) unvaccinated individuals (Kruskal-Wallis test, pre-exposed subjects after one (P < 0.0001) or two (P = 0.0014) doses of vaccine; Mann Whitney test, pre-exposed unvaccinated P = 0.00002). c) Neutralising antibody titres against B.1 and Delta SARS-CoV-2 variants among vaccinated naïve individuals (Kruskal-Wallis test, vaccinated naïve after one (P = 1) or two (P = 0.15) doses of vaccine). Asterisks indicate *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. GT: ground truth, infected Vo’ population.

Discussion

Monitoring the serological response to SARS-CoV-2 infection and vaccination over time is crucial to estimate the persistence of circulating antibodies, their neutralising efficacy, and to inform vaccination policies. Due to the continuous emergence of new viral variants, it is critical to assess the extent to which previous immunity, developed from natural infection or vaccination, protects against the new circulating strains.

The Vo’ cohort is a highly characterised population including a core of individuals identified as exposed to SARS-CoV-2 back in February/March 2020, which has been followed-up through time in sequential swab and serological surveys until June 2021, roughly 15 months after viral exposure, thus offering unique insights into the long term antibody dynamics. The results presented in this study confirm the trends observed in our previous follow-up, performed at 9 months since the first wave in Vo’ with strong variability observed among serological tests, especially for the two assays targeting the N viral antigen.

Of the identified SARS-CoV-2 cases who acquired the infection in February/March 2020, only 11.8% (95% CI 5.6-21.3%) tested positive by Abbott while 93.4% (95% CI 85.3-97.8%) tested positive by Roche after 15 months. This discrepancy could be due to differences in the employed antigens and to the fact that the N epitopes recognised by antibodies might change with time. In perspective, given that these two tests could
allow to discern recent from past infections, they could be employed in future seroprevalence studies to
assess the attack rate in vaccinated subjects and thus provide new data on the frequency of breakthrough
infections as well as re-infection.

We found that all individuals infected at the start of the pandemic and tested 15 months later are positive
to at least one serological assay, although the decreasing trend of antibody levels against both S and N
antigens is confirmed, independently from the type of test used. In the absence of vaccination, the
neutralising titres of the infected subjects drop almost completely below the 1:80 (1/dil) threshold.

While the observed decrease in antibody titres is in line with other recent reports, it does not necessarily
translate into an impaired immunity in these subjects, since humoral response is one arm of the adaptive
immune response, which also includes cellular immunity and reactivation upon stimulation of memory B
and T cells. 8

Unexpectedly, we found a significant difference in the amount of circulating N targeting antibodies
between vaccinated and unvaccinated subjects pre-exposed to SARS-CoV-2. To investigate this pattern we
retrospectively analysed the differences in N targeting antibodies present in the two groups in the
November serosurvey, before the beginning of the vaccination campaign. We found that the two groups
are significantly different in terms of age, with vaccinated subjects being older than unvaccinated subjects
(Suppl. fig. 2e). The age difference can be explained by the vaccination strategy and agrees with our
previous observation that antibody levels were higher with increasing age in this cohort. 8

We found that the response to vaccination is different among subjects vaccinated after pre-exposure and
when naïve: while a marked increase in S-targeting antibodies is observed in all individuals, antibodies
induced by vaccination are higher in pre-exposed subjects. In vaccinated pre-exposed subjects, a single
dose of vaccine saturates the dynamic range of DiaSorin assay and is shown to boost a strong neutralisation
response, as confirmed in other studies. 32-33. This suggests that a single dose of vaccine in pre-exposed
patients induces a robust immune response in support of the vaccination strategy implemented in
Germany, France, Italy, and Israel among other countries. It has been shown that B cell maturation due to
somatic hypermutation, possibly stimulated by long-term persistence of viral antigens in specific body
niches, can produce stronger and more specific antibodies. 37

By comparing the antibody levels in vaccinated naïve subjects (in June 2021) with those of patients who
recovered from natural infection in May 2020, we demonstrate that a complete vaccination course confers
stronger immunity than natural infection alone, at least in terms of serum antibodies as detected by both
DiaSorin and neutralisation.

