TITLE: **Impact of exposure to vaccination and infection on cellular and antibody response to SARS-CoV-2 in CVID patients through COVID-19 pandemic**

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ABSTRACT:
Purpose: To investigate the kinetics of response against SARS-CoV-2 elicited by vaccination and/or breakthrough infection (occurred after 3 doses of BNT162b2) in a cohort CVID patients.
Methods: We measured humoral and cellular immunity using quantitative anti-Spike antibody (anti-S-IgG) and neutralization assay and specific interferon-gamma release assay (IGRA) before and after the third or fourth dose of BNT162b2 and/or after COVID-19.
Results: In CVID 58.3% seroconverted after 2 doses, that increased to 77.8% after 3 doses. Between the 2nd and 3rd dose there was a decline in humoral compartment that led to titers below the cutoff of 1:10 (MNA90%) in CVID. This was paralleled by a significantly lower proportion (30%) and reduced magnitude of the residual cellular response among CVID.
The third dose achieved a lower titer of anti-S and nAb against the Wuhan strain than HC and significantly decreased the rate of those showing solely a positive neutralizing activity and those with simultaneous negativity of IGRA and nAbs; the differences in IGRA were overall reduced with respect to HC.
At further sampling after breakthrough SARS-COV-2 infection, mostly in the omicron era, or 4th dose), six months after the last event, the residual nAb titer to Wuhan strain was still significantly higher in HC, while there was no significant difference of nAbs to BA.1. The rate of IGRA responders was 65.5% in CVID and 90.5% in HC (p=0.04), while the magnitude of response was similar. None of CVID had double negativity to nAbs and IGRA at the last sampling.
Conclusion: This data shows an increase of adaptive immunity in CVID after mRNA vaccination in parallel to boosters, accrual number of exposures and formation of hybrid immunity.

Keywords: COVID-19, vaccination, CVID, coviferon, interferon-gamma release assay, BNT162b2, hybrid immunity

1. Introduction

The COVID-19 pandemic has been the main focus of the scientific community in the last three years due to the high rate of morbidity and mortality directly related to SARS-CoV-2 infection and the virus' impact on global health and society. Vaccination is currently the main truly effective mean of containing the viral spread and disease severity.

The Pfizer-BioNTech COVID-19 (BNT162b2) vaccine consists of a lipid nanoparticle-formulated mRNA vaccine that ~~which~~ encodes the SARS-CoV-2 spike protein (S-protein), a large class I trimeric fusion protein. Its use was authorized in Europe and the U.S. in late 2020, and data of immune response to BNT162b2 against the original strain have been reported [1] said vaccine induces durable SARS-CoV-2 specific spike-protein (and/or its RBD) B-cells and neutralizing antibodies and generates polyspecific CD8+ and CD4+ T-cell clones [2].
The profile of the immune response of the BNT162b2 vaccine remains to be investigated beyond the short-term especially in patients affected by primary immunodeficiencies (PID), which rendered them vulnerable during the pandemic period [3-5].
In patients with primary immunodeficiency, including genetically characterized inborn errors of immunity (IEI), vaccination is regarded as the most effective and safest preventive approach [5, 6].

Individuals with CVID, the most common clinically relevant primary immunodeficiency, are at greater risk of COVID-19-associated hospitalization and mortality, as well as extended or breakthrough SARS-CoV-2 infections [3] [7]. Age and comorbidities represent relevant risk factors for severe COVID-19 in addition to PID status [8, 9].
Therefore, since a considerable fraction of patients with common variable immunodeficiency disorder (CVID) have displayed insufficient responses to the primary cycle [10, 11], throughout the pandemics vaccine booster doses have been recommended to achieve adequate protection also for this susceptible group according to "waning immunity" and immune escape of Variants Of Concern (VOC) that emerged [6, 12, 13].
Previous longitudinal studies in healthy SARS-CoV-2 naïve individuals revealed a gradual decline in antibody and neutralization levels in parallel to a continued increase in the frequency of Spike+ and Spike+RBD+ memory B-cells from 3-6 months post-vaccination (2 doses) [3], reducing protection against symptomatic COVID-19 notwithstanding a strong positive impact on severe disease and hospitalization [14, 15].
Studies on patients with PAD and CVID showed a trend towards a decay of nAbs below cut-offs for protection after vaccination as compared to HC and positive impact of 4th dose [16, 17], but most studies focused on the first 2 or 3 doses mostly within a timeframe of 1-3 months [3, 11, 18-21].
Cellular response to SARS-CoV-2 is one of the critical determinants of severe disease protection, especially months after viral and/or vaccine exposure in the context of declining or absent humoral immunity [22-24]. In addition to antibodies and memory B cells, memory T cells can contribute to protection upon exposure to the virus. T cells have also been shown to be less affected by VOC ability to overcome the protective effect of neutralizing antibodies produced as a result of natural infection and/or vaccination[25]. Nevertheless, even in the presence of spike-specific CD4+ or CD8+ response, B-cell depleted patients with IMID may still show increased severity rates in case of breakthrough infection [26].
This may also be important in a context such as CVID where memory B-cells and Tfh responses are impaired, potentially leading to weak or no nAbs and short-term antibody production [19] [27] and thus leading to defective components of layered defenses against SARS-CoV-2 [28].
Breakthrough infections and the resulting hybrid immunity may further shape the immune response to SARS-CoV-2 and increase protection in both healthy and immunocompromised subjects [29].
To investigate the kinetics of immunity against SARS-CoV-2 in a cohort of CVID we evaluated the

cellular and humoral response to SARS-CoV-2 elicited by vaccination and/or subsequent infection throughout the course of pandemics up to early 2023.

