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Neutralizing antibody titers six months after Comirnaty vaccination: kinetics and comparison with SARS-CoV-2 immunoassays

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Abstract

Objectives: mRNA vaccines, including Comirnaty (BNT162b2 mRNA, BioNTech-Pfizer), elicit high IgG and neutralizing antibody (NAb) responses after the second dose, but the progressive decrease in serum antibodies against SARS-CoV-2 following vaccination have raised questions concerning long-term immunity, decreased antibody levels being associated with breakthrough infections after vaccination, prompting the consideration of booster doses.

Methods: A total number of 189 Padua University-Hospital healthcare workers (HCW) who had received a second vaccine dose were asked to collect serum samples for determining Ab at 12 (t_{12}) and 28 (t_{28}) days, and 6 months (t_{6m}) after their first Comirnaty/BNT162b2 inoculation. Ab titers were measured with plaque reduction neutralization test (PRNT), and three chemiluminescent immunoassays, targeting the receptor binding domain (RBD), the trimeric Spike protein (trimeric-S), and surrogate viral neutralization tests (sVNT).

Results: The median percentages (interquartile range) for decrease in antibodies values 6 months after the first dose were 86.8% (67.1–92.8%) for S-RBD IgG, 82% (58.6–89.3%) for trimeric-S, 70.4% (34.5–86.4%) for VNT-Nab, 75% (50–87.5%) for $PRNT_{50}$ and 75% (50–93.7%) for $PRNT_{90}$. At 6 months, neither PRNT titers nor VNT-Nab and S-RBD IgG bAb levels correlated with age (p=0.078) or gender (p=0.938), while they were correlated with previous infection (p<0.001).

Conclusions: After 6 months, a method-independent reduction of around 90% in anti-SARS-CoV-2 antibodies was detected, while no significant differences were found between values of males and females aged between 24 and 65 years without compromised health status. Further efforts to improve analytical harmonization and standardization are needed.

Keywords: binding antibodies; BNT162b2; Comirnaty; COVID-19; immunoassays; immunological response; kinetics; neutralizing antibodies; SARS-CoV-2 vaccine; serology.

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Introduction

The coronavirus disease 2019 (COVID-19) pandemic caused by SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) continues to sustain a global public health crisis, despite the availability of vaccines that have dramatically reduced severe disease, hospitalization and mortality [1]. Several studies have demonstrated that mRNA vaccines, such as Comirnaty [BNT162b2 mRNA, BioNTech-Pfizer, Mainz, Germany/New York, United States (US)], elicit high IgG and neutralizing antibody (NAb) responses, especially after administration of the second 30 μg dose [2, 3]. However, the decrease in serum antibodies against SARS-CoV-2 have raised questions concerning long-term immunity,

since decreased antibody levels have been associated with breakthrough infections, prompting the consideration of additional vaccine booster doses [4].

Although initial studies demonstrated that Ab titers elicited by Comirnaty can persist for more than 6 months after the second boost dose [5, 6], there is a pressing need for further data on time-dependent kinetics and persistence of immunization [5, 7]. Recent papers in the literature have reported lower antibody levels 3 months post-vaccination, with 6 months values being comparable to those of subjects vaccinated with one dose, thus indicating a progressive waning of immune response over time [7]. Moreover, analogous to studies evaluating Ab levels in convalescent individuals [8], further findings suggest that the dynamics of decrease in Ab elicited by vaccines might be not homogeneous across individuals [5, 9]. Antibodies may decrease earlier in the elderly, and in chronic renal disease, underweight, solid malignancy patients as well as in those on immunosuppressive medication, whereas they can increase in females [5, 7, 9]. On the other hand, in some individuals (4%), Ab slowly increase after the second dose, peaking 3 months later (late-responders) [7].

The humoral response and dynamic after vaccination has mainly been demonstrated by measuring binding antibody (bAb) using commercially available assays, often based on chemiluminescent technology targeting different forms of Spike proteins or its receptor binding domain (RBD) moiety [10, 11]. Only a few studies have focused on the short- and long-term dynamic of NAb [5, 12], found to correlate with protection from infection [13]. The assessment of neutralizing assays in clinical practice is cumbersome, since this technique is labor-intensive, has long turnaround time and needs bio-safety level 3 (BSL-3) containment, which is unavailable in most laboratories. Therefore, anti-S IgG immunoassays closely correlated with neutralizing antibody titers could be used to determine the relationship with protection, and for evaluating a vaccinated or Covid non-naïve individual level of immunity.