We tested the ability of antibodies developed against SARS-CoV-2 strains circulating early in the pandemic
to neutralise the Delta variant of concern (VOC B.1.617.2), which is characterised by several mutations in
the spike protein and an increased transmissibility that allowed this variant to become prevalent
worldwide. 38. We observed a decrease in neutralising reactivity across all immunity profiles (naturally
exposed and unvaccinated, vaccinated pre-exposed and vaccinated naïve). The reduction in neutralising reactivity is more evident in the vaccinated pre-exposed subjects (Fig. 6a), despite the neutralising titres remained above 1:40 (1/dil). Since neutralising IgG antibodies are the best current indication for protection against reinfection and correlate well with virological response and survival\textsuperscript{17,39}, this finding is of particular importance in consideration of the efforts and resources that have been invested in the vaccination campaign in Italy and worldwide. Our results show that the vaccines currently deployed in Europe, although developed on a viral strain that is no longer circulating, are conferring strong and durable protection against the most prevalent strain (as of October 2021). These results confirm that vaccination is a safe and effective strategy to generate immunity against SARS-CoV-2. At the same time, it is critical to maintain and strengthen epidemiological and genomic surveillance, to monitor the potential emergence of new, immune-escaping variants in the future.

Methods

Ethical approval statement

All the serosurveys of the Vo’ population were approved by the Ethics Committee for Clinical Research of the province of Padova (May survey approved on 30th April 2020, protocol number 0026971; November survey and additional follow up approved on 11th November 2020, protocol number 0068830). Study participation was by consent. For participants under 18 years of age, consent was provided by a parent or legal guardian.

Laboratory methods

Oro-nasopharyngeal swabs

Swab test were performed as previously described\textsuperscript{8,28}. Briefly, swabs were inserted into the posterior pharynx first, rubbed over tonsillar pillars and posterior oropharynx and then over the nasal wall in the nostrils. SARS-CoV-2 genome was searched with an in-house real-time RT–PCR method targeting the envelope gene (E), according to Corman et al.\textsuperscript{40}

Serum antibodies detection

IgG anti-SARS-CoV-2 were searched in venous blood collected in 5 ml BD Vacutainer Serum Separation Tubes (SST) and centrifuged for 10 min at 1000–1300 RCF (g). Serological tests were performed by trained laboratory staff using the same commercial kits employed in previous serosurveys\textsuperscript{8} and produced by Abbott\textsuperscript{41}, DiaSorin\textsuperscript{42}, and Roche\textsuperscript{43}, applying the detection thresholds provided by the manufacturer (Table 1). For DiaSorin, both the new TrimericS and the previous S1/S2 kits were used for comparison.

Micro-neutralisation assay
Two independent assays were set up in parallel to assess the neutralisation ability of patients’ seric antibodies against two viral isolates, a third passage B.1 strain isolated in March 2020 (GenBank accession MW468415) and a third passage B.1.617.2 strain from August 2021 (GenBank accession …(waiting for the release of the accession number)). Heat-inactivated serum samples (30 min at 56 °C) were diluted 1:10 with DMEM FBS Free medium and filtered (0.22 µm pore size). 50 µl of viral isolate, diluted in DMEM FBS Free to the final concentration of 100 median tissue culture infective dose (TCID50), were mixed with an equal volume of two-fold serial dilutions of sera in 96-wells microplates and incubated for 1 h at 37 °C in a humidified atmosphere with 5% CO2. Following incubation, 100 µL of VERO E6 cells suspended in DMEM 6% FBS were added to each well and incubated at 37°C. After 72h, cytopathic effect was assessed; the supernatant was removed and 120 µl of 5% formaldehyde Gram’s crystal violet 40% m/v were added to each well, followed by 30 min of incubation. After a washing step with water, plates were allowed to dry and the absorbance was read at 595 nm. The neutralisation titre was determined as the highest serum dilution showing an optical density (OD) of 90% or more with respect to the control sera.

Definition of COVID-19 recovered patients (ground truth, GT)

Multiple rounds of mass testing, that included oropharyngeal swabs and serological assays, allowed for the identification of all the residents in the municipality of Vo’ who were infected and recovered from SARS-CoV-2 infection during the first wave, between February and March 2020. To be included among COVID-19 recovered individuals, one of the following criteria had to be satisfied: i) a positive swab, ii) a viral neutralization titre greater than 1:40, or iii) serum reactivity against two serological tests with different antigen targets. We refer to this group as baseline ground truth (GT). It included 125 subjects, a size that perfectly fitted the seroprevalence estimated through a multinomial likelihood model. These subjects were followed up at several time points to monitor the presence and persistence of antibodies against both the spike (S) and the nucleocapsid (N) antigens (Figure 1), as well as to investigate the presence of virus neutralising antibodies (Tables 1 and 2). We previously reported that all subjects belonging to the GT were positive to at least one serological assay in May 2020, about two months after the time of their infection (Fig. 1). On occasion of a second serological survey conducted in November 98.8% of GT subjects were still positive nearly 9 months after the infection, although with strong differences depending on the test.