2. Material and Methods:

2.1. Study Design

We have measured humoral and cellular immunity using quantitative IgG anti-SARS-CoV-2 Spike antibody (anti-S-IgG) and neutralization assay, and specific interferon-gamma (IFN-g) release assay (IGRA) before and after the third dose of BNT162b2, to investigate the responses in a cohort of CVID patients. We also measured the humoral and cellular immunity after SARS-CoV-2 exposure after the third dose in terms of the fourth vaccine shot and/or infection. The responses were compared with those of healthy controls.

2.2. Patient selection

We enrolled prospectively consecutive subjects aged >18 years, who had received the COVID-19 vaccine Pfizer/BioNTech BNT162b2 (two doses 21 days apart of 30 µg mRNA vaccine Comirnaty by Pfizer Inc, NY, USA) and deliberately gave their consent to participate. For each patient enrolled in the study, we collected demographic and clinical variables, including sex, age, date, and type of vaccine; the number of SARS-CoV-2 infections. CVID diagnosis was in accordance with the ESID registry working party with at least 18 months of follow-up since diagnosis [30]. Chronic immunosuppressive treatment was defined as currently receiving steroids and/or DMARDs or biologics for immunosuppressive purposes. According to Chapel's definition the two different clinical phenotypes "infection only" and "complicated'' were identified [31]. We also compared data with a control group of subjects enrolled among healthy healthcare workers (HC) recruited at our hospital, not affected by immune-mediated diseases, no evidence of immunodeficiency or taking relevant medications.

To ensure the subjects status, given the differences of immune response determined by a previous SARS-CoV-2 infection, we checked the results of nasal swabs (PCR and antigenic tests) and of serological testing for surveillance of 2020-2022 of CVID and HC for IgM and IgG with the 2019-nCov chemiluminescent analytical system (CLIA) assay (Snibe, Shenzhen, China) on MAGLUMI platform, which detects antibodies of natural infection to SARS-CoV-2 Spike-(S) protein and N-protein with high sensitivity and specificity.

At the time of completion of the immunological studies related to the third mRNA vaccine dose, both CVID patients and matched HC were still naive to SARS-CoV-2. Two CVID subjects were not included due to COVID-19 before the completion of the three doses. At the following timepoints of the study, both patients and controls were enrolled either after having had vaccine booster or SARS-CoV-2 infection or their combinations. At T5 timepoint the patients' samples were collected months after infection, thus no interference could be due to therapeutic mAbs for COVID-19 (2/6 of treated patients) as previously described [32]; no patient underwent the long half-life mAb tixagevimab/cilgavimab for treatment or prophylaxis.

2.3. Sample Collection and Storing

10 mL of peripheral blood was obtained by venipuncture immediately before and after the third dose, or after fourth dose of BNT162b2 and/or after COVID-19 (Fig.1). The serum was separated by centrifugation (2,000 x g for 15 minutes) within 3 hours of collection and aliquots were stored at -80º C until use.

2.4. Serological studies

The primary outcome was the rate of seroconversion and anti-S-IgG and 90% protective response of neutralizing Abs at week 3 after the third vaccine dose.
Secondary outcomes included the assessment of residual response in a whole blood SARS-CoV-2 IGRA before the third dose and the kinetics thereafter.
The anti-S-IgG was assessed with the LIAISON SARS-CoV-2 TrimericS IgG assay (DiaSorin, Saluggia Italy), a chemiluminescence immunoassay that measures the anti-trimeric spike glycoprotein of SARS-CoV-2 in serum samples on the LIAISON XL (DiaSorin, Saluggia, Italy). The measuring range is 4.81 to 2080 BAU/mL, and, per the manufacturer's instructions, values more than 2080 BAU/mL were diluted 1:20; values > 33.8 BAU/mL were considered positive [33]. This was performed also on IVIG products similarly to previous studies [34].
SARS-CoV-2 Microneutralization assay. MNA was performed in a Biosafety Level 3 (BSL-3) laboratory (Section of Microbiology and Virology, University of Cagliari). Serum samples were diluted (1:2; 1:5, 1:10,1:40; 1:160; 1:640) in triplicates and mixed with 100 $TCID_{50}$ of SARS-CoV-2 virus (clinical isolate, strain VR PV10734, kindly donated by the Lazzaro Spallanzani Hospital of Rome, Italy) and BA.1 variant (clinical isolate, strain EPI_ISL_13398512) at 37°C, serum /virus mixes were transferred to 96-wells containing 5×10^5/ml adherent Vero E6 (ATCC, Manassas, Virginia, United States) cells seeded the day before in. Monolayers were incubated at 37 ◦C for 72 h or 96 (BA.1 variant) prior evaluation of CPE via microscope and then fixed and stained with Gram's crystal violet solution. The neutralization percentage of individual dilutions was calculated by setting the mean OD_{595} of the serum control equal to 100%. Virus dilution used for infection was titrated in each experiment. Cell growth and serum controls were run in each experiment. Neutralization titers of serum samples were determined by the highest serum dilution protecting 90 % of the infected wells.