In this study we describe the dynamics of neutralizing response of sera from HCW without and with prior SARS-CoV-2 infection after 6 months from administration of a primary cycle of Comirnaty/BNT162b2 vaccine, measured with plaque reduction neutralization test (PRNT), which is considered the gold standard for anti-SARS-CoV-2 Nab measurement [14]. The antibody kinetics was evaluated further with two commercially-available CLIA assays, having as targets either the RBD portions or the trimeric form of the viral Spike protein, and a surrogate viral neutralization test (sVNT) measuring specific interactions between SARS-CoV-2 and host cells with high affinity to Ab neutralization activity.

Materials and methods

This study included a cohort of 189 Padua University-Hospital healthcare workers (HCW) who underwent a primary cycle of vaccination (first dose, followed by a second after 21 days) between December 26th 2020 and March 10th 2021. HCW were consecutively enrolled from the Emergency Department, and the Infectious Disease and the Laboratory Medicine wards of the University-Hospital of Padova. All subjects underwent weekly nasopharyngeal swab testing from March 2020 to September 2021, while their immunological status for SARS-CoV-2 was determined weekly between April 8th and May 29th, 2020, as described elsewhere [11]. Thirty-five post-graduate medical trainees are included in the cohort. Seventeen HCW were previously diagnosed with COVID-19 natural infection on the basis of at least one positive nasopharyngeal swab test and clinical confirmation; the time elapsed after infection ranged from 3 to 9 months. Overall, the numbers and percentages of subjects within the age classes <30 years, 30/40 years, 40/50 years, 50/60 years and >60 years were: 32 (16.9%), 55 (29.1%), 41 (21.7%), 48 (25.4%) and 13 (6.9%), respectively.

Of the whole cohort, 179 underwent a second vaccine administration after 21 days from the first dose; the remaining 10, who were non-naïve to SARS-CoV-2 infection, had a single dose. All HCW were asked to undergone collection of serum samples for determining Ab at 12 (t_{12}) and 28 (t_{28}) days after the first Comirnaty/BNT162b2 inoculum; a pre-vaccination sample (t_0) was collected 24–0 h only from the 35 resident staff before vaccination. Around 6 months (t_{6m}) after the first vaccine administration (median time from first dose 185 days, min–max 13–214, 25th and 75th percentiles 180–195 days), a further blood sample for Ab assessment was obtained from all patients.

PRNT assays were performed with Vero E6 cells, in 96-wells plates as described elsewhere [15], using hCoV-19/Italy/PD_20VIR1935i-P4-L/2020 virus. The serum neutralization titer was defined as the reciprocal of the highest dilution resulting in a reduction of the control plaque count >50% ($PRNT_{50}$) or >90% ($PRNT_{90}$). A titer of ≥1:20 was considered the seropositive threshold. An evaluation was also made of the levels of binding antibodies against SARS-CoV-2 by different chemiluminescent assays (CLIA) (Table 1). SARS-CoV-2 trimeric-S IgG was determined using Liaison XL (DiaSorin, Saluggia, VC, Italy), SARS-CoV-2 S-RBD IgG by Maglumi 2000 plus (Snibe Diagnostics, Shenzhen, China), sVNT NAb by Maglumi 2000 plus (Snibe Diagnostics, Shenzhen, China). Due a limited availability of reagents, different numbers of samples were evaluated for each CLIA assay (Table 1). The sensitivity and specificity of the assays, as declared by manufacturers (using the values after 15 days post positivity for SARS-CoV-2), ranged from 91.7 to 100% and from 99.5 to 100%, respectively (Maglumi anti-SARS-CoV-2 RBD IFU SARS-CoV-2 S-RBD IgG-en-EU V1.2 2020-08, DiaSorin Trimeric-S IFU IT-54286-2021-02, Maglumi VNT-NAb IFU CoV-2 NAb-en-EU, V1.0 2020-12). The GraphPad Prism version 9.1 for Windows (GraphPad Software, LLC) was employed, using non-parametric tests (Kruskall-Wallis test and Spearman's correlation). Stata 16.1 (Statacorp, Lakeway Drive, TX, USA) was employed for multivariate analyses, performed using log transformed PRNT titers, and Ab values. MedCalc Statistical Software version 19.5.1 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2020) was used for methods comparison. All subjects gave their written, fully informed consent to participate in the study, which was conducted in accordance with the Declaration of Helsinki, and the Institutional Review Board of the University of Padova (protocol nr 7862).

Table 1: Characteristics of the chemiluminescent SARS-CoV-2 binding antibodies assays investigated in this study, given by the manufacturers.