Table 1. Commercial assays employed in the study to identify IgG anti-SARS-CoV-2 antibody levels.

<table>
<thead>
<tr>
<th>Test</th>
<th>Manufacturer</th>
<th>Recognised antigen</th>
<th>Method</th>
<th>Manufacturers’ thresholds</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIAISON® SARS-CoV-2 S1/S2 IgG</td>
<td>DiaSorin</td>
<td>S1/S2</td>
<td>CLIA²</td>
<td>Negative: &lt;12.0 AU/mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Equivocal: 12.0 ≤ x &lt;15.0 AU/mL</td>
</tr>
</tbody>
</table>
Table 2. Observed positivity rates by assays across the three serosurveys, stratified by vaccination status and dose for June 2021.

<table>
<thead>
<tr>
<th>Test</th>
<th>Detected antigen</th>
<th>Positive May 2020 (%)</th>
<th>Positive November 2020 (%)</th>
<th>Positive June 2021 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Not vaccinated (%)</td>
<td>1 dose (%)</td>
<td>2 doses (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abbott</td>
<td>N</td>
<td>86/92 (93,5)</td>
<td>28/93 (30,1)</td>
<td>0/76 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3/76 (3,9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6/76 (7,9)</td>
</tr>
<tr>
<td>DiaSorin</td>
<td>S</td>
<td>85/101 (84,2)</td>
<td>72/93 (77,4)</td>
<td>20/35 (57,1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>21/21 (100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20/20 (100)</td>
</tr>
<tr>
<td>Roche</td>
<td>N</td>
<td>92/92 (100)</td>
<td>90/93 (96,8)</td>
<td>33/76 (43,4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>19/76 (25,0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>19/76 (25,0)</td>
</tr>
<tr>
<td>Neutralisation</td>
<td>S</td>
<td>44/98 (44,9)</td>
<td>23/93 (24,7)</td>
<td>2/35 (5,7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>21/21 (100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20/20 (100%)</td>
</tr>
</tbody>
</table>

Statistical methods

Estimates of antibody decay rate and association analysis

The antibody decay rate was estimated at the individual level as the logarithmic change in antibody values observed between May 2020 and June 2021 (within the same subject) divided by the number of days between the two serosurveys (400 days). The antibody half-life was estimated as the natural logarithm of 0.5 divided by the antibody decay rate, and was calculated on all subjects testing positive in May 2020 serosurvey and without doubling antibody levels in November 2020 and June 2021 (Abbott n=65, DiaSorin n=29, Roche n=53, neutralisation n=9).
The associations between antibody levels and symptom occurrence, hospitalisation, sex, age-group and BMI and between vaccination and pre-exposure, symptom occurrence and sex were assessed using the Kruskal-Wallis test. We used Fisher’s exact test to assess the association between vaccination and previous hospitalisation.

Data availability statement

The dataset is available at https://github.com/MedCompUnipd/Vo-Serology.git

Ethical approval statement

The third serosurvey of the Vo’ population was approved by the Ethics Committee for Clinical Research of the province of Padova. Study participation was by consent. For participants under 18 years of age, consent was provided by a parent or legal guardian.

Competing interests

The authors declare no competing interests.

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Performed laboratory testing: MP, CB, MC, CDV, MCV, VL, MA, IG, CZ, MP, AP.

Sampling logistics and collection: EL, FC, GC, MN, EN, ES, BL, LF, LM, MG, FB, MS.

Performed swab and blood sampling: FC, GC, MN, EN, ES, BL, LF.

Statistical analysis: EL, LM, ID, ST, ARB.

Funding acquisition: EL, ST, ID, GT, AC.

Methodology: EL, LM, ST, ID, ARB.

Visualisation: EL, LM, ID.

Writing - original draft: EL.

Writing - review & editing: EL, ID, LM, ST, GT, ARB, AC.
Verified the underlying data: EL, LM, ST, ID, AC.

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