2.5. SARS-CoV-2 specific Cellular Immunity

We assayed cell-mediated immunity by measuring IFN-g secreted by T cells in response to SARS-CoV-2 antigens, using a specific IGRA (Covi-FERON ELISA, SD Biosensor, Suwon, Republic of Korea). Whole blood from the participants was injected into each Covi-FERON tube (Nil tube, SARS-CoV-2 spike protein antigen (Sp)1 tube, Sp2 tube, and phytohemagglutinin mitogen tube). The Sp1 tube contained spike protein antigens derived from the original SARS-CoV-2 (Wuhan/Hu-1/2019) and 20I/501Y.V1 variant, while the Sp2 tube contained those derived from the B.1.351 (20H/501.V2 or "Beta") and P.1 (20J/501Y.V3 or "Gamma") variants. After incubating at 37°C for 16–24 h, plasma was collected after centrifugation at 2200–2300g for 15 min. IFN-g was detected by ELISA and the measured optical density was converted to IFN-g concentration (IU/mL) using ELISA Report Software (SD Biosensor). The positive cut-off for S and N tubes minus that of the Nil tube was >0.25 IU/ml and~~,~~ ≥0.5 for positive control, according to manufacturer specification. Samples where the mitogen minus negative control was < 0.5 IU/ml or the negative control was > 8 IU/ml should be considered as indeterminate.

2.6. Statistical analysis

Patient characteristics were summarized using appropriate medians, IQR, ranges, and percentages. Chi squared tests of independence and Fisher's exact tests were used for categorical data. Mann-Whitney U and Kruskal-Wallis tests were used for unpaired continuous data and nonparametric Spearman's rank for correlation test. Linear regression was used to evaluate the relationship between the dependent variable (e.g. antibody titer) and the clinical and demographic characteristics of patients as independent variables. All reported p-values represent 2-tailed tests, with $p \leq 0.05$ considered statistically significant. All variables were analyzed using SPSS and Analyse-IT softwares.

2.7. Ethical aspects

Patients were recruited and enrolled in the study protocol at the University Hospital of Cagliari. Written informed consent was obtained from all patients and controls in accordance with the ethical standards (institutional and national) of the local human research committee. The study protocol, including informed consent procedures, conforms to the ethical guidelines of the Declaration of Helsinki and was approved by the responsible ethics committee (Ethics Committee of the Cagliari University Hospital approval May 27th, 2020; protocol number GT/2020/10894 and extension approved Jan 27th, 2021). Records of written informed consent are kept on file and are included in the clinical record of each patient.

3. Results:

We have collected complete data and results of immunity assays throughout all the exposures of 29 CVID patients and 21 HC, both groups included subjects naive to SARS-COV-2 infection until T4 (Figure 1).
The median age of HC was 45 years (IQR 23.3) and 41.5 (IQR 32.1) for CVID (p=0.056). There was no difference in gender, analysis time after the vaccine booster, and last event type when comparing these groups (p>0.05). Baseline characteristics and clinical manifestations related to CVID of enrolled subjects, vaccinated with at least 3 doses of BNT162b2 are shown in Table 1. A single patient previously underwent rituximab courses for autoimmune cytopenia and GLILD in 2020.
Over the course of COVID-19 pandemics, no HC was hospitalized due to infection, instead 2 patients in the CVID group were hospitalized (p=0.24). No HC was treated with antiviral drugs while 6 out of 26 (23.1%) were treated with antiviral drugs or mAbs (p=0.03).
As shown in Table 2, the duration of SARS-CoV-2 swab positivity was similar (7 days in CVID and 8 days in HC, p=0.77) and the duration of symptoms was not significantly different (5 days in CVID and 3.5 days in HC, p=0.45).

3.1 Response to BNT162b2 vaccine before and after the third dose

Based on a prior determination of IgG antibody response to S protein, measured one month after the first 2 dose course of mRNA vaccine, the 58.3% of CVID and 93.3% of HC seroconverted (Diasorin SARS-CoV-2 TrimericS IgG; cut-off: >33.8 BAU/mL). After the 3rd dose the 77.8% of CVID and 100% of HC seroconverted.
The proportions and type of positivity of the immunological assays performed in each group before and after 3rd dose are~~is~~ provided in Table 3. Passive antibody transfer via IGRT with IVIg or SCIg could influence this analysis through the presence of anti-SARS-CoV-2 antibodies in the products; however assessment of specific trimeric anti-S or anti-RBD on some batches of Privigen (CSL Behring) or IgVena (Kedrion S.p.A) until late 2021 gave negative results on all samples (n=6, data not shown).
Among responders, the neutralizing titer to Wuhan strain before the third dose was not significantly different between the groups (CVID median 1:5, IQR 7.8; HC median 1:10, IQR 0; p=0.2), hence most of CVID fell below the 1:10 (MNA 90%) titer cut-off for positivity of nAbs.
One month after 3rd dose there was a significant difference between groups for neutralization titer (CVID median 1:10, IQR 34.2 and HC median 1:40, IQR 120; p=0.0059) (table 3; figure 2). Similarly, the trimeric anti-S serum titer (Diasorin) before 3rd dose was not significantly different (CVID 233 BAU/mL, IQR 513; HC 314 BAU/mL, IQR 325; p=0.82). After the 3rd dose the anti-S among responders was 1690 BAU/mL (IQR 1150) in CVID and 4710 BAU/mL (IQR 5596) in HC (p=0.002).
The rate of subjects with a residual positive response in IGRA before the third dose was 30% in CVID and 85% in HC (p=0.05), although after 3rd it increased to 40% in CVID and 100% of HC (p=0.0006).
In addition, the rate of double positives to neutralizing antibodies and IGRA before 3rd dose was of 10% in CVID and 85.7% in HC (p<0.0001); subsequently, after 3rd dose, it grew to 40% in CVID and 100% in HC but the proportion remained significantly different between groups (p=0.0006).
The rate of subjects who tested positive only for nAbs before the third dose was 30% in CVID and 6.7%