Manufacturer	DiaSorin Inc.	Snibe Diagnostics	
Commercial name	Liaison SARS-CoV-2 Trimeric-S IgG	SARS-CoV-2 S-RBD IgG	SARS-CoV-2 neutralizing antibody
Platform	Liaison XL Analyzer	Maglumi series	Maglumi series
Method	Chemiluminescent immunoassay (CLIA)	Chemiluminescent immunoassay (CLIA)	Chemiluminescent immunoassay (CLIA)
Detection	IgG antibodies	IgG antibodies	Neutralizing antibodies
Antigen target	Trimeric Spike protein	Spike RBD portion	Spike RBD-ACE2 protein interaction
Results	kBAU/L	kBAU/L	mg/L
Interpretation	<33.8 Negative	<4.33 Negative	<0.3 Negative
	≥33.8 Positive	≥4.33 Positive	≥0.3 Positive

Results

Among the HCW included in the study, 58 (30.7%) were males, and 131 (69.3%) females. The overall mean value for age, which did not significantly differ by gender (Student's t=−0.562, p=0.574), was 42.3 (range, 24–66) years with a standard deviation (SD) of ±11.8 years. Seventeen individuals (8.9%) presented one or more comorbidities [11 had cardiovascular diseases without or in association with diabetes (n=1), respiratory diseases (n=1) or severe obesity (n=7); three had respiratory diseases; one had diabetes; two had past or current cancer]. Of the 17 individuals with previous SARS-CoV-2 natural infection, 8 (47.0%) were females and 9 (53.0%), males. Figure 1 shows the differences in bAb, VNT-NAb and NAb by gender. Multivariate regression analysis demonstrated that S-RBD IgG bAb levels at 6 months were neither correlated with age (p=0.079) nor with gender (p=0.466), while they were correlated with previous infection (p<0.001). Differently, trimeric-S IgG bAb levels at 6 months correlated with age (p=0.013) and with previous infection (p<0.001), but not with gender (p=0.723). VNT-Nab levels at 6 months were neither correlated with age (p=0.078) nor with gender (p=0.938), whereas they were correlated with previous infection (p<0.001). Likewise, $PRNT_{50}$ and $PRNT_{90}$ titers were neither correlated with age (p=0.064 and p=0.674, respectively) nor with gender (p=0.356 and p=0.563, respectively), while they were correlated with previous infection (p<0.001 for both). Figure 2 reports the kinetics of bAb and VNT-NAb in all HCW, and Figure 3 shows $PRNT_{50}$ and $PRNT_{90}$ titers at 12 days (t_{12}), 28 days (t_{28}) and 6 months (t_{6m}) after the first vaccine administration. The median and IQR of bAb and PRNT at different time points are reported in Table 2 for the entire cohort, as well as for those with and without previous COVID-19. In order to ascertain the decrease in bAb and PRNT over time in individuals with Ab measured both at t_{28} and t_{6m}, the difference (in percentage) between levels at 6 months and t_{28} were calculated. Figure 4 shows the