in HC (p=0.11), while after 3rd dose this rate decreased to 0% in HC and 30% in CVID, remaining significantly different between groups (p=0.023).
Furthermore, the rate of those double negatives for neutralizing antibodies and IGRA before 3rd was 50% in CVID and 0% in HC (p=0.0022), reduced to 27% in CVID and 0% in HC after three doses.
The magnitude of IGRA response to OSP (Original Spike) before the 3rd dose was significantly reduced in CVID than HC (Figure 3), respectively 0.25 IU/mL (IQR 0.08) and 0.43 IU/mL (IQR 1.61) (p=0.02).
Among the 40% responders, one month after the 3rd dose no significant difference was found for OSP (CVID median 6.4 IU/mL, IQR 7; HC median 2.030, IQR 9.25; p=0.29).
Similarly, the responder rate and magnitude of residual VSP (Variant Spike) response before the 3rd dose was lower in CVID than HC (respectively 0.13 IU/mL, IQR 0.077 and 0.39 IU/mL, IQR 0.963; p=0.03), but there was no significant difference after 3rd dose (p=0.68).

3.2 Response after further exposures to vaccine booster or SARS-CoV-2 infection
During the development of this study, in early 2023 (T5), the last event before sampling was the infection in about 50% of both CVID and HC (p=0.55); it occurred at a median of 212.5 (IQR 214.6) days, mainly in the omicron wave (for CVID 253 days, IQR 215; for HC 167 days, IQR 158.2; p=0.21). Overall, at this timepoint, the median of total exposures (vaccine shots and/or infection) was of 4 events (p=0.65), and both CVID and HC frequently had the SARS-COV-2 infection after the third dose.
In early 2023 the residual neutralizing titer against the Wuhan strain after the last event (additional SARS-CoV-2 infection or booster) was significantly higher among HC (CVID median 1:10, IQR 30; HC median 1:100, IQR 400; p=0.0188). Interestingly, at the same timepoint, there was no significant difference between neutralization titers against the Omicron (BA.1) variant of CVID group compared to HC (CVID 1:40, IQR 70; HC 1:40, IQR 150; p=0.28).
Theoretically, a progressive increase in SARS-CoV-2 nAbs could also be determined by the ongoing IGRT in the majority of CVID patients; at the end of 2022 the Ig batches used in clinical practice started to be more consistently positive for anti-S antibody or nAbs [35], but with limited or absent neutralization of Omicron variants. Thus, the differences we measured at this T5 timepoint in our cohort (lower nAbs to Wuhan and similar to BA.1 with respect to controls) may mostly underlie the true response of CVID subjects after multiple exposures rather than a major effect given by IGRT.
In January 2023 the rate of IGRA responders ~~was~~ increased to~~:~~ 65.5% in CVID and 90.5% in HC but was still significantly different between the two groups (p=0.04). The rate of double positives to nAbs and IGRA was 58.6 % for CVID and 90.5 % for HC (p=0.013).
The magnitude of IGRA response to both OSP and VSP was not significantly different between CVID and HC (for OSP median of CVID 1.19 (IQR 2.5) vs HC 1.19, IQR 2.6; p=0.8); for VSP (median CVID 2.02, IQR 6.2, median HC 1.13, IQR 2.4, p=0.4) (Table 4).
In early 2023 (T5, at a median of 223 days after the vaccine/or infection) 31% of CVID and 9.5% of HC (p=0.07) resulted positive for neutralizing antibodies (IGRA negative), but the rate of both CVID and HC simultaneously negative for both neutralizing antibodies and IGRA decreased to 0%.
Among CVID we found that those patients with hybrid immunity (n=13) had significantly higher levels of neutralizing titers against the BA.1 variant (p=0.05) but not against Wuhan (p=0.27), higher OSP and VSP response (p=0.05 and p=0.01, respectively) and there was a higher rate of double positives, compared to CVID that received a 4th dose (n=13).

4. Discussion:

The ongoing COVID-19 pandemic is currently being limited by an unprecedented vaccine program and innovative use of vaccine technologies, which include using mRNA especially in subjects at risk of severe outcomes [36] [3] [37].
Several real-world data have reported a-variably reduced proportion or failure ~~reduction~~ of humoral and

cellular response in patients with CVID after vaccination against COVID-19 [38]. Patients with IEI and PADs, including CVID, were not included in the clinical trials that have led to the approval of the vaccines. This issue has raised public health concerns and has resulted in main changes in vaccination strategies, also in the context of SARS-CoV-2 VOC emergence. In this scenario, providing data about immunogenicity in this population to guarantee informed health policies is of the utmost importance.

Our study aimed to analyze the development of humoral and cellular response and its longevity throughout the recent pandemics after repeated exposures to SARS-CoV-2 vaccination or after infection in a cohort of patients affected by CVID.

In CVID patients naive to SARS-CoV-2 infection we found that 58.3% seroconverted after 2 doses, later increased to 77.8% after 3 doses. Our findings largely agree with an analysis of 19 studies on a total of more than 800 CVID patients about seroconversion after the second dose in 64.92% and 78.27% after the third dose [39] [40, 41] [20], and with more recent reports [16, 18, 42].

Between the 2nd and 3rd dose there was a waning in humoral compartment that in our study among responders led to nAb titers below the threshold of 1:10 MNA90% in CVID at the time of 3rd dose; as in a previous report, the antibody titer peak of CVID is lower than controls but declines slower, ~~independently~~ and the decay is not ~~of~~ influenced by ongoing IgRT [16]. We observed that this was paralleled by a significantly lower proportion and reduced magnitude of the residual cellular response rates among CVID assessed by a SARS-CoV-2 specific IGRA before 3rd dose. With the limit of not having performed for this study the IGRA assay after the second dose due to the unavailability of this method at that time, we~~the~~ observed a rate of residual responders to IGRA of 30%, comparable to similar studies [10, 20, 42]

The third dose increased the rate of responders (seroconverters) among CVID, albeit achieving a lower titer of anti-S and nAb against the Wuhan strain than HC. However, the booster significantly decreased the rate of those showing solely a positive neutralizing activity and those with simultaneous negativity of IGRA and nAbs (27%), close to other reports [16, 18]. The booster dose has previously been reported to increase the proportion of PAD patients having nAbs and, albeit transiently, the titer against Wuhan, Delta and Omicron variants in naïve patients [43, 44].

After the 3rd dose the differences in IFN-gamma response were overall reduced with respect to HC, confirming recent reports [16, 42, 45]. Similar findings regarding IGRA, anti-S antibodies and surrogate virus neutralizing titers have been described in CVID subjects compared to controls[16, 19, 46], showing also that anti-S antibody avidity is reduced in CVID after 3rd dose but not after the primary cycle in the context of an impaired cTFH response [19]. Still, a defective development in germinal centers of memory B-cells (MBC) specific for RBD of Spike protein and non-canonical B-cell responses has been demonstrated [46-48].

Nevertheless, in CVID and IEI the partial response to the first course of 3 vaccine doses (mostly done with mRNA vaccines), the availability of specific antiviral treatments and the current prevailing omicron lineage subvariants have led to an attenuated severity even in the context of pre-existent comorbidities [27, 49] [8, 50].

Then, during the course of pandemic we sampled again the subjects that got breakthrough SARS-COV-2 infection, mostly in the omicron era, or got boosted (4th dose with the bivalent original/BA.1) during the vaccine campaign according to indications for subjects with PID. Six months after the last event, the residual nAb titer to Wuhan strain was still significantly higher among HC than CVID, while there was no significant difference in neutralization titers against BA.1 variant.

In HC the antibody responses in breakthrough omicron infection occurring after 3 doses derived mostly from cross-reacting B cells initially induced by vaccination, that generate B-cells binding both the Omicron and Wuhan spike protein [51]. This may be defective in CVID even after breakthrough infection [30], thus we measured a residual difference in nAbs to Wuhan [51, 52].

Memory B-cells recall response to SARS-CoV-2 main epitopes after vaccination has shown to be defective in CVID [48] and also partially after breakthrough infection in a subset of CVID that are lymphopenic with reduced CD19 cells and absent nAbs and S-specific MBC [47].

Considering the definition of response or presence of nAbs to SARS-CoV-2 in CVID, it could be theoretically influenced by IGRT, due to a progressive increase by passive transfer of antibodies in IGRT. However, consistently to previous literature our assessment of batches of IVIG during 2021 did not show the presence of such anti-S [34, 35, 53], thus it may not have influenced the evaluation of response to 3rd dose. At the end of 2022 the Ig batches used in clinical practice started to be more consistently positive for anti-S antibodies or nAbs, but with limited neutralization of Omicron variants [35]. Thus, the differences we measured at T5 timepoint in our cohort (residual lower nAbs to Wuhan and similar titers to BA.1 with respect to controls) may mostly underlie the true response of CVID subjects after multiple exposures including infection, rather than a major effect given by IGRT.

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Finally, at T5 the magnitude of IGRA response to both OSP and VSP, more than 6 months after the last event, was not significantly different between CVID and HC. The rate of IGRA positivity (65% of residual response) and that of double positivity to IGRA and nAbs was increased in CVID compared to 3 doses but remained still significantly different compared to HC. Furthermore, the rate of both CVID and HC simultaneously negative for both neutralizing antibodies and IGRA decreased to 0% at T5.

Hybrid immunity refers to the combined protection against infection (e.g. SARS-CoV-2) achieved by both natural infection and vaccination. For those who have already received COVID-19 vaccination but later have encountered the virus (in our and other cohorts a large proportion of CVID nowadays fits in this category, mostly infected with omicron variants), hybrid immunity becomes a crucial concept for evaluation of risk. Recent data showed a notable impact of boosters and of hybrid immunity in response among heavily immunosuppressed patients [54] and in PID [55].

Our data suggest that vaccinated CVID patients show the progressive formation of a quite robust response, in particular if we consider a "layered defense" model [28] and ~~we~~ confirm or extend over a longer time frame the results of previous studies [45, 47, 55, 56].

The progressively favorable clinical outcomes of COVID-19 achieved throughout the evolution of pandemic [3, 8, 9, 20] may correlate with our data in the clinical context of CVID patients having SARS-CoV-2 infection after a three doses vaccine course. These patients mounted, although probably not completely, the main layers of circulating (or tissue) adaptive immunity.

The main study limitations are the monocentric and non-randomized design, the absence of flow-cytometric analysis, in particular the memory B-cells recall response to SARS-CoV-2 and finally a potential effect of IGRT when considering measuration of nAbs at T5. The accurate assessment of the humoral immune response, including neutralization with validated neutralization assays and specific anti-S-IgG, together with the T-cell response in terms of specific IGRA over multiple timepoints strengthen the validity of our study. Moreover, said methods to detect SARS-CoV-2 specific responses are highly reproducible.