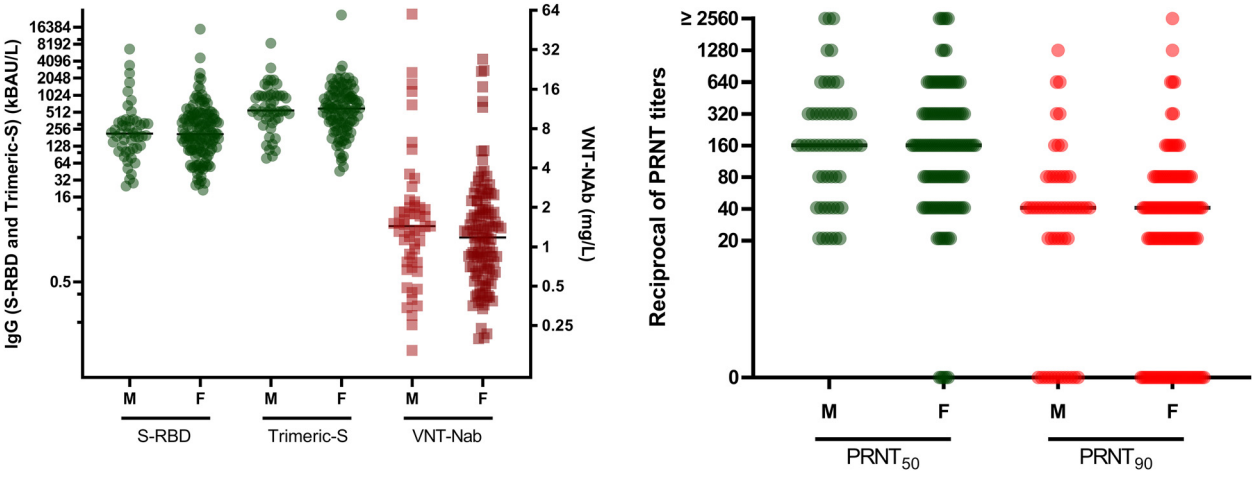

Figure 1: Dot-plots of antibody levels subdivided by gender.
Results for S-RBD IgG, trimeric-S IgG (in kBAU/L) binding antibodies (bAb) and viral neutralization test neutralizing antibodies (VNT-NAb) are reported in the left panel. Right panel shows the reciprocal of the plaque reduction neutralization test (PRNT) results at the two stringency thresholds $PRNT_{50}$ and $PRNT_{90}$.

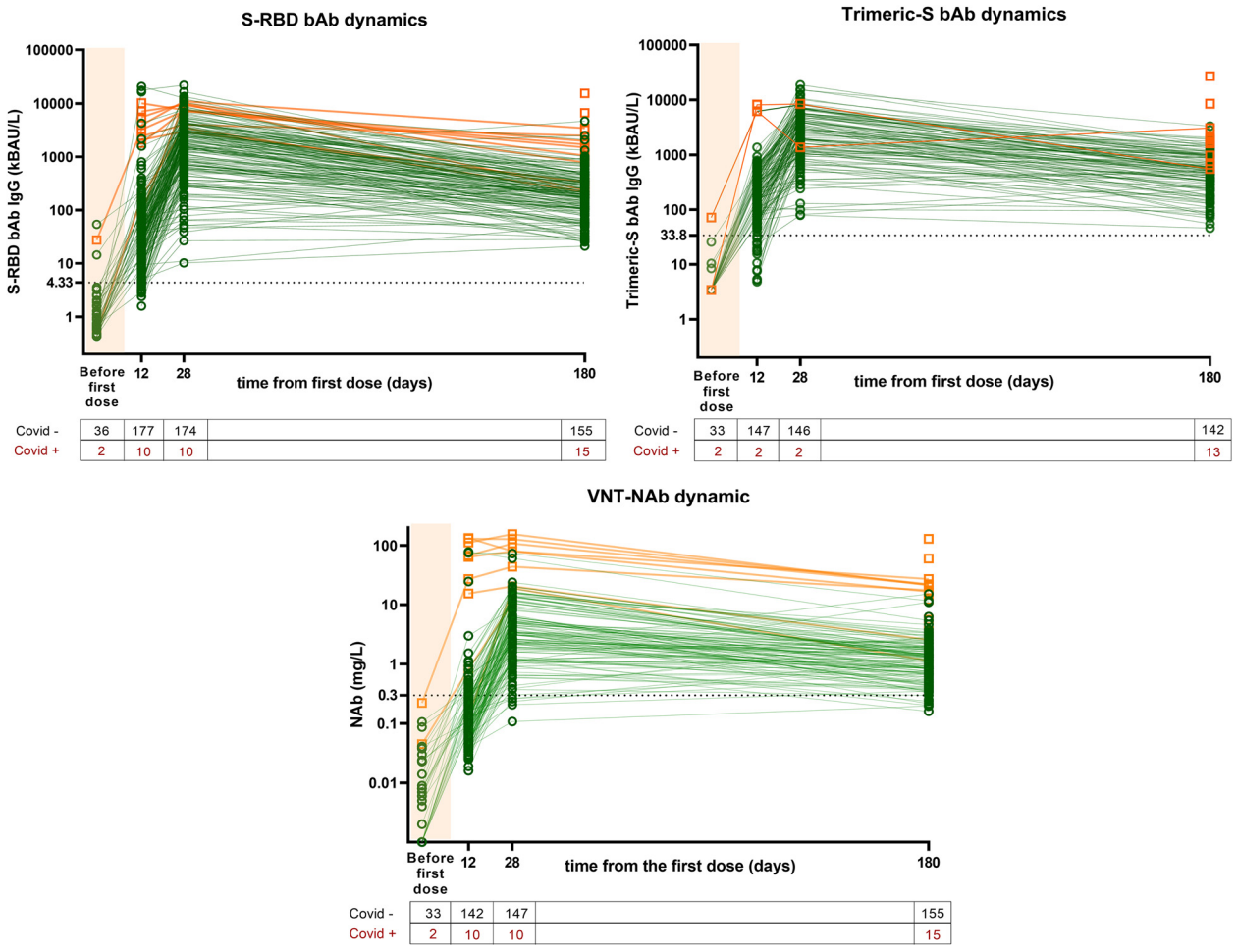

Figure 2: Spaghetti plots reporting the dynamic of S-RBD IgG, trimeric-S IgG (in kBAU/L) binding antibodies (bAb) and viral neutralization test neutralizing antibodies (VNT-NAb) (in mg/L) measured before the first dose (at t_0) and after 12 days (t_{12}), 28 days (t_{28}) and 180 days from the first dose.
The numbers of infection naïve (COVID–) individuals and subjects with previous SARS-CoV-2 infection (COVID+) with measured Ab are reported at the corresponding time points.

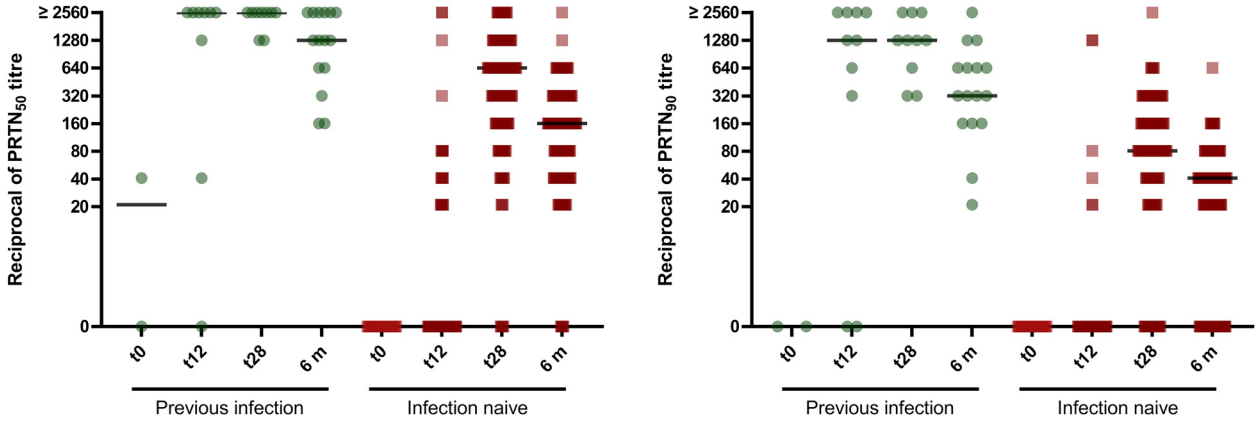

Figure 3: Reciprocal of $PRNT_{50}$ (left panel) and $PRNT_{90}$ (right panel) titers, measured at each time point, subdivided for infection naïve individuals and convalescent patients with previous infection.

Table 2: Median, 25th and 75th percentiles of binding antibodies (bAb) levels and plaque reduction neutralization titers (PRNT), at low ($PRNT_{50}$) or high ($PRNT_{90}$) stringency thresholds.

Anti-SARS-CoV-2 Ab	Median (25th and 75th percentiles) at t_0	Median (25th and 75th percentiles) at t_{12}	Median (25th and 75th percentiles) at t_{28}	Median (25th and 75th percentiles) at t_{6m}
S-RBD bAb IgG, kBAU/L				
Overall	0.7 (0.5–1.6)	37.5 (16.0–87.2)	1,944.8 (703.6–3,621.6)	215.4 (115.6–393.5)
Infection naïve	0.7 (0.5–1.6)	35.6 (14.9–78.8)	1,780.8 (650.6–3,407.8)	194.8 (107.6–357.4)
Previous infection	13.9 (0.5–27.5)	2,822.9 (1,795.6–55,269.4)	7,417.3 (4,016.2–9,920.0)	1,201.1 (380.6–2,489)
Trimeric-S bAb IgG, kBAU/L				
Overall	3.4 (3.4–3.4)	191.1 (76.2–398.0)	2,850.4 (1,215.0–5,360)	588.8 (329.0–1,000.0)
Infection naïve	3.4 (3.4–3.4)	185 (75.6–398.0)	2,810 (1,190–5,340)	540 (299–956)
Previous infection	37.5 (3.4–71.6)	3,820 (1,370–6,270)	8,340 (8,200–8,480)	1,890 (1,610–2,080)
VNT-NAb, mg/L				
Overall	0.07 (0.001–0.024)	0.121 (0.046–0.269)	3.62 (1.83–10.57)	1.31 (0.68–1.99)
Infection naïve	0.06 (0.01–0.023)	0.119 (0.06–0.242)	3.39 (1.58–7.96)	1.17 (0.65–1.82)
Previous infection	0.134 (0.045–0.223)	65.51 (15.49–111.80)	70.14 (20.33–107.60)	16.64 (5.38–22.2)
$PRNT_{50}$ titer				
Overall	0 (0–0)	0 (0–0)	640 (160–1,280)	160 (80–320)
Infection naïve	0 (0–0)	0 (0–0)	640 (160–1,280)	160 (40–320)
Previous infection	20 (0–40)	2,560 (1,280–2,560)	2,560 (2,560–2,560)	1,280 (640–2,560)
$PRNT_{90}$ titer				
Overall	0 (0–0)	0 (0–0)	80 (40–320)	40 (0–80)
Infection naïve	0 (0–0)	80 (40–160)	40 (0–40)	40 (0–40)
Previous infection	1,280 (320–2,560)	1,280 (640–2,560)	320 (160–640)	320 (160–640)