Our data show that after ~~a~~ priming with 3 doses of mRNA vaccine, the infection with Omicron (or the second booster) may further enhance the proportion of those CVID patients with a measurable response, as well as the magnitude and the duration of specific SARS-CoV-2 immunity. This may therefore protect against future exposures of SARS-CoV-2 or from severe outcomes and should be further assessed in specific studies.

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Availability of Data and Material: data is available upon reasonable request to the corresponding author.

Ethics approval: the study has been approved by the Ethical Committee of the A.O.U. di Cagliari University Hospital (CORIMUN STUDY: May 27th, 2020 protocol n. GT/2020/10894 and extension approved Jan 27th, 2021).

Consent to participate: written informed consent is recorded for each patient

Consent for publication: not applicable.

Data availability:
The dataset may be available upon reasonable request

Acknowledgements: § contributed equally to this work.

List of Abbreviations: CLIA, chemiluminescent analytical system; COVID-19, coronavirus disease 2019; HC, healthy controls; MNA, Microneutralization test; RBD, receptor binding domain; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

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Table 1.

Table 1 caption: Characteristics of enrolled subjects and main data of COVID-19 of study

SUBJECTS CHARACTERISTICS	CVID n=29	HEALTHY CONTROL n=21	p-value
Age (years), median (IQR)	41.5 (32.1)	45 (23.3)	0.56
Female ~~%~~	63%	52%	0.46
Current Immunoglobulin replacement therapy	93%	n.a.	
Chronic lung disease	36%	n.a.	
Complicated phenotype	33%	n.a.	
History of autoimmune cytopenia	19%	n.a.	
Current immunosuppressive treatment	8%	n.a.	
IgG through level (mg/dl)	722 (580-886)	n.a.	

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Total events (vaccine and/or infection), median (IQR)	4 (1)	4 (0.6)	0.65
Infection after 3rd dose	12	13	0.18
Infection after 4th dose	5	0	0.11
Last event type (infection or vaccine)	50%	50%	0.55
Days since the last event to sampling at T5, median (IQR)	253 (215)	167 (158.2)	0.21

Legend:
Interquartile range: IQR

Table 2.
Table 2 caption: The main data of enrolled subjects and COVID-19.

	CVID n=29	HEALTHY CONTROL n=21	p-value
Hospital admission	2	0	0.24
Duration of COVID-19 symptoms, median (IQR)	5 (7.1)	3.5 (3.8)	0.45
Duration of swab positivity, median (IQR)	7 (13.1)	8 (9.1)	0.77
Treatment with antiviral or mAb	6	0	0.03

Legend: CVID: patients affected by primary immunodeficiency; HC: Healthy control. Data are expressed as median (IQR).

Table 3.

Table 3 caption: Data of humoral and cellular response in CVID and HC before and after the third dose of BNT162b2 and after SARS-CoV-2 infection or booster at T5 in subjects previously vaccinated with 3 doses of BNT162b2.

			Response before and after 3rd BNT162b2 vaccine dose							
	# of exposures	Days after 2nd dose	nAb titer [1:X] BA.1	nAb titer [1:X] Wuhan	IGRA OSP IU/mL	IGRA VSP IU/mL	Rate of responders to IGRA	Double positivity to nAb and IGRA	Rate of isolated nAb positivity	Rate of Double negativity to nAb and IGRA
		BEFORE 3RD DOSE (T3)								
CVID	2	172 (26)	n.a.	5 (7.8)	0.25 (0.08)	0.13 (0.077)	30%	10%	30%	50%
HC	2	280 (20.5)	n.a.	10 (0)	0.43 (1.614)	0.39 (0.88)	85%	85%	6.7%	0%
p-value	n.a.	<0.0001	n.a.	0.20	0.02	0.0027	0.05	0.0002	0.11	0.0022
		AFTER 3RD DOSE (T4)								
CVID	3	n.a.	n.a.	10 (34.2)	6.40 (7)	2.75 (6.47)	40%	40%	30%	27.3%
HC	3	n.a.	n.a.	40 (120)	2.03 (9.25)	1.6 (9.04)	100%	100%	0%	0%
p-value	n.a.	n.a.	n.a.	0.0059	0.29	0.68	0.006	0.0006	0.023	0.0267
	Residual response after further exposures (SARS-CoV-2 infection or booster), assessed in January 2023 (T5)									
	# of exposures	Last event to sampling (days)	nAb titer [1:X] BA.1	nAb titer [1:X] Wuhan	IGRA OSP IU/mL	IGRA VSP IU/mL	Rate of residual responders IGRA	Double positive to nAb and IGRA	Rate of isolated nAb positivity	Rate of Double negative to nAb and IGRA
CVID	4 (1)	253 (215.1)	40 (70)	10 (30)	1.19 (2.5)	2.020 (6.2)	65.5%	58.6%	31%	0%
HC	4 (0.6)	167 (158.2)	40 (150)	100 (400)	1.19 (2.60)	1.13 (2.4)	90.5%	90.5%	9.5%	0%
p-value	0.65	0.21	0.28	0.02	0.80	0.4	0.04	0.013	0.07	0%

Legend: Healthy Controls: HC; CVID: Common Variable Immunodeficiency; Interquartile range: IQR. nAb: serum neutralizing antibody titer assessed by SARS-CoV-2 Microneutralization assay (90% Protective activity of neutralizing Ab against the CPE induced by the virus, Wuhan strain); IGRA: Interferon gamma (IFN-g) release assay to SARS-CoV-2 Spike protein (Wuhan/Hu-1/2019 and 20J/501Y.V3 "gamma" variant), positive >0.25 IU/mL. BEFORE: residual response assessed before BNT162b2 booster (3rd dose) in subjects naive to SARS, previously vaccinated with two doses of BNT162b2. AFTER: response assessed 4 weeks after BNT162b2 booster (3rd dose) in subjects naive to SARS, previously vaccinated with two doses of BNT162b2. At T5: n=13/29 CVID having had 3 vaccine doses and COVID-19 afterwards; n=13/29 CVID having had 4 vaccine doses and naïve to COVID-19. Data are expressed as median (IQR) or percentage when appropriate.

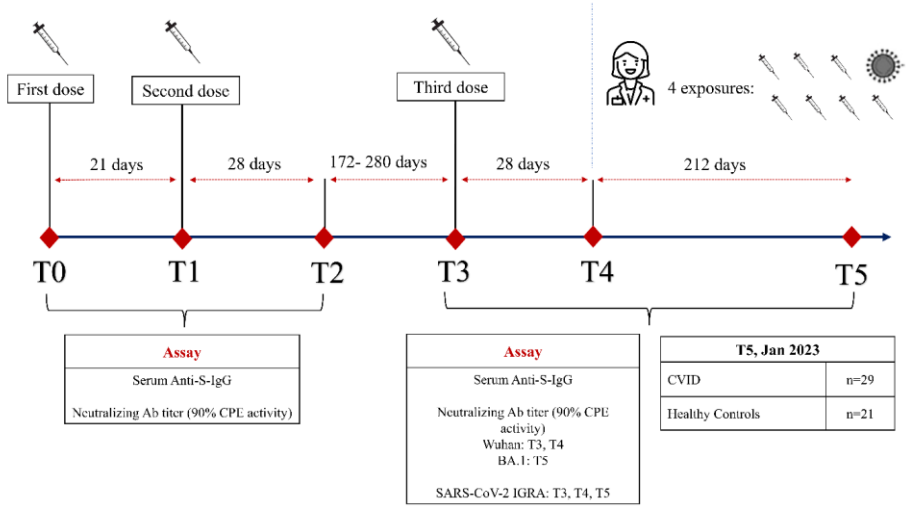
~~Table 4.~~

~~Table 4 caption: Response after SARS-CoV-2 infection or booster at T5 in subjects previously vaccinated with 3 doses of BNT162b2.~~

	~~Residual response after further exposures (SARS-CoV-2 infection or booster), assessed in January 2023 (T5)~~									
	~~# of exposures~~	~~Last event to sampling (days)~~	~~nAb titer [1:X] BA.1~~	~~nAb titer [1:X] Wuhan~~	~~IGRA OSP IU/mL~~	~~IGRA VSP IU/mL~~	~~Rate of residual responders IGRA~~	~~Double positive to nAb and IGRA~~	~~Rate of isolated nAb positivity~~	~~Rate of Double negative to nAb and IGRA~~
~~CVID~~	~~4 (1)~~	~~253 (215.1)~~	~~40 (70)~~	~~10 (30)~~	~~1.19 (2.5)~~	~~2.020 (6.2)~~	~~65.5%~~	~~58.6%~~	~~31%~~	~~0%~~
~~HC~~	~~4 (0.6)~~	~~167 (158.2)~~	~~40 (150)~~	~~100 (400)~~	~~1.19 (2.60)~~	~~1.13 (2.4)~~	~~90.5%~~	~~90.5%~~	~~9.5%~~	~~0%~~
~~p value~~	~~0.65~~	~~0.21~~	~~0.28~~	~~0.02~~	~~0.80~~	~~0.4~~	~~0.04~~	~~0.013~~	~~0.07~~	~~0%~~

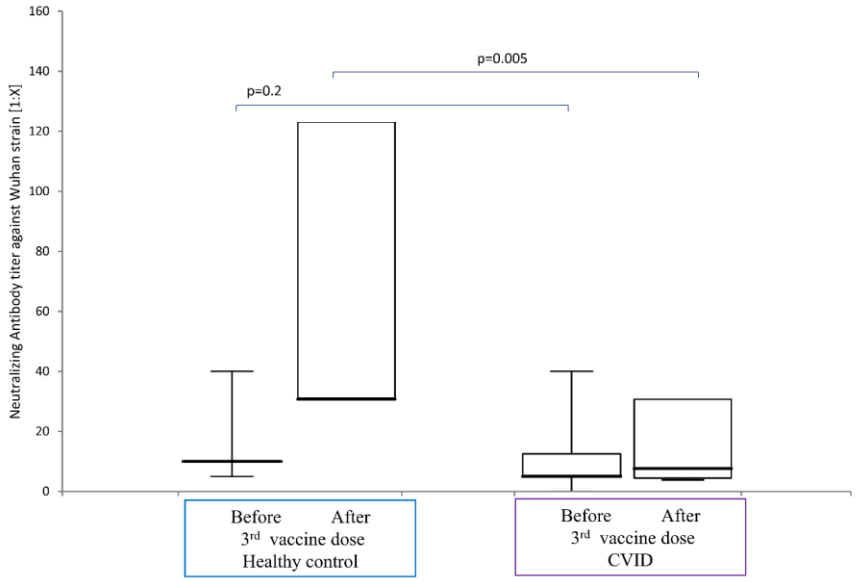
~~Legend: Healthy Controls: HC; CVID: Common Variable Immunodeficiency; Interquartile range: IQR. nAb: serum neutralizing antibody titer assessed by SARS-CoV-2 Microneutralization assay (90% Protective activity of neutralizing Ab against the CPE induced by the virus, Wuhan strain); IGRA: Interferon gamma (IFN-g) release assay to SARS-CoV-2 Spike protein (Wuhan/Hu-1/2019 and 20J/501Y.V3 "gamma" variant), positive >0.25 IU/mL. Data are expressed as median (IQR) or percentage when appropriate.~~

Figure 1. Study design.