Results of all the studied time points are presented overall (including all 189 subjects) of subdivided by individuals with or without previous COVID-19.

histograms of results obtained for the percentage of decrease. A descriptive analysis was made in order to estimate how Ab levels decreased after 6 months. The median percentages (and 25th and 75th percentiles) of decreases after 6 months from first dose were 86.8% (67.1–92.8%) for S-RBD IgG, 82.0% (58.6–89.3%) for trimeric-S, 70.4% (34.5–86.4%) for VNT-Nab, 75% (50–87.5%) for $PRNT_{50}$ and 75% (50–93.7%) for $PRNT_{90}$. A series of individuals presented higher Ab titers at 6 months than at t_{28}. This late-response was present in 12/176 (6.8%) for S-RBD IgG, 7/121 (5.7%) for trimeric-S IgG, 7/121 (5.8%), 21/140 (15.0%) for VNT-Nab, 15/158 (9.5%) for $PRNT_{50}$ and 7/140 (5.0%) for $PRNT_{90}$. Of the 12 individuals presenting S-RBD IgG at 6 months, 5 were males, and 7 females (age range 26–59 years).

Table 3 also reports the results of multivariate analyses, conducted in order to establish whether age and gender were significantly associated with the percentage of Ab decrease. Supplementary Figure 1 shows the dot plots of corresponding decreases levels by age and gender. Supplementary Figure 2 reports results at Passing and Bablok analyses (including equations, 95% CI of slopes and intercepts) across bAb, VNT-NAb and PRNT.

Table 3: Dependence of delta between levels at 6 months and t_{28} and age, gender and previous covid infection.

	Age	Gender	Previous COVID-19 infection
(log delta) S-RBD	0.546	0.368	0.613
(log delta) Trimeric-S	0.848	0.581	–
(log delta) VNT NAb	0.898	0.106	0.407
(log delta) $PRNT_{50}$	0.266	**0.042**	0.194
(log delta) $PRNT_{90}$	0.261	0.136	**0.038**

Multivariate robust linear regression analyses, performed using the absolute values of difference in percentage between levels at 6 months and t_{28} (log_{10}-transformed) as the dependent variable, while gender age and previous covid infection were used as predictors. Table reports p-values of the associations between predictors and dependent variable, and significant values are reported in bold.

Discussion

Vaccines against COVID-19 have been demonstrated to be effective in preventing severe disease, hospitalization and death [1]. However, studies evaluating Ab levels in response to vaccination have reported contradictory results. Yet it is of utmost importance to gain sound understanding of the extent and duration of protection following natural

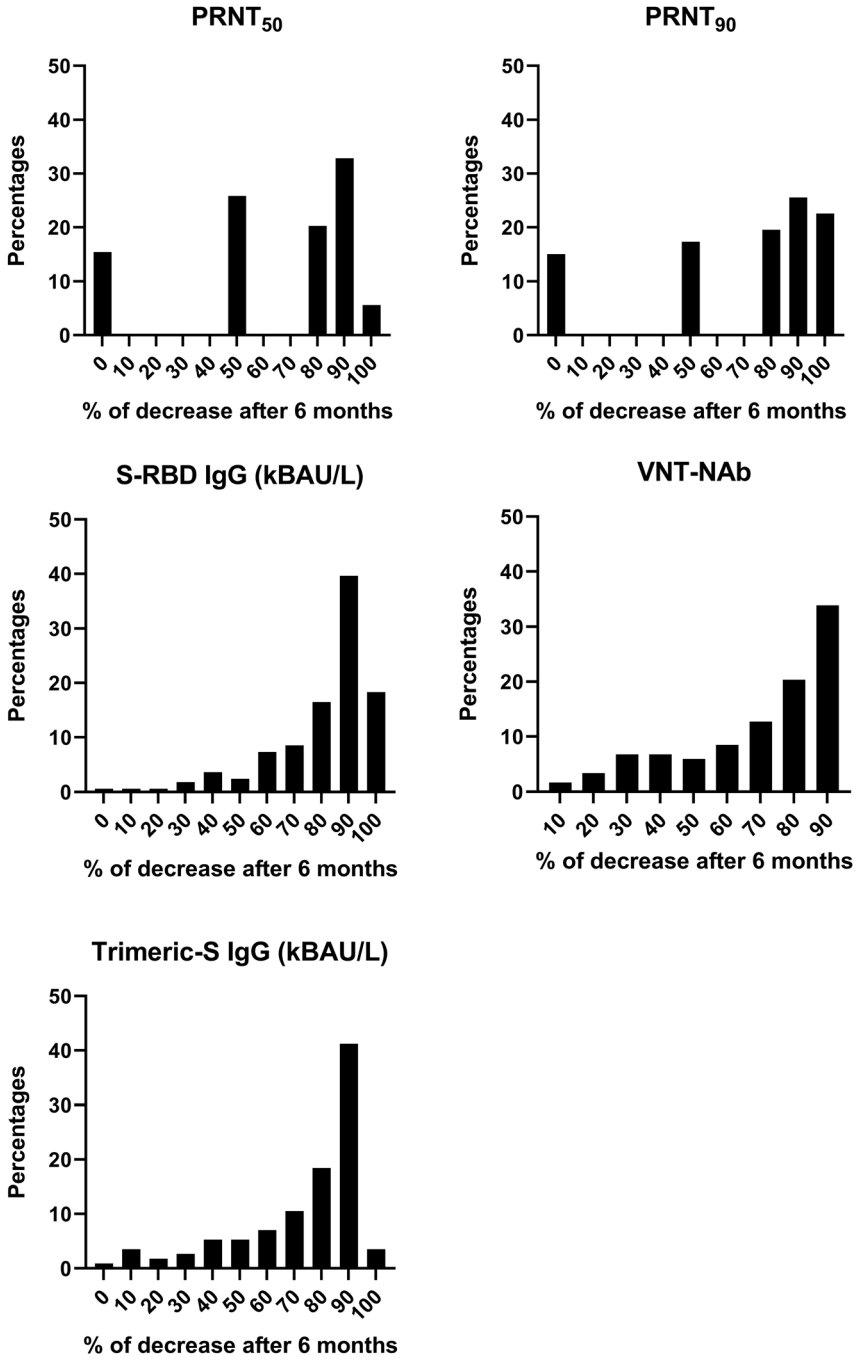

Figure 4: Frequency histograms of the distribution of values of the percentage of decrease of neutralizing antibodies at the two stringency thresholds $PRNT_{50}$ and $PRNT_{90}$, of binding antibodies (bAb) and of viral neutralization test neutralizing antibodies (VNT-NAb) levels after 6 months. The histograms show the distribution of the % of decrease of Ab levels after 6 months from the first inoculum, calculated by subtracting to t_{6m} Ab the t_{28} Ab, dividing the result by t_{28} Ab, and multiplying by 100.

SARS-CoV-2 infection, and vaccination. The waning of serum antibodies and neutralizing antibodies against SARS-CoV-2 has been widely documented [16, 17], although several studies have underlined that levels of vaccine-induced Ab persist also 6 months after the second dose [18]. In view of these results, the heterogeneous levels reported might be explained by the different Ab types evaluated [binding antibodies (bAb) or neutralizing antibodies (NAb)] and/or by age or gender dependent differences. Poor standardization of analytical methods, as well as different targets evaluated by commercial assays, might also explain the reported discrepancies. Overall, NAb are considered the most accurate available method for ascertaining protection against COVID-19, since they correlate with the capability of immune response of neutralize the entry of virus into host cells [13].