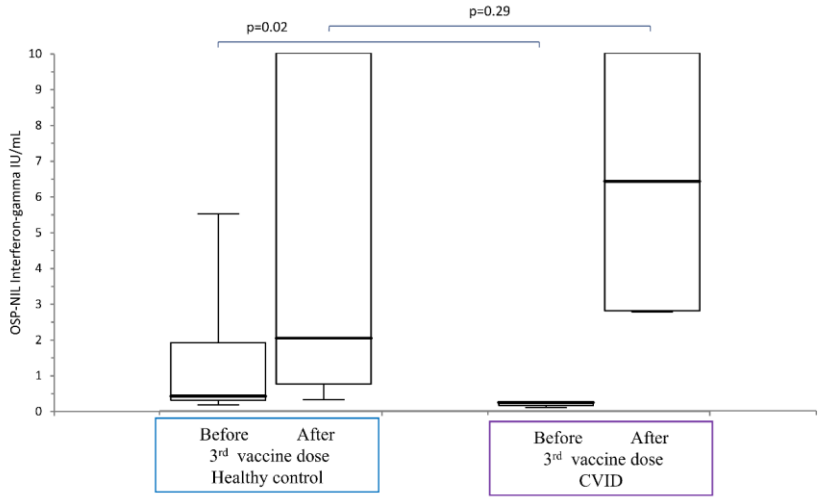
Legend:
CVID: Common Variable Immunodeficiency, anti-S-IgG levels: serum SARS-CoV-2 trimeric anti-spike assay; Neutralizing ab: serum neutralizing antibody titer assessed by SARS-CoV-2 Microneutralization assay (90% Protective activity of neutralizing Ab against the CPE induced by the virus); IGRA: Interferon-gamma (IFN-g) release assay to SARS-CoV-2 Spike protein (Wuhan/Hu-1/2019 and 20J/501Y.V3 “gamma” variant).

Figure 2.
Figure 2 caption: Neutralizing Antibody titer against Wuhan strain, before and after the third dose of BNT161B2.



Legend: CVID: Common Variable Immunodeficiency; nAb: serum neutralizing antibody titer assessed by SARS-CoV-2 Microneutralization assay (90% Protective activity of neutralizing Ab against the CPE induced by the virus, Wuhan strain), serum titer 1:X.

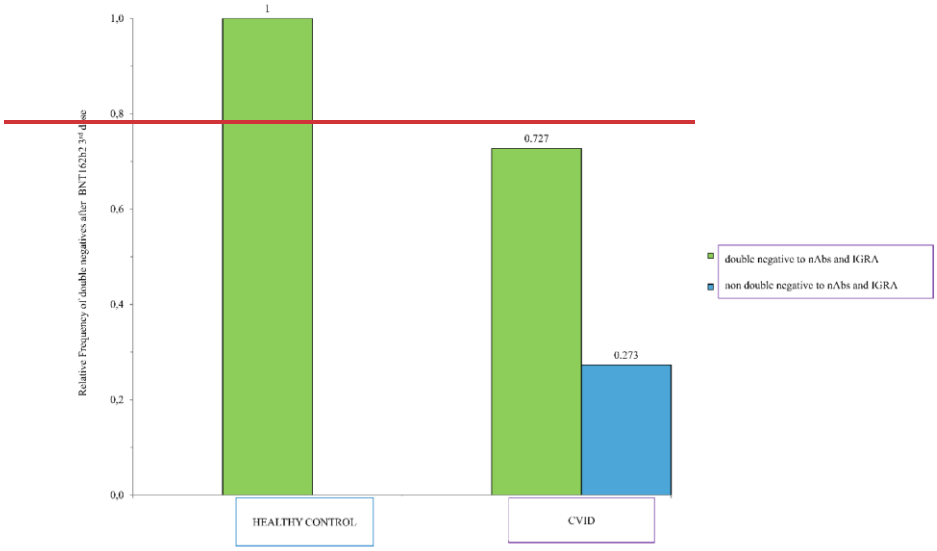
Figure 3.
Figure 3 caption: The IGRA response to Original Spike protein in HC and CVID before and after the third BNT162b2 dose.



Legend: IGRA: Interferon gamma (IFN-g) release assay to SARS-CoV-2 Spike protein (Wuhan/Hu-1/2019 and 20J/501Y.V3 "gamma" variant), positive >0.25 IU/mL. Data are expressed as median (IQR).

Figure 4.

Figure 4 caption: The rate of double negative subjects for IGRA response to original spike protein and neutralizing antibodies before and after three doses of BNT162b2 vaccine, in subjects naive to SARS-CoV-2.



Legend: CVID: Common Variable Immunodeficiency; nAb: serum neutralizing antibody titer assessed by SARS-CoV-2 Microneutralization assay (90% Protective activity of neutralizing Ab against the CPE induced by the virus, Wuhan strain).