In this study, a cohort of HCW was followed up for 6 months after vaccination with Comirnaty (BNT162b2, BioNTech/Pfizer). Using vital virus, NAb titers were measured by the gold standard, the plaque reduction neutralization test (PRNT) [14], at low ($PRNT_{50}$) or high ($PRNT_{90}$) stringency

thresholds, and the results compared with those obtained with three different CLIA assays: two assays measured bAb, and one sVNT measured VNT-NAb. Investigation into SARS-CoV-2 previous infections is of utmost importance in understanding the humoral response. In this cohort, all HCW underwent repeated nasopharyngeal swabs as from March 2020, but were also further interviewed to collect data on previous infection and comorbidities. A total of 17 subjects were not infection naïve, since they previously had COVID-19, while comorbidities were only present in a limited number of individuals. Multivariate analyses were performed to evaluate the correlation of Ab levels at 6 months adjusted by age, gender and previous infection. Our results demonstrate that for all the investigated methods, neither bAb nor NAb correlated with gender (Figure 1), while only trimeric-S was slightly associated with age. These results contradict findings made by other Authors, who have reported age- and gender-dependence of bAb and/or NAb at 6 months [5, 16, 19], while they are in agreement with the finding of an absence of correlation of Ab levels and age or gender found by us and other groups [2, 11, 20]. The different detectable levels for gender, as well as for age, could be attributed to heterogeneity of individuals included in the study. However, contrasting results for the significance between Ab levels, age and gender are reported also by other Authors. Levin et al., for example, studied Ab dynamics, and found that bAb levels did not differ by gender, but were associated with age classes; likewise, NAb varied by gender and age groups [5]. A different pattern was reported by Khoury et al., who underline that gender differences are appreciable only for individuals above 50 years of age [16]. The slight differences found in the our enrolled cohort were not statistically significant. The limited analytical standardization might also explain reported differences among published studies, as confirmed by our data when comparing different methods (Supplementary Figure 2).

Feng et al. demonstrated that increasing Ab levels proportionally reduce the relative risk of symptomatic COVID-19. Interestingly, on the basis of results from this group, it is reasonable to assume that a vaccine efficacy of 80% could be achieved with anti-RBD IgG levels above 506 kBAU/L [21]. In our study 23/172 (13.4%) infection naïve and 12/17 (70.1%) previously infected individuals, presented bAb above this level. However, a detailed analysis showed that 50/172 (29.1%) infection naive and 14/17 (82.3%) previously infected individuals, presented $PRNT_{50}$ above 1:160, which is the threshold recommended by the FDA for convalescent plasma therapy administration [22]. Overall, 175/189 (92.6%) had positive (>1:10) $PRNT_{50}$ titers.

The evaluation of Ab dynamics revealed that, independent of the assay used to determine bAb of NAb levels, at 6 months the majority of subjects had about 90% decrease in their anti-SARS-CoV-2 Ab levels (Figure 4). These results were independent of age and gender and previous infection, as confirmed at multivariate analyses, with exception of a slight significance found for $PRNT_{50}$ and $PRNT_{90}$ (Table 3). Interestingly, a limited number of individuals presented Ab levels at 6 months higher than after 1 month from the first dose. Accordingly to Naaber et al., these individuals could confidently be considered "late responders", rather than statistical outliers, as they slowly developed Ab after vaccination [7].

The present study has some limitations. First, the number of HCW with a previous infection is limited, although our data confirm previously reported patterns [2]. Second, to elucidate the waning of humoral response, a longer study period is required. Third, the full spectrum of analytical performances of CLIA methods (except for Maglumi SARS-CoV-2 S-RBD IgG [15]) was not verified. The strength of this study was its characterization of the cohort of HCW individuals, who were followed weekly, undergoing molecular testing to identify any early infection. Further, NAb titers are developed using vital virus (PRNT), as this method is consensually accepted as a valuable tool for appropriately estimating the risk of re-infection and protection against SARS-CoV-2.

Conclusions

The findings made in the present study demonstrate a method-independent reduction of 90% of anti-SARS-CoV-2 antibodies occurs 6 months post-vaccination, and in individuals aged 24–65 years without severe health issues, no significant differences between males and females are to be expected. However, SARS-CoV-2 antibody assays still present contradictory results, and there is an urgent need for comparability and standardization, particularly in view of the fact that PRNT determination is labor-intensive, has a long turnaround time and calls for bio-safety level 3 (BSL-3) containment, which is unavailable in most clinical laboratories, thus precluding its widespread utilization in clinical practice.

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Informed consent: Informed consent was obtained from all individuals included in this study.
Ethical approval: The study was conducted in accordance with the Declaration of Helsinki, and the Institutional Review Board of the University of Padova (protocol nr 7862).

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